Research Article

Rapid Adaptation of Cellular Metabolic Rate to the MicroRNA Complements of Mammals and its Relevance to the Evolution of Endothermy

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SUMMARY

The metabolic efficiency of mammalian cells depends on the ability of microRNA to attenuate intrinsic translation noise. We devised a metric of cellular metabolic rate (*cMR)*, *rMR* optimally fit to the number of microRNA families (*miRNA.Fam*), that is robust to variation in mass and sensitive to body temperature, consistent with the heat dissipation limit theory of Speakman and Król (2010). Relatively abrupt increases in *miRNA.Fam* and *cMR* in later-branching, permanently homeothermic Boreoeutheria led us to postulate the emergence of new optimum in the adaptive landscape. With *miRNA.Fam* as predictor, simulation of an Ornstein-Uhlenbeck process of stabilizing selection, together with an adaptive shift between Atlantogenata and Boreoeutheria, accounted for 95% of the variation in *cMR*. Branchwise rates of evolution of *cMR*, *miRNA.Fam* and body temperature concurrently increased 6- to 7-fold, independent of the evolution of mass. Cellular *MR* variation across placental mammals was also predicted by the energy spared by model conserved microRNA-target interactions, revealing an unexpected level of integration of the microRNA-target apparatus into the energy economy of the mammalian cell.

INTRODUCTION

Phylogenetic models envision adaptive landscapes that change relatively slowly or quickly (Simpson, 1944; Eldrege and Gould, 1972; Eldredge, 1989; Hansen, 1997; Pagel, 1999; Venditti et al, 2011; Bartoszek et al, 2012; Rabosky et al, 2013; Bastide et al, 2017; Landis and Schraiber, 2017; Pagel et al, 2022). The mechanism for traversing the landscape can be random and clock-like, simulated by a Brownian motion process (Felsenstein, 1985), or deterministic and adaptive, such as the Ornstein-Uhlenbeck model of stabilizing selection (Hansen, 1997; Bartoszek et al, 2012). One candidate for an adaptive shift is the combination of permanent homeothermy, fast specific metabolic rates (*sMR*s), and body temperatures (T_b's) > 36ºC among Euarchontoglires and those orders of Laurasiatheria that diverged more recently than Chiroptera. This energy regime contrasts with that found in Atlantogenata and early-branching orders of Boroeutheria (Eulipotyphla, Chiroptera), with T_b's < 36ºC that overlap those of marsupials (Supplementary Figure S1), and that generally exhibit daily torpor and/or behavioral thermoregulation, reliant as they are on low-energy insectivory or folivory (McNab, 2008; Lovegrove and Génin, 2008; Boyles et al, 2013; Ruf and Geiser, 2015; Lovegrove, 2017; McNab, 2019; Muramatsu et al, 2022). The emergence of permanent homeothermy associated with fast *sMR*s, in birds as well as mammals, can be understood as an endpoint in the evolution of the regulation of body temperature and the ultimate safeguarding of development from external perturbations, thereby allowing a more forward-looking and efficient allocation of energy among somatic maintenance, reproductive reserves, and parental care (Koteja, 2000; Maino et al, 2014; Swanson et al, 2017; Bernhardt et al, 2020).

In large mammalian trees, the rate of evolution of basal *MR* (*bMR*) was found to be more variable than the rate of evolution of T_b, and better correlated with ambient temperature (T_a) (Avaraia-Llautureo et al, 2019),

making it a more likely target of selection. Overall *bMR* depends on mass, hence *sMR*, as well as mass-independent factors, such as thermal conductance (Naya et al, 2013b; Fristoe et al, 2015). Cellular *MR* accounts for 90% of *bMR* (Rolfe and Brown, 1990), and depends on the rate of protein synthesis, which drives 20% of ATP turnover (Buttegereit et al, 1995). The rate of protein synthesis is, in turn, dictated by cell size, which is extremely conserved in mammals (Ginzberg et al, 2018; Knapp et al, 2019; Neurohr et al, 2019) (for reviews, see Björklund, 2019 and Zatulovskiy and Skotheim, 2020). Matching of the rate of protein synthesis to cell size is predicted by models in which the rate of translation in exponentially growing cells is limited by competition among mRNAs for ribosomes (Kafri et al, 2016; Lin and Amir, 2018). A slow rate of translation relative to transcription minimizes energy losses due to both error correction by kinetic proofreading (Hopfield, 1974; Ninio, 1975; Bennett, 1979; Barato and Seifert, 2015; Banerjee et al, 2017; Mallory et al, 2019; Yu et al, 2022), and to intrinsic translation noise (Ozbudak et al, 2002; Lin and Amir, 2021).

We propose that the fitness benefits of higher T_b's and faster *sMR*s would have been offset without compensatory adaptations to limit increases in energy dissipation due to the cost of kinetic proofreading and translation noise. The interactions between microRNAs and targeted messenger RNAs constitute a well-established mechanism to attenuate intrinsic translation noise (Bartel, 2018; Shang et al, 2023). The distribution of the rates of translation of low- and high-abundance mRNAs conform to the predicted outcome of global acitivity of microRNAs (Vogel et al, 2010; Schwannhäusser et al, 2011), which preferentially repress the translation of low-abundance transcripts (Schmiedel et al 2015). Furthermore, these relative rates of translation appear to have evolved under an energy efficiency constraint (Schwannhäusser et al, 2011), which can be understood in light of the 10- to 100-fold higher thermodynamic cost per gene of translation relative to transcription in eukaryotes (Wagner, 2005; Lynch and Marinov, 2015). Assuming conservatively that global microRNA activity reduces the global rate of translation by 25% (Krek et al, 2005; Baek et al, 2008; Selbach et al, 2008; Spies et al, 2013; Schmiedel et al, 2015; Lu et al, 2023), a 10-fold increase in cellular *MR*, without a corresponding increase in global microRNA activity, would incur a 6% loss of efficiency of cellular *MR* (5.4% of overall resing *MR, rMR*) due to protein overproduction, as well as add to the cost of protein degradation (Peth et al, 2013; Snoberger et al, 2017). While not directly comparable, it is worth noting that losses of fitness of up to 3% were found when the intrinsic translation noise of individual, constitutive proteins in yeast was doubled (Schmiedel et al, 2019).

Consistent with an adaptation to maintain energy efficiency, the complements of conserved microRNAs and density of conserved microRNA target sites in placental mammals are larger than those of metatheria, with notable increases among permanently homeothermic late-branching Boreoeutheria (Agarwal et al, 2015; Tarver et al, 2016; Fromm et al, 2020) (Supplementary Tables S1 and S2). We postulated that the emergence of permanent homeothermy by thermoregulation in fast *sMR* Boreoeutheria represented the

appearance of a new optimum in the adaptive landscape, characterized by coordinated increases in microRNA repertoires, microRNA-target interactions, cellular *MR*s and T_b. By assuming that global microRNA activity is proportional to the number of microRNA families (*miRNA.Fam*), we devised a new metric to compare cellular *MR* across species. Rather than inferring *sMR* from the slope of log *rMR*/log *M*, we used variable allometric scaling to optimally fit rMR/M^{exp} to the distribution of *miRNA.Fam*. We interpret this rMR/M^{opt} as the global rate of microRNA-mediated repression of translation, which we assume is proportional to cellular metabolic rate and designate as *cMR*, in order to avoid confusion with conventional *sMR*. We demonstrate below that this metric of cellular *MR* has desirable properties compared to inference from the ratio log *rMR*/log *M*, including greater robustness to variation in mass and increased sensitivity to the implied ratio of surface area (a measure of the of rate heat dissipation), to mass or volume (a measure of the rate of heat generation) (Porter and Kearnery, 2009; Ballesteros et al, 2017). Importantly, the variation across speces of rMR/M^{opt} with respect to *miRNA.Fam* (or any other covariate) is homoscedastic (Supplementary Figure S2), and thus compatible with regression models (Benson et al, 2022) that have allowed us to test the following predictions:

i) The evolution of *cMR* with respect to *miRNA.Fam* will conform better to a deterministic (Ornstein-Uhlenbeck) model of adaptation than to a model of random drift by Brownian motion;

ii) The distribution of optimally-scaled *cMR* will be best simulated by an OU process with an adaptive shift corresponding to the divergence of Boreoeutheria;

iii) Consistent with selection, an adaptive shift to permanent homeothermy will be evidenced by simultaneous shifts in the rates of evolution of *miRNA.Fam*, *cMR* and T_b;

iv) The relative increase in *cMR* among placental mammals will correspond to approximately 20% of the reduction in translation cost that can be attributed to the sum of all model microRNA-target interactions.

v) The allometric scaling of *cMR* will depend more on surface area as body temperature increases and temperature-dependent variation will be constrained at the upper end of the range of *cMR*, as predicted by the heat dissipation limit (HDL) theory of Speakman and Król (2010). These authors proposed that species encounter two kinds of ecological constraint on metabolic rate: when the food supply is limited, competition will favor animals with low *rMRs*, but when fuel energy is abundant, then a physiological contraint, specifically the maximal rate of heat dissipation, will limit the evolution of faster *cMRs* (Speakman and Król, 2010).

RESULTS

Allometric scaling of vertebrate MRs by maximum likelihood

In conventional studies of the evolution of metabolic rate, constraints such as mass, temperature, aridity, and diet have been inferred from the slope and residual analysis of the regression of log *rMR* on log *M* (see, for example, Kleiber, 1932; McNab, 2008; McNab, 2009; Naya et al, 2013a; Ballesteros et al, 2018; McNab, 2019). We first established that our sample of 20 vertebrate species in MirGeneDB was broadly representative of vertebrates with regard to the distributions of mass and *rMR*. As shown in

Figure 1A, the *rMR*s of the 14 endotherms and 6 ectotherms exhibited a conserved relationship to mass, with slopes (0.75 and 0.88 respectively) very similar to those reported in previous, large-scale studies (White et al, 2006; Gillooly et al, 2017). When M^{exp} was fit by maximum likelihood to the distribution of *rMR* (with resolution $M^{0.1}$) (White, 2011), the optimal regression yielded a common slope of 0.80 for both ectotherms and endotherms, normally distributed residuals, and a smaller residual error than the log-based method for either endotherms or ectotherms (P = 6.36e-32) (Figure 1B).

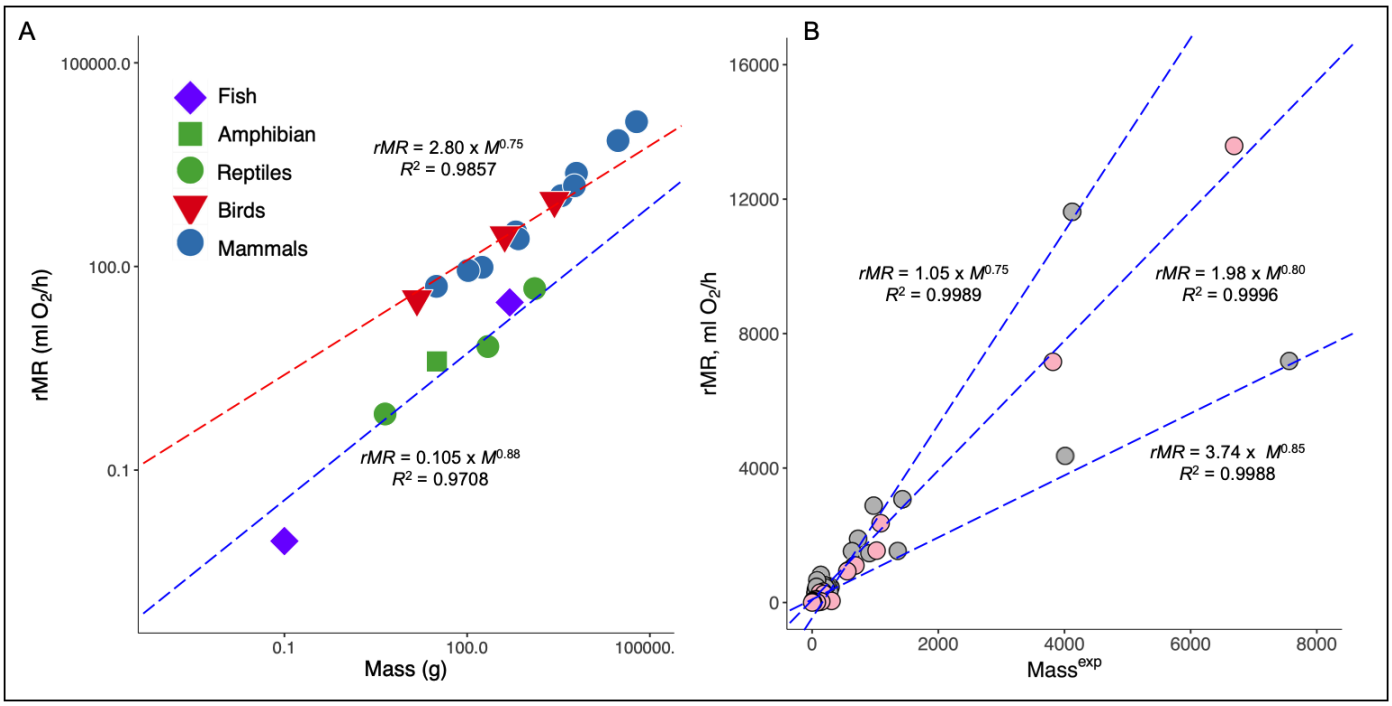

Figure 1. Comparison of the allometric scaling of the *rMR*s of 20 vertebrates by (A) the conventional power law method, or (B) maximum likelihood scaling according to the optimal fit ($M^{0.80}$: pink circles) and two suboptimal fits ($M^{0.75}$, $M^{0.85}$: gray circles).

Importantly, even though the variation of *rMR* with respect to M^{exp} is heteroscedastic (Figure 1B), when the scaling of M^{exp} is optimized in order to match the ratio of rMR/M^{exp} to the distribution of a third covariate, such as *miRNA.Fam*, the variation is homoscedastic (Figure 4 and Supplementary Figure S2), and therefore suitable for comparing the dependence of *cMR* on covariates across a wide range of species sizes (Benson et al, 2022).

As shown in Figure 2, maximum likelihood scaling of *cMR* across the arithmetic range of *rMR* is more robust to variation in mass and more sensitive than the log-based method to the ratio of surface area/mass, thereby providing more information regarding the biophysical nature of the relationship. For example, when scaled by maximum likelihood, the optimal slopes of *cMR* with respect to *miRNA.Fam* for early-branching mammals (mean T_b = 34.8ºC) and late-branching mammals (mean T_b = 37.6ºC) were $M^{0.78}$ and $M^{0.70}$, respectively (P = 1.54e-02, ANCOVA) (Supplementary Table S3 and Supplementary Figure S3). However, when the corresponding *sMR*s were derived from the slopes of

log *rMR*/log *mass* ($M^{0.75}$ and $M^{0.74}$, respectively) they were not significantly different (P = 0.2240, ANCOVA). The difference between the two methods of inferring cellular *MR* can be attributed to the fact that the relationship between *rMR* and mass is a function of many interacting factors, while the relationship between *rMR* and *miRNA.Fam* presumably reflect a single mechanism. Being more robust to variation in mass and more sensitive to variation in body temperature (Figure 2A), the ratio of surface area/mass indicated by optimal scaling of *cMR* is also more informative of relative rates of heat generation and/or dissipation (Naya et al, 2013b; Fristoe et al, 2015; Ballesteros et al, 2018).

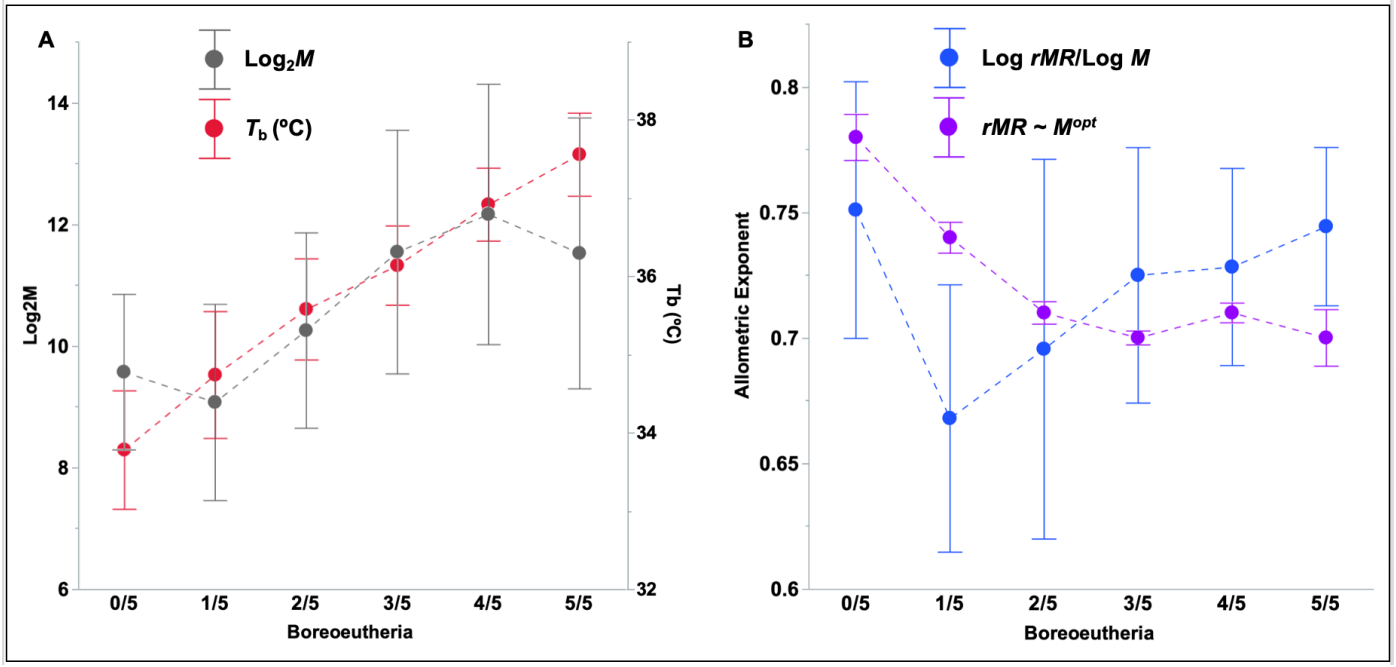

Figure 2. "Sliding Window" comparison of the inference of *sMR* and *cMR*.
Staggered groups of five mammals ranked by T_b were designated by the number of Boreoeutheria.
Panel A. The effect of group composition on the mean value of body temperature or mass (right axis).
Panel B. The effect of group composition on allometric exponent of *sMR* (the slope of log *rMR*/log *M*) or the maximum likelihood exponent of *cMR* (means ± ½ standard deviation).

There was a significant anti-correlation between the optimal *cMR* exponent and body temperature (P = 0.020). In other words, as mean T_b increased, the relationship between *miRNA.Fam* and *cMR* became more highly dependent on surface area. Furthermore, as predicted by the HDL theory, the optimal *cMR* exponent, and implied ratio of surface area/mass, asymptotically approached a limiting value of 0.70 despite increasing T_b. In contrast, the observed ratio of log *rMR*/log *M increased* with temperature.

The acquisition of microRNA families accelerated in late-branching mammals

The number of families of conserved, microRNA-encoding genes (*miRNA.Fam*) increased steadily in the order of divergence of vertebrate clades, except among Archosauria (Figure 3A)(Fromm et al, 2020; Álvarez-Carretero et al, 2022). The variation in *miRNA.Fam* was unrelated to genome size or number of

coding genes (Supplementary Table S3), but was 85% correlated with *cMR.75* ($rMR/M^{0.75}$) across 16 non-avian vertebrates (P = 1.02e-07, Kendall's rank correlation) (Figure 3B).

The acquisition of shared microRNA families accelerated in placental mammals, with increases of 46 in the 64 million years between the divergences of Theria and Placentalia, and 27 in the 8.7 million years between the divergence of Atlantogenata and Euarchontoglires (Álvarez-Carretero et al, 2022). Among the latter, fewer than ten microRNA families are uniquely expressed in the placenta (Fromm et al, 2020).

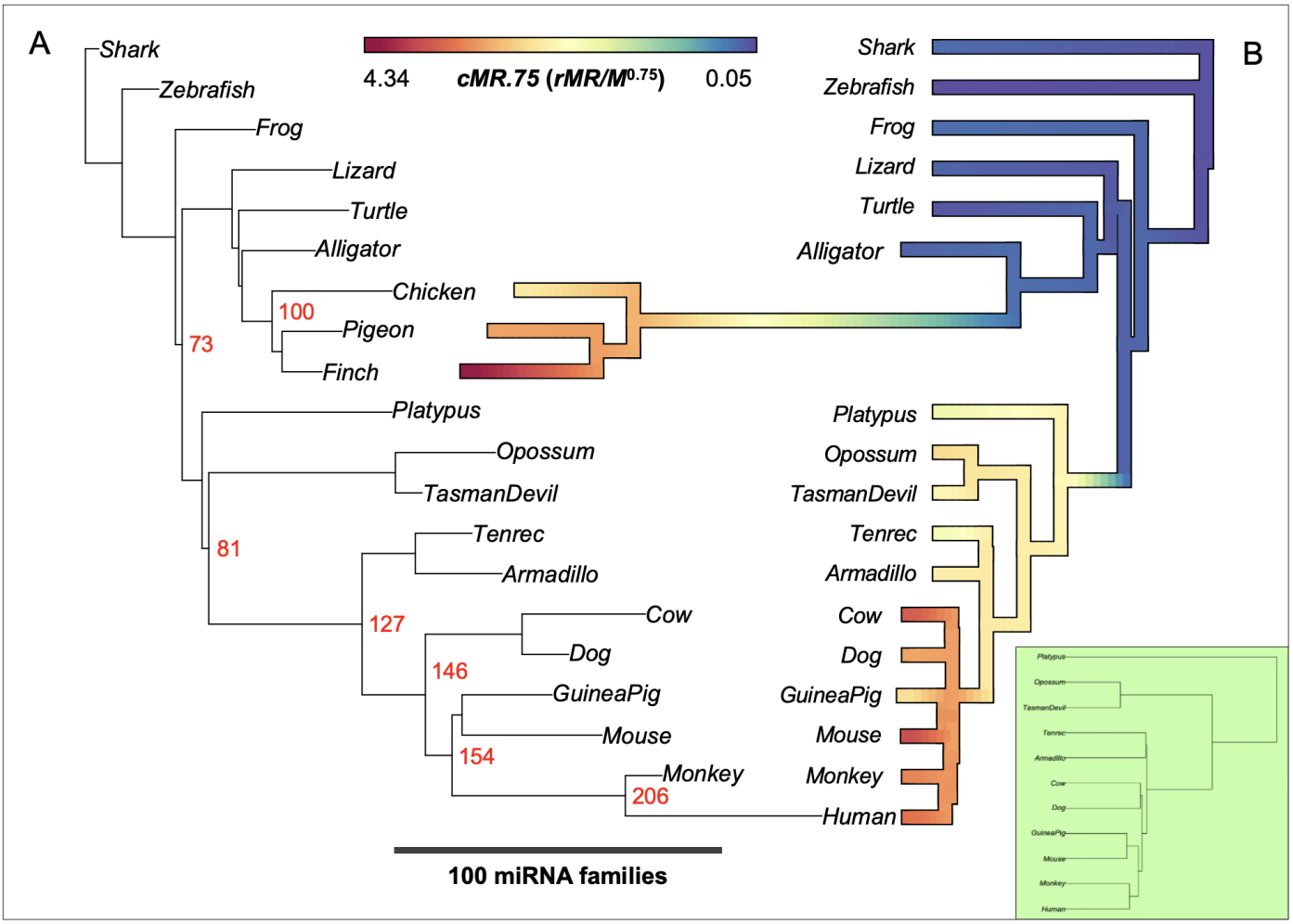

Figure 3. Evolution of the number of microRNA families and cellular metabolic rate (*cMR*).

A. Distance tree of microRNA families. Branch lengths represent the total number of families. Node values represent the number of shared microRNA families in each clade.

B. An ultrametric reference phylogeny (inset) was assembled based on divergence times drawn from Álvarez-Carretero et al 2022) for mammals and Irisarri et al (2017) for earlier-branching vertebrates. The reference tree was rescaled (BayesTraits) according to the rate of evolution of *cMR.75*, which corresponds to the optimal scaling in a model of stabilizing selection of *rMR* with respect to the number of microRNA families in tetrapods and mammals (see Figure 5). Values of *cMR.75* inferred at ancestral nodes (BayesTraits) were then mapped onto this variable rate tree (see Methods).

These differences correspond to gains of 0.92% per million years in pre-placental mammals *versus* 2.24% per million years among placentalia. Consequently, the number of microRNA families common to all four Euarchontoglires (154) is approximately twice the number shared by all Theria (81), as is the number of genes (Figure 1A) (Fromm et al, 2020).

The rate of evolution of *cMR.75* ($rMR/M^{0.75}$) increased markedly at each stage in the bird lineage, in an apparently inverse fashion to the number of microRNA families. In mammals, comparison of the internal branch lengths between the variable rate tree for *cMR.75* and the reference constant rate tree (inset) indicated compression of the terminal branches and the branches leading to the ancestral therian and placental mammal, and expansion of the branch leading to the ancestral Boreoeutherian, corresponding to a marked increase in *cMR* (Figure 3B; for additional analysis, see Figure 7).

The association between cMR and miRNA.Fam is independent of phylogenetic distance.

Phylogenetic linear regression indicated that the distribution of *miRNA.Fam* across 14 non-avian tetrapods accounted for 84% of the variation in optimally scaled *cMR* ($rMR/M^{0.68}$, P = 2.26e-06), independent of phylogenetic distance (Pagel's *lambda* = 0.00) (Figure 4A and Supplementary Table S3). The optimal allometric slope, 0.68, was significantly different from 0.78, the slope of log *rMR*/log *M* (P = 8.02e-03, ANCOVA). Similar results were obtained when only mammals were considered, which yielded optimal exponents of 0.71 for *cMR* and 0.77 for log *rMR*/log *M* (Supplementary Table S3).

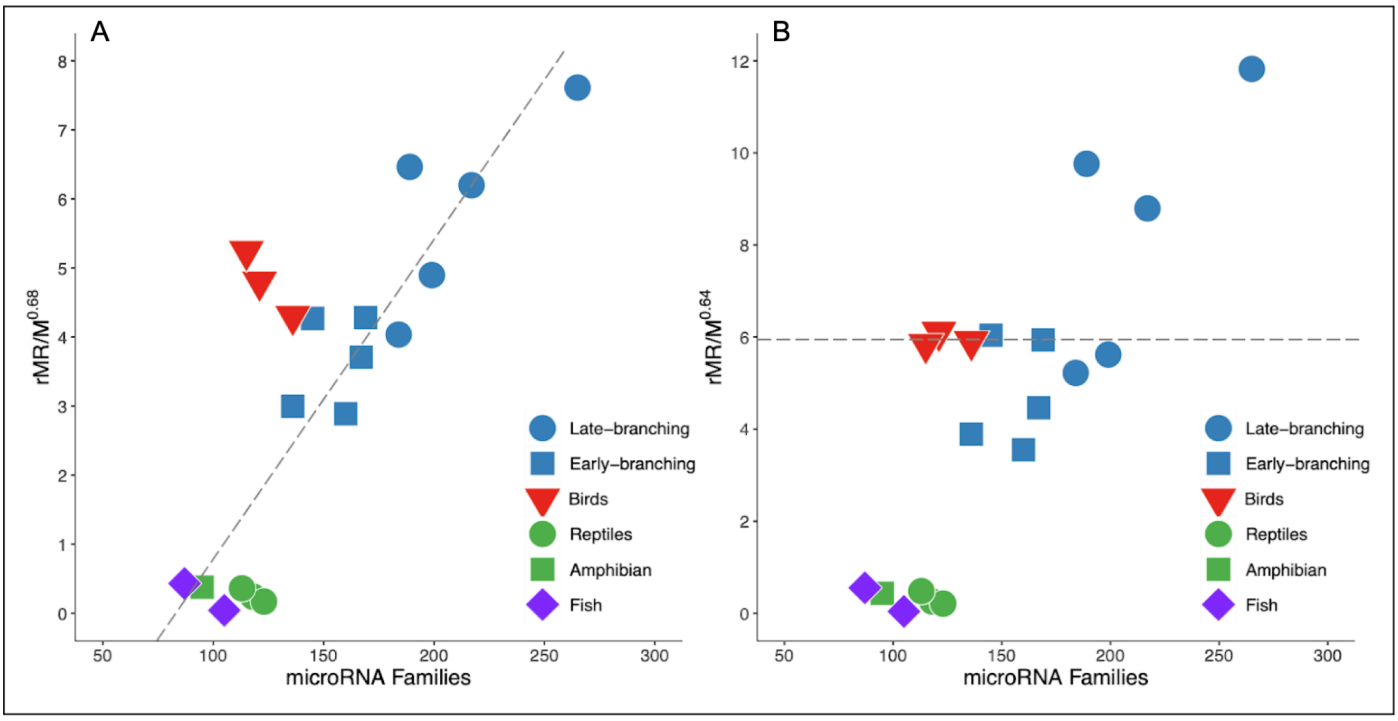

Figure 4. Phylogenetic regression of *cMR.68* ($rMR/M^{0.68}$) on *miRNA.Fam*.
A. The number of microRNA families in non-avian tetrapods accounted for 84% of the variation in *cMR*68, independent of phylogenetic distance (see Supplementary Table S2).
B. The same data scaled to the slope of log *rMR*/log mass (0.64) in 211 birds (Fristoe et al, 2015).

The variation of *cMR* with respect to the number of microRNA genes (*miRNA.Gen*) was weaker and, in the case of mammals, the association appeared to be secondary to phylogenetic distance (Pagel's *lambda* = 0.617, Supplementary Table S3).

The previous analysis excluded the three bird species in our sample (chicken, dove and finch) since the absolute number of avian microRNA families decreases in phylogenetic order (Figure 3A). However in large datasets, the log-based allometric exponent for *rMR* in birds is markedly lower than that found in mammals, ~ $M^{0.64}$ *versus* $M^{0.73}$ (White et al, 2006; McNab, 2009; Fristoe et al, 2015). When scaled to $M^{0.64}$, the slope of the regression of the bird *cMR* on the number of miRNA families was 0.00 (Figure 4B), and turned positive for any allometric exponent < 0.64. That is, when scaled with an allometric exponent < 0.64, bird *cMR*s *do* vary positively with *miRNA.Fam*. Thus, as in the case of mammals, the optimal allometric scaling of *cMR* with respect to *miRNA.Fam* in birds may be displaced toward a greater dependence on surface area *i.e.* lower allometric exponent, than indicated by the slope of log *rMR*/log *M* (Supplementary Table S3).

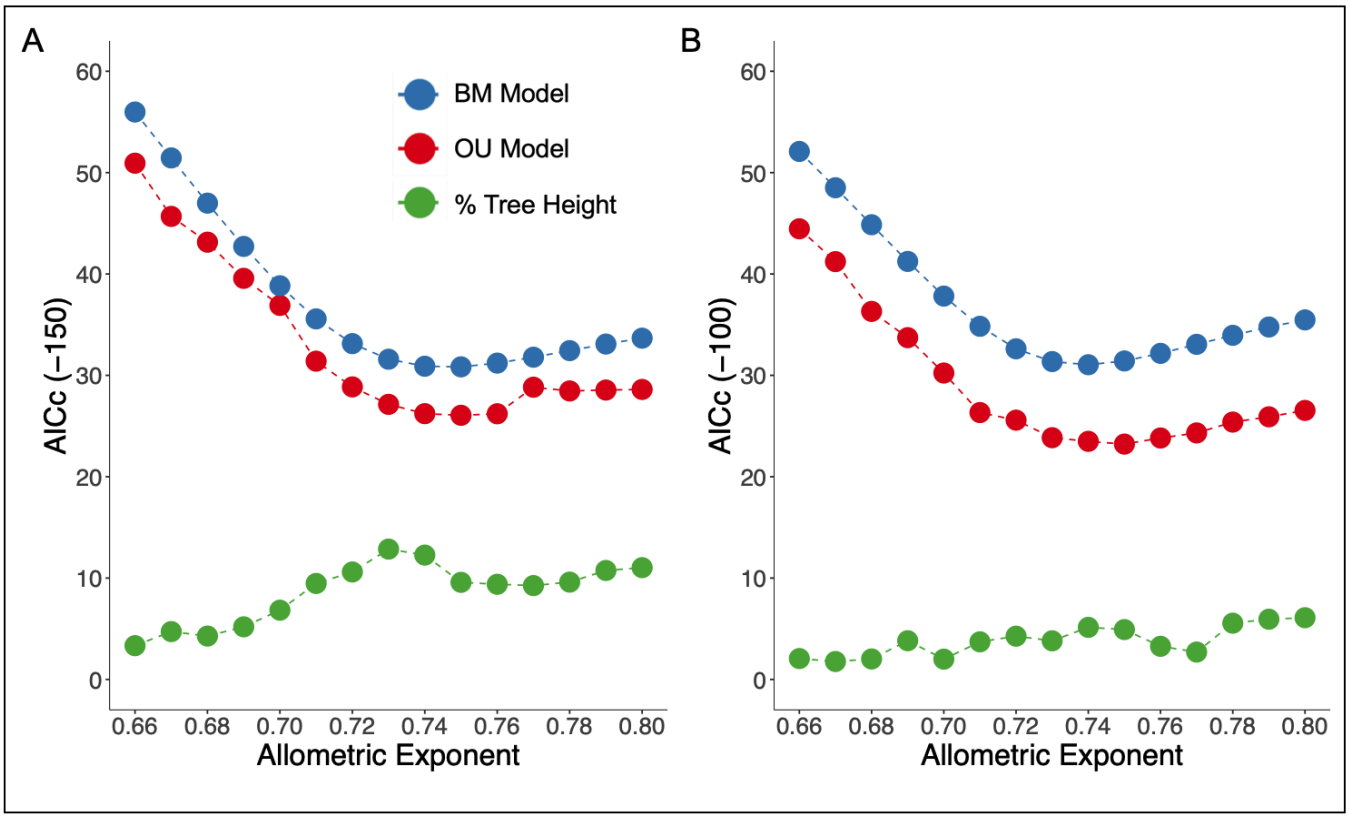

Figure 5. Comparison of BM and OU models for the variation of c*MR* with respect to *miRNA.Fam.* Curves represent the corrected values of the Akaike information criterion (AICc) for each model, and the rate of adaptation (% tree height) for the OU models. Note the difference in range of the ordinate axes. A: 14 tetrapods (excluding birds and cow). B: 10 mammals.

Cellular MR variation in relation to the number of microRNA families conforms to an OU model of stabilizing selection

We compared two hypotheses for the evolution of metabolic rates in relation to the microRNA complement: a Brownian motion (BM) process, where *cMRs* diverge as a function of phylogenetic distance, *versus* an Ornstein-Uhlenbeck (OU) process, according to which *cMR*s, under the influence of stabilizing selection, converge asymptotically toward the distribution of an independently varying predictor (Bartoszek et al, 2012), in this case, the number of microRNA families. We used two complementary approaches: first, we compared how well each model fit the relationship of *cMR* to *miRNA.Fam* across the range of allometric exponents 0.66 - 0.80; second, using the same phylogeny and distribution of microRNA families, we compared the actual distribution of *cMR* values with those generated by BM and OU simulations that included an adaptive shift at different stages of mammalian evolution.

For any *cMR* scaling in the range $M^{0.66}$ - $M^{0.80}$, the BM model for the evolution of *cMR* with respect to *miRNA.Fam* was inferior to the OU model (Figure 5A): at $M^{0.75}$, the minimal ∆AICc (BM-OU) value was +2.68 before correction (Supplementary Figure S4) and +6.88 after empirical correction of the bias recorded in 40 OU and 40 BM simulations on the same phylogenetic tree (Supplementary Table S4). The inferred rate of adaptation of *cMR* with respect to *miFam* at $M^{0.75}$ corresponded to 12.63% of the tetrapod tree (Figure 4A), or 4.92% when only the 10-mammal tree was considered (Figure 5B). Cellular *MR*s exhibited a similar ∆AICc optimum with respect to the number of microRNA genes (*miRNA.Gen*), however no such inflection was observed in models of *cMR* variation in relation to genome size (*Gnm*) or number of coding genes (*Cdg*), and AICc values declined monotonically (Supplementary Figure S4).

In our dataset there was an abrupt increase in the number of microRNA families between the two Atlantogenata (Tenrec: 160, Armadillo: 169) two Laurasiatheria (Dog: 184; Cow: 212), suggesting the emergence of a new optimum in the adaptive landscape (Simpson, 1944; Eldredge and Gould, 1972; Eldredge, 1989). We therefore simulated the evolution of *cMR* with respect to *miRNA.Fam* in 14 tetrapods by either a BM or OU process, assuming two adaptive shifts (Bastide et al, 2018): the first corresponding to the divergence of mammals, and the second shift corresponding to the divergence of either Placentalia, Boreoeutheria, or Euarchontoglires (Figure 6A). The best model, an OU process with an adaptive shift at the divergence of Boreoeutheria, accounted for 95% of the variation in *cMR.75* (P = 4.10e-09), compared to 88% for the corresponding BM model (Figure 6B). Estimates of the rate of adaptation increased in the order of the phylogenetic position of the second adaptive shift, corresponding to decreasing distance from the tips of the phylogenetic tree (P = 5.19e-06, ordinal logistic regression) (Figure 6C). In sum, for both OU and BM models of the variation in *cMR* across mammals, the optimal allometric scaling with respect to *miRNA.Fam* corresponds closely to the conserved ¾ power law model for the overall dependence of *rMR* on mass in mammals. It remains to be determined whether in birds, by analogy, the optimal *cMR* scaling in BM and OU models of *cMR*

dependence on *miRNA.Fam* corresponds to the ~ ⅔ power law model that fits avian resting metabolic rates (White et al, 2006; Fristoe et al, 2015; Ballesteros et al, 2018).

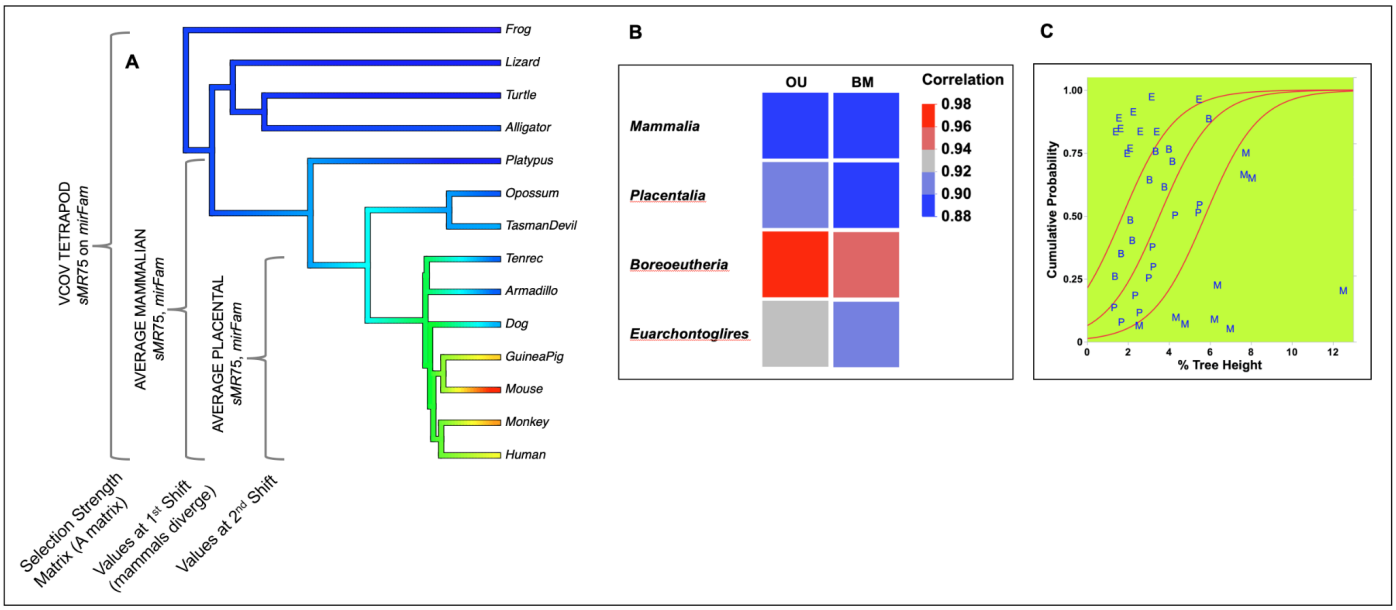

Figure 6. Design and analysis of simulated adaptive shifts in the evolution of *cMR* in relation to the predictor *miRNA.Fam.*

A. The variance/covariance matrix for the regression of *cMR.75* on *microRNA families* was used as the selection strength matrix to generate pairs of values for these traits. The average values of these traits across all mammals, or across placental mammals, were assigned to the first shift (divergence of mammals) and second shift, respectively. Phenogram colors correspond to the log of the *cMR.75* values scaled to the original, 36-fold range. B. Correlations with the actual *cMR.75* distribution of the average of ten simulations of the OU or BM process in 14 tetrapods. Adaptive shifts were modeled at the divergence of Mammalia (single shift), with a second shift at the divergence of either Placentalia, Boreoeutheria or Euarchontoglires (*P* = 1.23e-05 for OU *versus* BM, Boreoeutheria shift). C. Validation of the estimated rates of adaptation: prediction of shift positions according to the % tree height obtained from ten OU models. Shift positions are indicated by the first letter of each diverging clade (M, P, B, E; *P* = 5.19e-06, ordinal logistic regression).

The divergence of Boreoeutheria was associated with a step increase in the rates of evolution of *cMR.75, miRNA.Fam and body temperature.*

Based on the evidence for an adaptive shift in the dependence of *cMR* on *miRNA.Fam* and its relationship to body temperature, we predicted increases in the branch-wise rates of evolution of all three traits. Using a Bayesian variable rates algorithm, we generated phylogenetic trees in which branch lengths were scaled in proportion to the rate of evolution of each trait, relative to an expectation of a Brownian motion process (Venditti et al, 2011; Baker et al, 2016) (Figure 7).

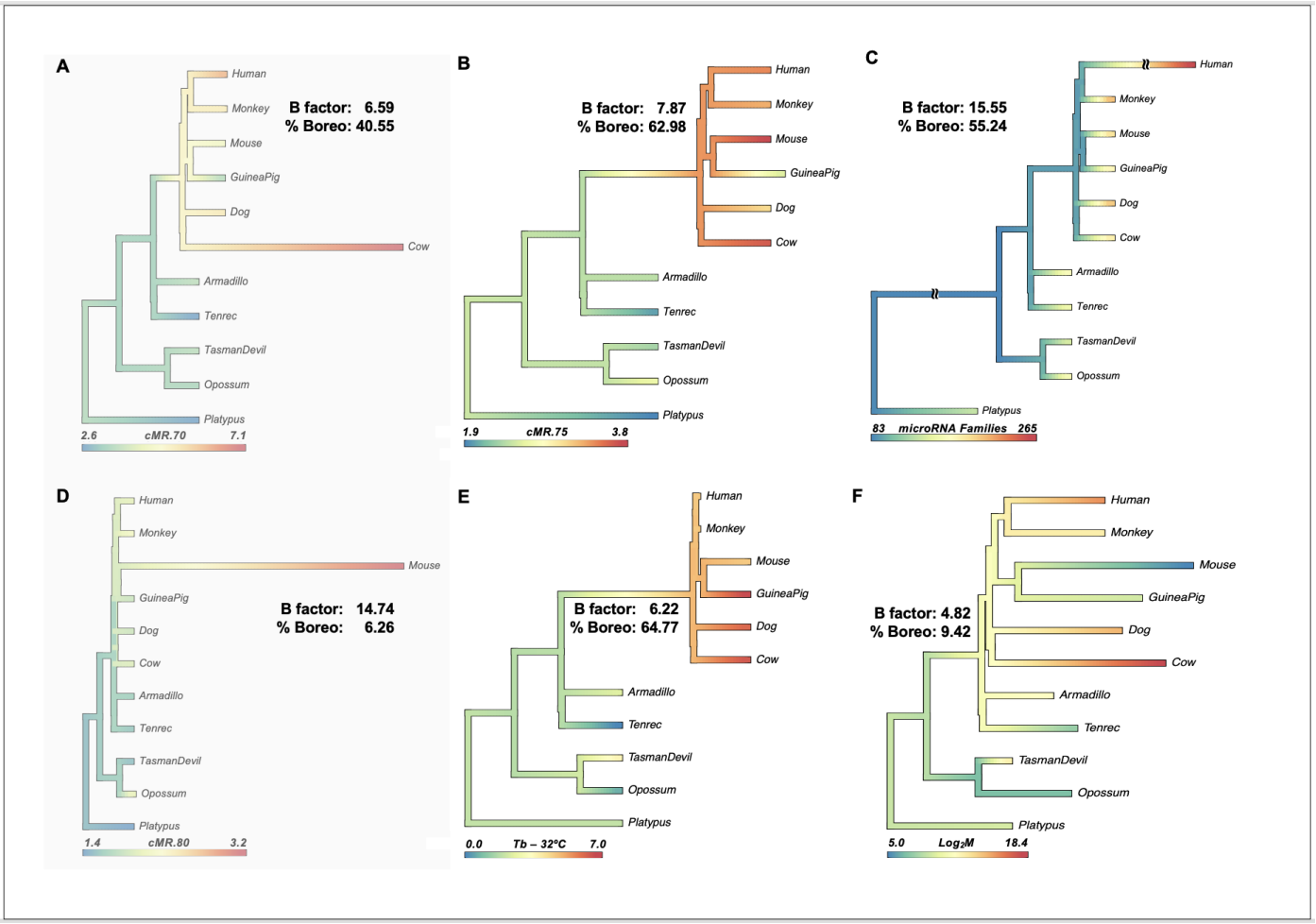

Figure 7. The ultrametric reference phylogeny of mammals (constant rate model) was rescaled according to the rate of evolution of each of six traits (A - F): *cMR.70*, *cMR.75*, *cMR.80*, *miRNA.Fam*, T_b(ºC) and $\log_2 M$. The length of the branch for the divergence of Boreoeutheria was compared (%) to the distance between the divergence of Theria and the divergence of Primates. Bayes factors were computed by comparing the median log likelihoods of the 100 most likely trees to the median log likelihoods of the trees with the smallest non-zero sum of changes to branch *i.e.* those trees that differed the least from the constant rate model. This criterion was adopted in lieu of comparing the marginal likelihoods of the variable and constant rate models, which reflect the overall fit of all of the branches. The range of ancestral node values inferred in a Brownian motion context were mapped onto each tree (see Methods). The value at each node represents the median of the ten reconstructions with the highest likelihood. In panel C (*miRNA.Fam*), the lengths of the branches leading to humans and to the ancestral therian have been reduced by half.

As shown in panels A, B and D, arbitrarily changing the allometric exponent changes the relative magnitudes of root-tip branch lengths of the variable rate trees for *cMR*: lower slopes (lower exponents/higher ratios of rMR/M^{exp}) magnify the difference at the upper end of the range of mass, and *vice versa*. Based on comparison of the median log likelihood of the 100 trees with the greatest (1%) likelihood and the 100 trees with the smallest (1%) sum of adjustments to branch lengths, *i.e.* those trees most similar to the reference constant rate model, the variable rate trees representing *cMR.75*, *miRNA.Fam* and T_b (Bayes factors > 6) and mass (Bayes factor > 4) were strongly supported (Raftery,

1996; Baker et al, 2016). The lengths of the branch representing the relative rate of evolution between Atlantogenata and Boreoeutheria were 6 - 7 times the lengths of the corresponding branch in the tree representing the evolution of $\log_2$ mass (Figure 7), and the branch lengths representing the descent of Primates from Theria were 99% correlated (Supplementary Table S5). If the variable rate tree for *sMR.75* depicts the outcome of adaptation, then the remaining phylogenetic variance ought to reflect only genetic drift and the difference in credibility between adaptive OU and random BM models based on this ree ought to be minimal, as was the case: ΔAICc (BM-OU) from 2.68 (before correction) to -0.53 (Figure 5, Supplementary Figure S5).

As previously reported, losses have been rare in the evolution of microRNA repertoires (Tarver et al, 2018), and only gains were noted in the terminal branches of the *miRNA.Fam* tree, with the human lineage exhibiting an exceptional increase (Figure 7C). On the other hand, accelerated rates of size evolution and marked decreases in body size were inferred in the three smallest mammals: opossum, tenrec, mouse and, to lesser extent, guinea pig (Figure 7F). The other adaptations associated with the reductions in size differed between the two permanent homeotherms (mouse, guinea pig) and the two heterotherms (opossum, tenrec). In the homeotherms, the adaptations were consistent with thermoregulation designed to limit maximal heat generation and the risk of hyperthermia: in the mouse, a severe reduction in size and increase in the surface area/volume ratio appears to have facilitated an increase in cellular *MR*, but not T_b. In the guinea pig, with 20 times the mass of the mouse, an increase in body temperature appears to have been offset by a reduction in cellular *MR*. (We have assumed that mouse and guinea pig have similar thermal conductances.) In contrast to the two homeotherms, the two heterotherms exhibited concurrent reductions in both size and body temperature, indicative of an energy constraint, but opposite changes in *cMR* (Figure 7E, B). The interpretation of these responses is complicated by the fact that both opossums and tenrecs exhibit daily torpor (Lovegrove and Génin, 2008; Ruf and Geiser, 2015; Dausmann et al, 2020).

Since the acquisition of microRNAs and increase in *cMR* in mammals were generally associated with higher body temperatures and larger body sizes (Supplementary Table S1; Baker et al, 2015), we used two approaches to further analyze the interdependence among these traits. Conventional analysis of covariance indicated that the dependence of *cMR.75* on *miRNA.Fam* in mammals was still significant after controlling for mass (P = 0.0267), but not after controlling for body temperature (P = 0.2133) (Supplementary Table S6). Bayesian regression confirmed that the inclusion of T_b improved the overall validity of the model, while in the model including mass, there was a lack of support for the regression coefficient for ($\log_2$) mass (Supplementary Table S6).

Faster sMRs and homeothermy in late-branching placental mammals are associated with the number, not kind, of microRNAs, and their conserved 3'-UTR targets

Of the 146 microRNA families common to all six Boreoeutheria in this study, 19 were not shared by both Atlantogenata, tenrec and armadillo: 10 were present only in tenrec, 8 were present only in armadillo, and only a single microRNA family, miR-370, was absent from both genomes. Moreover, for three of these 19 microRNAs there were no conserved target sites: miR-105, miR-370 and miR-6715 (Supplementary Table S7). It cannot be excluded that the difference in energy regimes between early- and late-branching placental orders is related to the activities of some combination of the remaining 16 microRNAs, affecting targets unique to, or differentially expressed in later-branching placental mammals (Christmas et al, 2023). However, any such discovery would not shed light on the central question of this study, the nature of the broad correlations across mammals between cellular metabolic rate, the size of the microRNA repertoire, and the predicted number of microRNA-target interactions.

We have assumed that global repression of translation by the combinatorial activity of microRNAs is proportional to the number of microRNA-target interactions and target sensitivity to repression. Since the TargetScan database lacks data from Atlantogenata, we tested this by comparing late-branching mammals (all Boreoeutheria) with a marsupial (opossum). Based on the number of conserved 3'-UTR sites (TargetScan database) of genes targeted by conserved microRNAs (MirGeneDB), *i.e.* those sites most likely to have been maintained by natural selection, we evaluated the following model to predict the energy cost saved by the sum of all conserved microRNA-target interactions per cell:

ΔTranslation cost = Median Sensitivity to Repression x Sites/Cell x AA Cost/Site

Given that the cost of protein synthesis accounts for ~20% of cellular metabolic rate, we predicted that the ~ 30% increase in *cMR*.75 between marsupials and late-branching placental mammals would be associated with an increase in overall microRNA-mediated repression of translation of ~ 150%. Figure 8 depicts the ratios of the value for each factor in the model to the corresponding value in the opossum. Relative to the opossum, the proportion of coding genes targeted and the number of 3'-UTR sites/mRNA were both approximately 50% higher in the placental mammals (Supplementary Tables 2). The total number of predicted microRNA-target site interactions per cell increased 100%, 140% and 250% in dog, mouse and human while the corresponding median values for sensitivity to repression by combinatorial microRNA activity (C_n) decreased by 11%, 12% and 16.7% (Figure 8). After taking into account the species distribution of target proteins 500 - 5,000 AA in length, the predicted ratios of overall translation cost were 180%, 192% and 269% (Figure 8). These results are in reasonable agreement with our prediction, considering that we made no correction for the relative abundance of each protein, and that the C_n values (combinatorial repression strengths), based on the most effective microRNA from each family, are likely to be overestimates (Agarwal et al, 2015; McGeary et al, 2019).

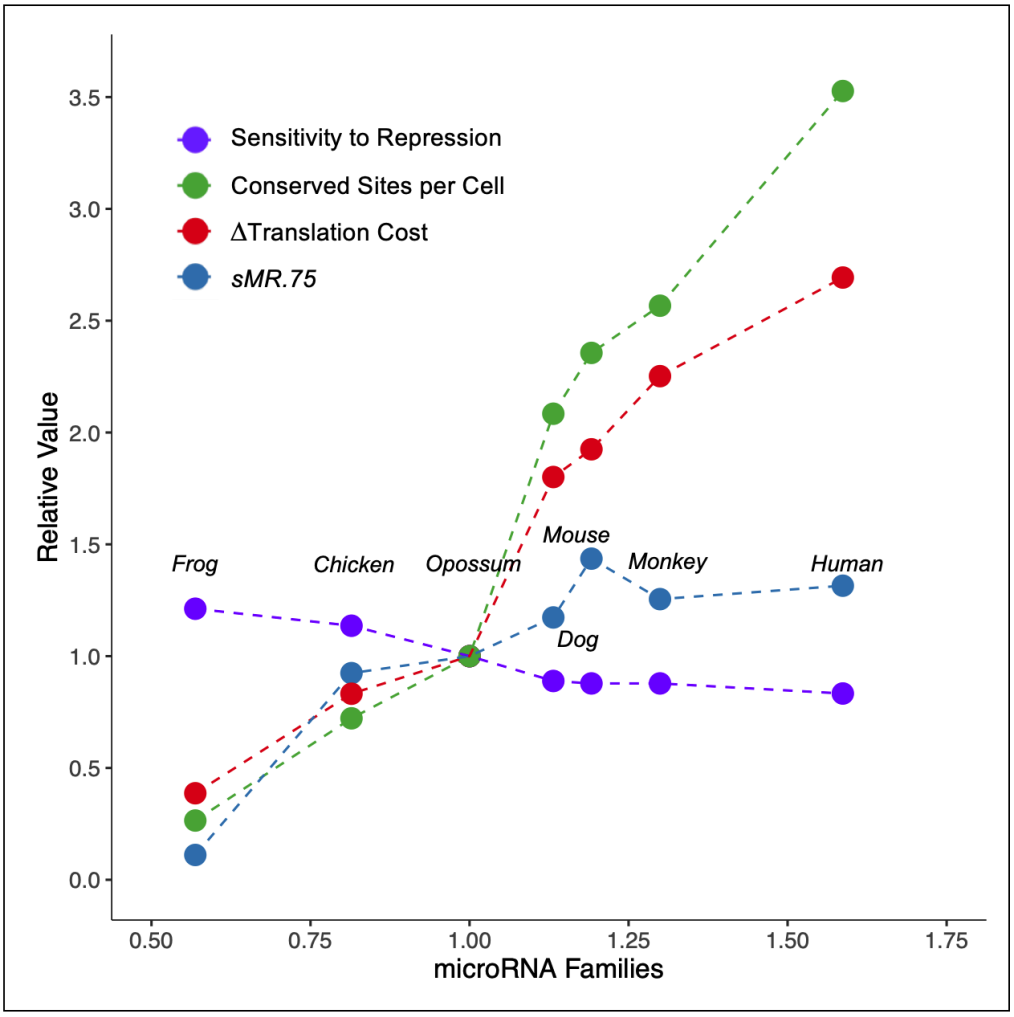

Figure 8. Evolution of factors in a model for energy cost saving by global microRNA activity.
Sensitivity to Repression: the median value of the distribution of cumulative weighted context scores for all models of the conserved targets of conserved microRNAs (see Methods).
Conserved Sites per Cell: the total number of sites was standardized with respect to the number of coding genes in each species.
AA cost/site: the weighted sum of 500 amino acid-length bins for the distribution of a non-redundant set of target proteins between 500 - 5,000 amino acids (see Methods).

DISCUSSION

Variable allometric scaling: a new method to explore the evolution of cellular MR

Given the high metabolic cost of protein synthesis and its strict relationship to cell size (Ginzberg et al, 2018; Björklund, 2019), we used variable allometric scaling in conjunction with phylogenetic comparative methods to estimate *cMR* by fitting the rMR/M^{exp} ratio to the size of the microRNA repertoire, assuming that it is proportional to the global attenuation of intrinsic translation noise. This study thus complements the inference of *cMR* from separate analyses of the variation of log mass and log *rMR* (Venditti et al, 2011; Baker et al, 2015; Avaria-Llautureo et al, 2019; White et al, 2019). The greater resolution of the arithmetic range of *rMR* for fitting rMR/M^{exp} to *miRNA.Fam*, and the method's

robustness to variation in mass made it possible to detect changes with body temperature in the surface area dependence of the *cMR-miRNA.Fam* relationship, even between small groups of animals. The method itself, fitting the distribution of the rMR/M^{exp} ratio to that of a third relevant trait, simulates a process of selection.

MirGeneDB is a new reference for comparative biology research.

Previous comparative studies have referenced molecular traits, such as genome size, the number of protein-coding genes, or quantity of non-coding DNA (for example, Choi et al, 2020). With the availability of the manually curated microRNA gene database, MirGeneDB, we are able to extend this approach to conserved microRNA families and genes in a wide range of vertebrate species and organs (Fromm et al, 2020; Fromm et al, 2021). A major concern in microRNA research in the past has been the quality of the online repository miRBase. This database contains many false-positives while at the same time missing a large number of *bona fide* microRNAs from undersampled species, which were misinterpreted as secondary losses (Chiang et al, 2010; Langenberger et al, 2011; Wang and Liu, 2011; Jones-Rhoades, 2012; Meng et al, 2012; Tarver et al, 2013; Castellano and Stebbing, 2013; Taylor et al, 2014; Fromm et al, 2015; Ludwig et al, 2017; Axtell et al, 2018; Fromm et al, 2020). Consequently, the conservation and utility of microRNAs as phylogenetic markers was questioned in the past (Thompson et al, 2014). However, these omissions were clearly technical artifacts, since losses of microRNA families have rarely been observed in well-annotated repertoires (Tarver et al, 2018). We and others have recently successfully employed MirGeneDB complements in a range of comparative (Fromm et al, 2021; Hu et al, 2021), phylogenetic (Ma et al, 2021; Rosani et al, 2021), developmental (Taylor et al, 2023; Jenike et al, 2023) and evolutionary studies (Peterson et al, 2022; Zolotarov et al, 2022), as a validation cohort for experimental findings (Kang et al, 2021; Baronti et al, 2020), and as a reference for the development of MirMachine, a novel, machine learning-based bioinformatics tool (Umu et al, 2023).

Exhaustive sequencing and genome curation have revealed that the number of families of conserved, microRNA-encoding genes has grown steadily over the course of vertebrate evolution, with very low rates of loss (Fromm et al, 2015; Tarver et al, 2018; Fromm et al, 2020; Fromm et al, 2021). Notably, the acquisition of shared microRNA families (Fromm et al, 2020) and conserved 3'-UTR target sites (Agarwal et al, 2015) accelerated in placental mammals, doubling the potential number of conserved microRNA-target interactions (Supplementary Tables S2). The acquisition of microRNA families and targets coincided closely with an elevation in *rMR*, body temperature and propensity for homeothermy among those mammalian orders that diverged more recently than Chiroptera (Ruf and Geiser, 2015; McNab, 2019): out of six mammalian orders with mean body temperatures significantly higher than that of bats, the only clade that diverged earlier than Chiroptera, Macroscelidea (Supplementary Figure S1), was a lineage characterized by an extreme reduction in size (Pagel et al, 2022). Conversely, out of eight orders with lower

average body temperatures, the only one that diverged later than Chiroptera, Pholidota, is characterized by species with relatively large masses, low *MR*s and body temperatures close to ambient (Clarke et al, 2010; Supplementary Figure S1), indicative of a limited capacity to dissipate heat (Speakman and Król, 2010). Since the order Pholidota has undergone relatively few speciation events since the Cretaceous (Foley et al, 2023), this observation is a reminder that faster mechanisms of heat dissipation must have had to evolve hand in hand with faster rates of metabolic heat generation (Speakman and Król, 2010: Naya et al, 2013b; Fristoe et al, 2015).

Cellular MR variation with respect to miRNA.Fam fits a model of stabilizing selection

For a BM process, between-species trait covariances are proportional to their shared ancestry, and a BM model will be favored if most of the crown species diverged recently relative to the total age represented by the phylogeny, as in the case of the mammalian tree used in this study (Álvarez-Carretero et al, 2022). Furthermore, for a small sample and phylogeny the mvSLOUCH algorithm for the comparison of OU and BM models exhibits a bias towards BM that has been attributed to a difference in the effective population size for each process (Bartoszek, 2016). For example, in a reanalysis of Darwin's data for 13 species of finch (Harmon et al, 2008), measures of effective population size yielded correction factors of ~ 0.5 and ~ 0.9 for the BM and OU models, respectively (Bartoszek, 2016). However, even before applying an empirical correction for bias, we found that an OU process was consistently favored over a BM process as a model for the evolution of *cMR* with respect to *miRNA.Fam*, in both the 14-tetrapod and 10-mammal datasets.

Assuming constant rates of evolution, the optimal slopes obtained by phylogenetic linear regression for the dependence of *cMR* on *miRNA.Fam* across tetrapods or mammals were $M^{0.68}$ and $M^{0.71}$, respectively (Supplementary Table S3). However, the AICc information criteria for both BM and OU models reached their minimal values when *cMR* was scaled to $M^{0.75}$, where BM and OU processes most precisely simulated the biphasic evolution of *cMR* (Supplementary Figure S6). This example illustrates an aspect of the scaling of *MR* that is generally overlooked when comparing allometric exponents inferred from the slopes of log *rMR*/log *M*, namely that differences in allometric slopes generally represent shifts in the rank order of the ratios between *sMR* (or *cMR*) and a third covariate. For example, among 10 mammals included in this study, the dog has the highest ratio of *cMR*/*miRNA.Fam* when *cMR* is scaled to $M^{0.70}$, second highest at $M^{0.75}$ and fourth lowest at $M^{0.80}$.

The analogous models with respect to the number of microRNA genes exhibited the same $M^{0.75}$ optimum, but no optimum was observed for *cMR* variation in relation to genome size or the number of coding genes (Supplementary Figure S4). Thus, *cMR* variation is most directly linked to the number of microRNA families. Given that the mechanism by which microRNAs conserve metabolic energy is well established, the specific association between *cMR.75* and *miRNA.Fam* supports our assumption that

the number of microRNA families is proportional to global microRNA activity and has evolved under similar constraints.

Three-quarter power scaling corresponds approximately to the conserved slope of log *rMR*/log mass reported for mammals in countless reports since Kleiber (1932) and Brody and Proctor (1932) (see also Savage et al, 2004). The present study indicates that this allometry also represents the optimal ranking of the ratios of *cMR/miRNA.Fam*, supporting the assumption that *miRNA.Fam* is a useful proxy for global microRNA activity, hence cellular *MR*. It also implies that conserved ~ ¾ power allometry is the outcome of selection primarily on the efficiency of cell metabolism, and only secondarily on mass or *rMR* (White et al, 2019). This comports with the observation that *sMR.75* and T_b and *miRNA.Fam* underwent concurrent 6- to 7-fold increases in evolutionary rate between the divergence of Atlantogenata and Boreoeutheria, independent of the rate of evolution of mass. An increase above the background rate of evolution of 2-fold or more is considered evidence of positive phenotypic selection (Baker et al, 2016). According to this conjecture, the relative rates of translation of low- and high-abundance transcripts, which is dictated by global microRNA activity, ought to be optimized for energy efficiency, as has previously been demonstrated (Schwannhäusser et al, 2011). A further implication for future studies is that rather than inferring cellular *MR* from the slope of log *rMR*/log *M*, it may be more relevant to infer the power law relationship between *rMR* and mass from the optimal scaling of *cMR* with respect to *miRNA.Fam*.

Optimally scaled MRs may also predict the microRNA complement in birds

In linear phylogenetic regressions, the optimal *cMR* scaling with respect to *miRNA.Fam* in tetrapods ($M^{0.68}$), mammals ($M^{0.71}$) and Boreoeutheria ($M^{0.70}$) exhibited greater dependence on surface area than did the log-based dependence of *rMR* on mass ($M^{0.78}$, $M^{0.77}$ and $M^{0.74}$, respectively). These observations may shed light on the correlation between vertebrate morphological patterns and microRNA complement size reported by Deline et al (2018). These authors demonstrated that morphological types, a function of aspect ratios, were significantly related to the number of microRNA families, but not to genome size or the diversity of protein domains. Furthermore, the morphospace occupied by birds was significantly displaced from a reptilian-mammalian axis (Deline et al, 2018), an interesting parallel to the shift we have predicted in birds, where *cMR*s are positively related to *miRNA.Fam* only when the allometric exponent is set lower than the ~ ⅔, *i.e.* slope of log *rMR*/log *M* (White et al, 2006; McNab, 2009; Fristoe et al, 2015; Ballesteros et al, 2018).

Another factor that will impact the allometric scaling of *cMR* is the relative cost of protein synthesis. Muramatsu (1990) compared specific rates of protein synthesis in the chicken and a range of domestic mammals, all homeothermic Boreoeutheria. After correcting for egg production (14% of total protein synthesis, 3% *bMR;* Hiramoto et al, 1989, Sakomura, 2004), the specific rates of protein synthesis in

chicken were 40 - 78% higher than in mouse, rat or rabbit, while the specific *MRs* of chicken were only 17 - 44% higher (Supplementary Table S8). If *miRNA.Fam* in birds is calibrated to a greater fraction of cellular *MR* than in mammals, then maximum likelihood fitting of the two distributions should yield a lower allometric exponent, hence higher rMR/M^{opt}.

There is an important difference between birds and mammals in the regulatory context in which microRNAs operate. Ancestral birds underwent a reduction in genome size, from ~ 2 Gb to ~ 1 Gb, primarily by jettisoning transposable elements (TEs). The massive loss of TEs in the ancestral avian genome contrasts with the steady evolutionary accumulation of TEs in mammals, where, with the exception of bats, they occupy 40 - 50% of the genome (Zhang et al, 2014; Kapusta et al, 2017; Platt et al, 2018). Retrotransposon sequences have been identified in the 3'-UTRs of 25% of mammalian coding genes and serve as binding sites for inhibitory KRAB-ZnF transcription factors (Faulkner et al, 2009). The loss of TEs in the ancestral bird genome was accompanied by the nearly complete loss of KRAB-ZnFs (Liu et al, 2014; Imbeault et al, 2017), implicating microRNA activity as the principal mechanism for the downregulation of gene expression. Interestingly, bat genomes are also characterized by smaller size, loss of transposable elements (Kapusta et al, 2017; Jebb et al, 2020) and shorter introns (Zhang and Edwards, 2012) relative to non-volant placental mammals, suggesting that these reductions represent adaptations for the faster rates of energy expenditure and signaling associated with flight (Hughes et al, 1995; Andrews et al, 2009; Wright et al, 2014; Ji and DeWoody, 2017). For example, shorter introns enable faster mRNA splicing (Heyn et al, 2014), while compact genomes and nuclei, if found in cells of smaller size and higher ratio of surface area/mass, would allow more rapid exchange of nutrients, as well as a higher density of neurons in the brain (Olkowicz et al, 2016; Gregory, 2018).

The predicted energy saved by global microRNA activity increased faster than cellular metabolic rate in placental mammals

The classification of conserved microRNA genes in MirGeneDB is congruent with the classification of conserved 3'-UTR target sites in TargetScan database (Kitai et al, 2022), but the latter lists targets for microRNAs that are excluded from the reference database, MirGeneDB. For example, of the 265 thousand sites listed for the human in the conserved site database, only 173 thousand corresponded to the reference microRNAs listed in MirGeneDB (Supplementary Tables S2). In order to exclude false positives, we considered only the conserved targets of reference conserved microRNA families (see Methods).

For each species C_n, the cumulative combinatorial repression per site, was estimated from the median value of the distribution of "cumulative weighted context++ scores" (Agarwal et al, 2015). The CWCS model in the current version of TargetScan (.v8) accounts for 37% of the variation in messenger RNA

levels following microRNA transfection of Hela cells, and probably overestimates the repression, since for each site, only the interaction with the most effective member of a microRNA family is evaluated (McGeary et al, 2019). After controlling for the number of coding genes in each species and the distributions of the lengths of the targeted proteins, the estimated energy cost saved by global microRNA activity in placental mammals was double that found in the opossum, consistent with the ~ ⅓ increase in c*MR*.75. This is only a first-order approximation, as we did not try to account for the relative abundance of each protein. Even though the majority of mammalian-specific target sites appear to be evolving neutrally (Simkin et al, 2020), the agreement with the prediction suggests that there is an immediate energy-saving benefit proportional to the overall number of microRNA-target interactions.

An adaptive shift at the divergence of Boreoeutheria simulates the evolution of cMR

The advent of placentation allowed some orders to exploit high quality resources through an increase in energy expenditure and reproductive output, adaptations denied to marsupials with access to the same resources (McNab, 2008). This divergence in energy regimes was associated with differences in the relative prevalence of homeothermy *versus* heterothermy *i.e.* daily torpor. Heterothermy is achieved by lowering the T_b set point, and evolved as an energy-saving strategy in small (< 1 kg) birds and mammals, notably bats, that are reliant on foraging and vulnerable to changes in food supply. After accounting for mass, climate and geography, earlier-branching placental clades, including Afrotheria, Xenarthra and Chiroptera, generally exhibit T_b < 36ºC (Supplementary Figure S1), a higher incidence of heterothermy, a lower proportion of skeletal muscle mass and lower metabolic rates. In contrast, in Euarchontoglires (excepting Eulipotyphla) and Laurasiatherian orders that diverged after Chiroptera (excepting Pholidota), homeothermy and T_b > 36°C predominate (Supplementary Figure S1) (McNab, 2008; Cooper and Geiser, 2008; Boyles et al, 2013; Ruf and Geiser, 2015; Rowland et al, 2015; Lovegrove, 2017; McNab, 2019).

Our demonstration that the *cMR* distribution in mammals was best simulated by an adaptive shift at the divergence of Boreoeutheria, and its implication of a Simpsonian jump (Simpson, 1944), suggests a model with distinct adaptive optima for low- and high-T_b mammals, based on the present range of species in MirGeneDB (Fromm et al, 2021). Figure 9 illustrates estimated temperature gradients and optimal allometric exponents for the *cMR-miRNA.Fam* relationship based on five early-branching, low T_b mammals (including Atlantogenata) and five later-branching, high T_b mammals (Boreoeutheria) (Supplementary Table S3). A separate optimum is shown for birds, with a predicted allometric exponent shifted to below the allometric slope (0.64) of log *rMR*/log mass (White et al, 2006; Fristoe et al, 2015). The temperature gradients maintained by the low- and high-T_b mammals differ by approximately 8°C (Clarke et al, 2010; Supplementary Figure S7), corresponding to a theoretical difference of 2.5% in thermodynamic efficiency ("Carnot Efficiency"), potentially offsetting some of the additional cost of sustaining higher body temperatures (see Methods for data sources).

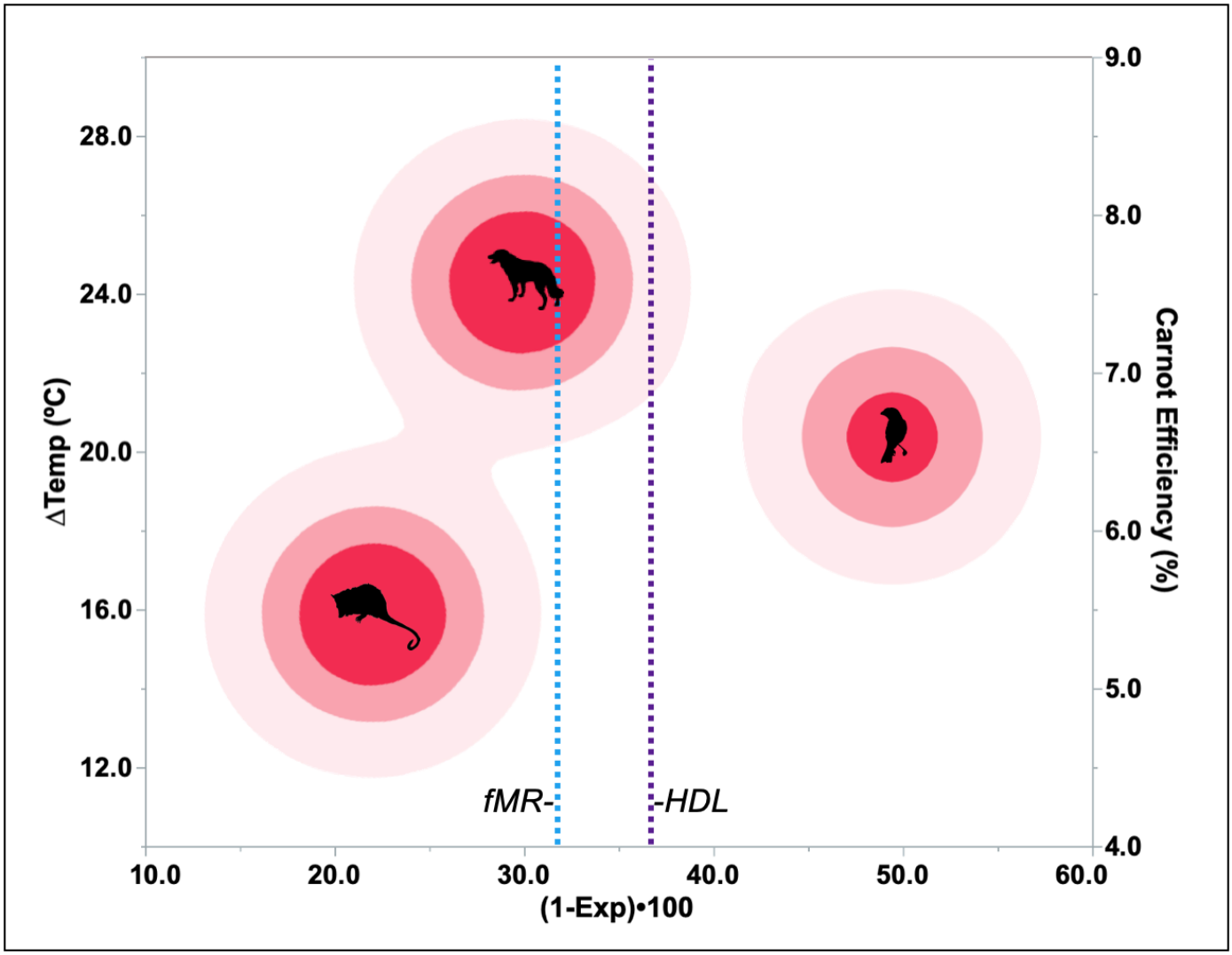

Figure 9. Contour plot of a hypothetical fitness landscape mapped onto the relationship between the core-surface temperature gradient and the optimal allometric exponent of the *cMR-miRNA.Fam* relationship (see Methods for sources).
Exp: Optimal *cMR-miRNA.Fam* scaling exponent. Δ**Temp** = Body temperature - Ambient temperature.
The dashed lines represent the allometric scaling of field metabolic rates and the predicted allometric scaling of the heat dissipation limit in mammals (Speakman and Król, 2010).
Silhouettes represent late-branching mammals (Boreoeutheria), earlier-branching mammals, and birds. (Phylophic © 2023: Marsupial, Sarah Werning; Dog, Tracy A. Heath; Bird, T. Michael Keesey).

The inference of distinct adaptive peaks is based on the present range of mammalian microRNA repertoires in MirGeneDB, which represents only two of the four possible combinations of ancestry and phenotype: earlier-branching mammals with low T_b's and later-branching mammals with high T_b's. The addition of definitive microRNA repertoires to intermediate branches of the mammalian tree, such as those of low T_b Eulipotyphla and Chiroptera and Lorises (heterothermic primates) may eventually bridge these hypothetical optima and better conform to a gradual model of genetic drift (Li et al, 2022; Pagel et al, 2022). On the other hand, body size evolution underwent a significant reduction in the rate of

directional change in Chiroptera, and reductions in the rates of both directional change and evolvability in early-branching orders of primates (Pagel et al, 2022) *i.e.* those orders of Laurasiatheria and Euarchontoglires that are heterothermic, suggesting that heterothermy is a derived condition in these orders while homeothermy, or at least the potential for permanent homeothermy, is the ancestral condition in Boreoeutheria. This interpretation is in keeping with earlier studies indicating that the incidence of heterothermy is strongly lineage dependent (McNab, 2008; Cooper and Geiser, 2008). Our model thus predicts a *loss* of microRNA families in heterothermic clades of Boreoeutheria relative to sister clades that are homeothermic, contrary to the overall trend in mammalian evolution (Tarver et al, 2018). It is interesting, in this regard, that In an ongoing survey using the machine-learning based algorithm MirMachine (Umu et al, 2023), the range of microRNA families in the three available reference genomes of lorises (164 - 167) is lower than that found in assemblies of similar quality (N50) for Platyrrhini (167 - 171), Catarrhini (178 - 182) and two other orders of Strepsirrhini (170, 183).

Furthermore, the evolution of T_b across 554 mammals has been found to be relatively static when compared to the rates of change of *MR* and ambient temperature, consistent with an adaptive barrier to the emergence of higher, but not lower body temperatures (Avaria-Llautureo et al, 2019). As mammals diversified in size (thus ratio of surface area to mass) in response to cooler environments (Clavel and Morlon, 2017; Baker et al, 2015; Avaria-Llautureo, 2019), multiple anatomic and physiological mechanisms were available to adjust overall *MR* without altering T_b or cellular *MR*, including changes in thermal conductance (Naya et al, 2013b; Fristoe et al, 2015) and relative organ size (Wang et al, 2012), particularly that of skeletal muscle (Rowland et al, 2015; McNab, 2019; Wright et al, 2021). However, shifting to a higher T_b and cellular *MR* while maintaining the strict relationship between cell size and rate of protein synthesis during the cell cycle would have depended on the ability to synchronize the expression of hundreds of regulatory genes with the activities of their target pathways. Just such a role has been proposed for microRNAs: microRNA activity was essential to buffer the effect of temperature fluctuation on photoreceptor development in Drosophila (Li et al, 2009). When fruit fly larvae were genetically engineered to decrease *MR*, doubling the time to eclosion, the entire microRNA repertoire was rendered dispensable for normal development. According to the mechanism proposed by Cassidy et al (2019), the effectiveness of transient signaling by a transcription factor depends on the timely decay of its concentration. Since this decay rate is inversely related to the rate of protein synthesis, as metabolic rate increases, greater repressor activity is required to maintain the same, evolutionarily conserved, rate of decay. By extension, increased repression by microRNAs may have been necessary to maintain the orderly unfolding of developmental programs in warmer, late-branching mammals.

Alternatively, bats are mammals that alternate between activity with thermoregulation to maintain homeothermy or torpor, a state in which they are essentially ectotherms (Fjelldahl et al, 2022). According to the mechanism proposed above, the timing of transient signaling that is

microRNA-dependent during homeothermy may not be disrupted in the transition to torpor if accompanied by a temperature-related decrease in the rate of Ago2-loading, which is normally rate-limiting for global microRNA activity (Gebert and MacRae, 2019; Reichhof et al, 2019). The implied ratchet effect, which would make decreases in body temperature more likely than increases, may inform the observation that on average in large datasets, birds and mammals evolved towards colder body temperatures from warmer-bodied ancestors (Avaria-Llautureo et al, 2019).

The majority of studies on the evolution of mammalian endothermy have focused on adaptations for the generation, conservation and dissipation of heat (*e.g.* Rowland et al, 2015; Legendre and Davesne, 2020), rather than on the mechanism of thermostatic control (Bernhardt et al, 2020). Our analysis of the relationships among *cMR*, *miRNA.Fam* and T_b in mammals has yielded evidence consistent with multivariate selection for stable and efficient thermoregulation. Speakman and Król (2010) have argued that evolutionary fitness must depend more on the realized rate of energy expenditure than on basal metabolic rate (*bMR*), and have proposed that variation in field *MR* (*fMR*) will better reflect the outcome of selection against the risk of hyperthermia. It follows that if variation at the upper end of the range of *cMR*s have been constrained (as we have demonstrated) by a limited ability to dissipate heat, this will be reflected better by the variation of *fMR* with respect to T_a than by variation of *bMR* with respect to T_b.

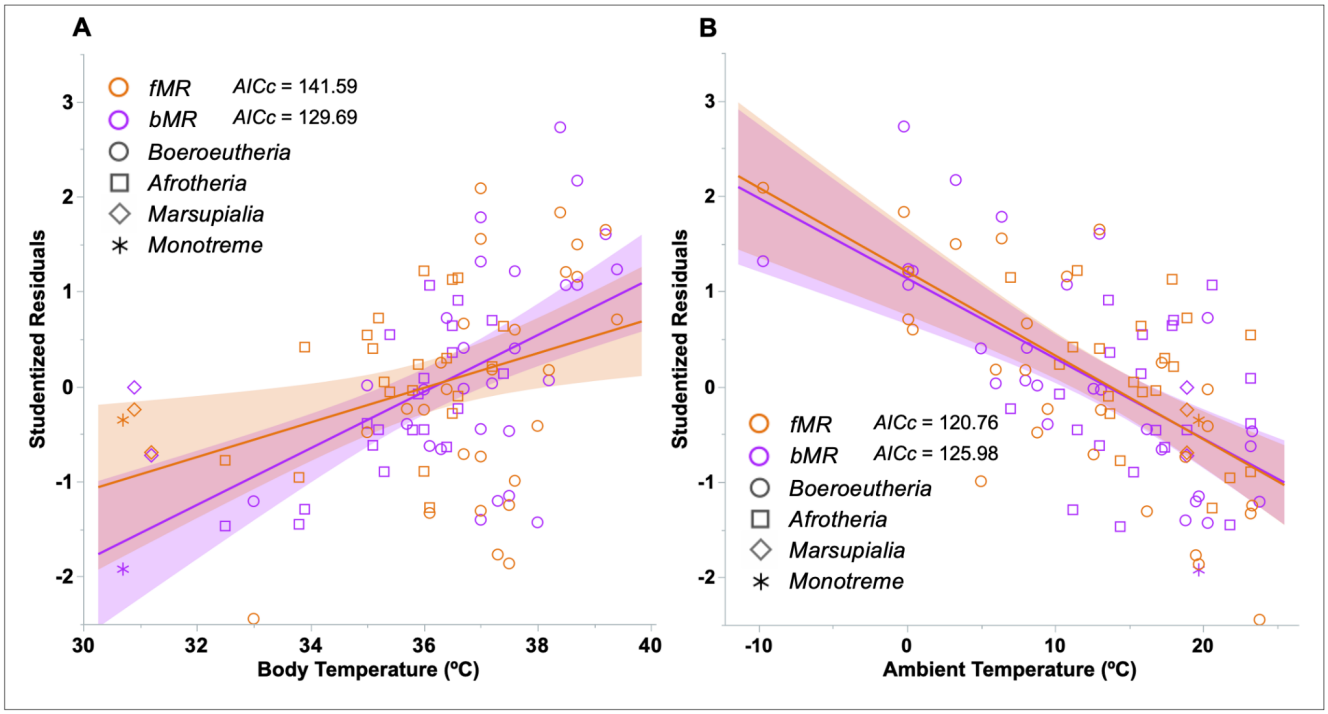

Figure 10. Variation in mass-independent residuals of *rMR* and *fMR* with respect to T_b and T_a. Species included one Monotreme, two Afrotheria, 20 Marsupials and 27 Boreoeutheria.

We compared these four parameters in 50 species common to the datasets of Clarke et al (2010) and Capellini et al (2010); in the latter, *fMR* had a lower phylogenetic signal than *bMR* in both Metatheria

and Eutheria, consistent with a greater role for selection (Figure 10). In our derived dataset, the log-based slope of *fMR* (0.5872) was shifted by 0.07 towards greater dependence on surface area than the slope for *bMR* (0.656) (P = 0.0191, ANCOVA), similar to the difference (0.06) that we found in mammals between the phylogenetic slope of log rMR/log M (0.77) and the optimal scaling of *cMR* with respect to *miRNA.Fam* (0.71). As expected, mass-independent residuals from log *bMR* exhibited less variance with respect to body temperature than did the residuals of log *fMR*, but the best predictor for both *bMR* as well as *fMR* was ambient temperature, not body temperature (Figure 10), in accord with the observations of Avaria-Llautureo et al (2019) in large mammalian trees. These authors found that the rates of evolution of *bMR* were coupled to T_b and T_a in 29% and 75% of all branches, respectively. Based on the credibility (AICc) of each model and the nearly identical slopes for *fMR* and *bMR* relative to ambient temperature (Figure 10B), we infer the following order of significance of factors that have shaped variation in mammalian metabolic rate: T_b < *bMR* < *fMR* < T_a. This order reconciles the observations of Avaria-Llautureo et al (2019) as well as our own with the HDL theory of Speakman and Król (2010).

There is additional biogeographic and physiological evidence for the notion that the maximal rate of heat dissipation and risk of hyperthermia have exerted a hard constraint on the upper end of variation in body temperature and metabolic rate. Phylogenetically-informed patterns of geographic distribution indicate that the diversification of mammalian morphology has been asymmetric, with sizes increasing in most orders (Baker et al, 2015), but evolving more rapidly at higher latitudes and during cooler geologic eras (Clavel and Morlon, 2017). In a complementary fashion, the diversification of the limits of tolerance to cold temperatures evolved faster than the limits of tolerance to heat (Araújo et al, 2013; Cooney et al, 2016; Qu and Wiens, 2020), and fit a biased OU model, *i.e.* stronger selection on upper temperature limits (Bennett et al, 2021). To some extent the asymmetry may reflect a smaller range of habitat variation in warm climatic niches together with more options for behavioral, as opposed to physiological adaptation, but additional evidence supports the operation of a hard physiological constraint on heat dissipation:

(i) T_a is a much better predictor of the thermodynamic gradient that mammals are able to maintain (Pie et al, 2021; Supplementary Figure S7);

(ii) After controlling for mass and phylogeny, the anti-correlation between basal metabolic rates and the upper limit of the thermoneutral zone (the temperature above which cooling mechanisms must be activated in order to prevent hyperthermia) was three times steeper than the anti-correlation with the lower temperature limit (below which cold-induced thermogenesis is activated) (Khaliq and Hof, 2018);

(ii) Relative to unselected lines, inbred lines of bank voles selected for higher *bMR*s under thermoneutral conditions exhibited elevated body temperatures at 34ºC, indicating a diminished capacity for effective thermoregulation and increased risk of hyperthermia (Stawski et al, 2017).

In the present study, sliding window analysis revealed an asymptotic, temperature-related decrease in optimal *cMR* scaling relative to *miRNA.Fam* in later-branching Boreoeutheria. The finding that the optimal allometric slope approached that of field metabolic rate (Figure 9), suggests that the *cMR* - *miRNA.Fam* relationship has been conditioned by physiological limits on the rate of heat dissipation (Speakman and Król, 2010) and implies that as evolving clades of Boreoeutheria increased in size (Baker et al, 2015) and/or rate of heat generation (Porter and Kearney, 2009), they were forced to negotiate a trade-off between gains in power and thermodynamic efficiency (ΔT_b-T_a) on the one hand, and an increased risk of hyperthermia, on the other. Such a trade-off would be a sufficient explanation for the asymmetric distributions of the physiological variables C_{min} (thermal conductance) and U_{CT} (upper limit of the thermoneutral zone) in Boreoeutheria (Figure 11) (see also Naya et al, 2013b; Khaliq et al, 2014; Fristoe et al, 2015).

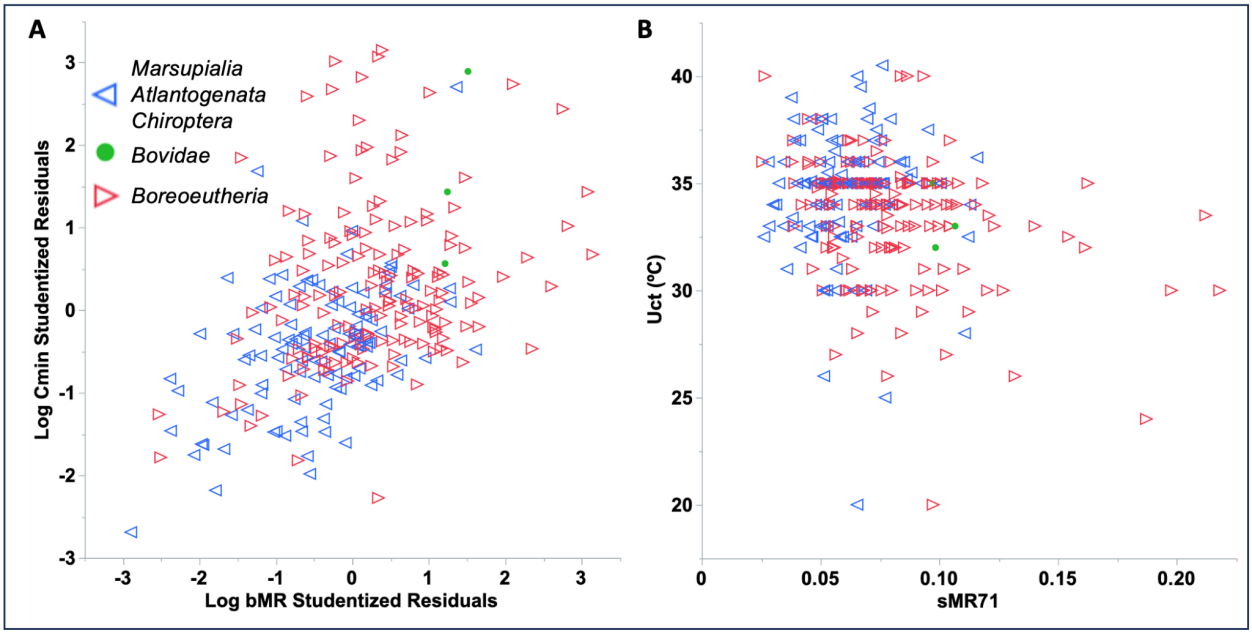

Figure 11. Thermoregulatory Status and the Distributions of Log C_{min} and U_{CT}.
276 mammals from the dataset of Khaliq et al (2014) were divided between Chiroptera and earlier-branching mammals (low incidence of homeothermy) and later-branching Boreoeutheria.

Homeothermic Boreoeutheria exhibited a combination of trends, higher C_{min} and lower U_{CT}, that are consistent with adaptation and constraint at the higher end of the range of mammalian T_b and *cMR*. Later-branching Boreoeutheria predominated in the upper one eighth of the distribution of Log C_{min} residuals (Figure 11A; P = 2.85e-05, □^{2}). This subset, with T_b's that were 0.75 ºC higher, and *cMR*s that were 29% higher than mammals in the lower seven/eighths of the distribution, had U_{CT}'s that were 2.7ºC lower, despite being exposed to similar ambient temperatures (Supplementary Table S9).

The adaptive value of repression by individual microRNAs operating in gene regulatory networks has been attributed to their ability to canalize phenotypic expression by buffering the effects of environmental or allelic variation (Hornstein and Shomron, 2006; Li et al, 2009; Cassidy et al, 2013)

and to set thresholds for cell fate determination (Mukherji et al, 2011; Ebert and Sharp, 2012). The ability of each microRNA seed to regulate many targets may have facilitated the evolution of regulatory networks of increasing complexity (Moran et al, 2017). Given the potential for microRNA regulation to increase the heritability of adaptive traits (Peterson et al, 2009), the enlargement of microRNA and target repertoires has been proposed as a key mechanism in the diversification of animal cell types and morphologies (Makeyev and Maniatis, 2008; Erwin et al, 2011; Deline et al, 2018; Dexheimer and Cochella, 2020; Peterson et al, 2021; Jenike et al, 2023; Taylor et al, 2023). However, relatively little attention has been paid to the nature of global microRNA activity (Reichholf et al, 2019; Kingston and Bartel, 2019; Tarbier et al, 2020; Kirstein et al, 2023) or its impact on energy metabolism (Schwanhäusser et al, 2011; Cassidy et al, 2019). Our model predicts that compensatory noise suppression by microRNAs should be proportional to body temperature, since temperature is the primary determinant of the rate of protein synthesis, and therefore of intrinsic stochastic translation noise (Thattai and van Oudenaarden, 2001; Ozbudak et al, 2002). Post-transcriptional regulation of gene expression ("mRNA processing") is the most constrained gene category in the evolutionary history of placental mammals (Christmas et al, 2023), which suggests a significant fitness cost for any loss in the efficiency of the processing of transcripts as mammals evolved faster and more complex regulatory networks.

In the present study we have shifted the focus of research on the evolution of metabolic rate from the adaptations of mass-independent to the efficiency of the cell mass-dependent rate of protein synthesis. Our findings indicate that the size of the microRNA-target apparatus exhibits an unexpected degree of integration with the cellular energy economy, which complements previous evidence for an energy constraint on the microRNA-dependent ratio of rates of translation of low- and high-abundance transcripts (Schwännhausser et al, 2011). These observations are consistent with a role for global microRNA activity in maintaining metabolic efficiency, thereby facilitating an adaptive shift in the Boreoeutherian lineage to higher body temperatures, faster metabolic rates, and permanent homeothermy.

DATA AVAILABILITY

The original data with accompanying references and the results of all analyses referred to in the text are provided in the Supplementary Information. Phylogenetic tree matrices are available upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization: BF and TS; Methodology: TS; Investigation: BF and TS; Writing – Original Draft: TS; Writing – Review & Editing: BF and TS; Visualization: BF and TS; Funding Acquisition: BF.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR METHODS

RESOURCE AVAILABILITY

LEAD CONTACT

For further information requests should be directed to Thomas Sorger (tsorger@rwu.edu).

MATERIALS AVAILABILITY

This study did not involve any physical material.

DATA AND CODE AVAILABILITY

The data compiled for this study are listed in Table S1 in the Supplementary Information.

All other data compilations are referenced in the main text.

This paper does not report original code.

METHOD DETAILS

MicroRNA Families, Genes and Targets

The numbers of conserved microRNA families and genes for 20 vertebrate species were obtained from MirGeneDB, a curated database of microRNA-encoding genes (Fromm et al, 2020). Conserved microRNAs were defined as those shared by two or more species. The number of conserved

mammalian mRNA targets was extracted from the TargetScan8.0 database (Agarwal et al, 2015; McGeary et al, 2019) and filtered for targets corresponding only to conserved microRNAs.

Other Genomic Variables

Values for genome size and number of coding genes were obtained from the NCBI genome assembly database (as of March, 2022).

Body Temperatures and Ambient Temperatures

Body temperatures and ambient temperatures for mammals, excluding 20 multivariate outliers (mass, *bMR*) were obtained from Clarke et al (2010): differences of 24ºC and 16ºC were estimated for Boreoeutheria (n = 318) and earlier-branching mammals (n = 94). A gradient of 20ºC for birds < 1,200 g was estimated from mean body temperatures of 40ºC (n = 203; Fristoe et al 2015) and a mean ambient temperature of 20ºC, the average of maximum and minimum temperatures across the entire avian geographical distribution (n = 157; Khaliq et al, 2014). Mass-independent residuals for log Cmin, log *bMR*, and upper and lower critical temperatures were drawn from Khaliq et al (2014), after excluding multivariate outliers (mass, *bMR*) (n = 242).

QUANTIFICATION AND STATISTICAL ANALYSIS

Resting Metabolic Rates and Temperature Correction

Rates of oxygen consumption and body temperature were drawn primarily from the compilations of White et al (2006) and Gillooly et al (2017). Where multiple values had been recorded at different temperatures, we used the value nearest to the standard body temperature (see below). The original reference for each species is listed in Table S1 of the Supplementary Information. Resting metabolic rates (*rMR*s) were corrected to 38˚C for endotherms, and 25˚C for ectotherms, as *per* White et al (2006) and Gillooly et al (2017). Since oxygen consumption data were available only for *X. laevis*, and *X. tropicalis* adults are approximately half the size of adult *X. laevis*, we assumed 0.54 the *rMR* of *X. laevis* (assuming $rMR_2/rMR_1 = M_2^{0.88}/M_1^{0.88}$ for amphibia).

Phylogenies

“Phytools” was used to generate a 20-species vertebrate tree and phenogram, 14-species tetrapod tree and 10-species mammalian tree (Revell, 2012) based on divergence times drawn from Álvarez-Carretero et al (2022) for mammals, and Irisarri et al (2017) for earlier-branching vertebrates. Trees were made ultrametric and scaled to unit height using Mesquite © (2021).

In order to model the evolution of *miRNA.Fam*, a matrix of interspecific distances was assembled based on the number of conserved microRNA families (MirGeneDB: March, 2022) (Fromm et al, 2020),

without considering losses or gains unique to each lineage. A distance tree was inferred by minimal evolution (LeFort et al, 2015).

Statistics

Phylogenetic linear regression was carried out using the *R: CAPER* package (Orme, 2013) using the maximum likelihood estimate between 0.0-1.0 for the *lambda* parameter (Pagel, 1999). All R^2 values were adjusted for the number of predictors.

Multivariate correlations, screening for outliers, ANCOVA and ordinal logistic regression were performed in JMP®16. For analysis of the fold variation by quintile in mass and cellular metabolic rate, Chiroptera and 21 outliers were removed from the original dataset of Clarke et al (2010). The cow was considered an outlier with respect to mass and, as in the case of all ruminants, metabolic rate (White and Seymour, 2005). All other statistical tests were performed in R (2020).

OU versus BM comparison and simulation

BM and OU models of the variation of *cMR* with respect to number of microRNA families were evaluated with the package mvSLOUCH (Bartoszek et al, 2012), with six repetitions of the hill-climbing algorithm for the OU process using two adaptation matrices (Diagonal and DecomposableReal). Preliminary validation based on the AICc criterion revealed that for the 14 tetrapod model, OU processes were misclassified as BM for 50% of the simulations, while 10% of the BM simulations were misclassified as OU. The corresponding rates of misclassification for the 10 mammal model were 75% and 5% (n = 40, Supplementary Table S3). The rates of correct classification for the BM and OU simulations were equalized by adjusting the AICc scores +/- 2.1 for the tetrapod model, or +/- 4.0 for the mammalian model (Supplementary Table S4).

The Phylogenetic EM package (Bastide et al, 2018) was used to simulate *cMR* and *miRNA.Fam* distributions using variance/covariance matrix for *cMR.75* on *miRNA.Fam* as the selection strength matrix, with the median setting (3) for selection strength (Supplementary Figure S4). The average values for all mammals *cMR.75* and *miRNA.Fam* were assigned to the first adaptive shift (divergence of mammals), and the averages for all placentals were assigned to the second adaptive shift (divergence of Atlantogenata or Boreoeutheria or Euarchontoglires) . Ten simulations were generated and averaged for each shift or pair of shifts.

Bayesian inference of the branch-wise rates of evolution

To generate variable rate trees for each trait, the VarRates algorithm of *BayesTraits* (version 4) was run for 12.5 million iterations with 20% burn-in and sampled every 1000 trees, yielding 10,000 trees fit to the distribution of either $\log_2$ mass, *cMR.75*, *miRNA.Fam* or T_b (Venditti et al, 2011). Bayes factors were

obtained by comparing median likelihoods of the 100 trees with the highest log likelihoods and the 100 trees with the smallest non-zero sum of scale changes (constant rate model). The latter was equivalent to the median log likelihood of random walk models for the reference tree (constant rate model). For the temperature data and reference tree, for example, the median log likelihood of 10,000 random walk models was -25.90, while the median log likelihood of the 100/10,000 variable rate trees with the smallest non-zero sum of scale changes was -26.62.

For each trait, correlations among the sets of branch lengths between the divergence of Theria and the divergence of Primates were obtained from the median branch lengths of the best 100 (1%) variable rate trees.

Variable Rate Regression and Inference of Internal Node Values

In order to evaluate the dependence of *cMR.75* on *miRNA.Fam*, while controlling for size (log mass) and body temperature, Bayesian regressions were run for 2.25 million iterations with 20% burn-in and sampled every 1000 trees (BayesTraits). Model statistics represent median values for the models with the 5% highest likelihood (100 trees).

Internal node values (BayesTraits) for $\log_2 M$, *cMR.75*, T_b and *miRNA.Fam* were inferred from the random variation model run for 8.0 million iterations with 20% burnin and sampled every 1,000 trees.

Quantification of Model Interactions between Reference microRNAs and their 3'-UTR Target Sites

The entries in the TargetScan database "Conserved Site Context Scores" (Agarwal et al, 2015; McGeary et al, 2019) were filtered for the reference microRNAs listed for each species in MirGeneDB (Fromm et al, 2020). For each distribution of CWCS scores, the median value was used to estimate sensitivity to repression. In each species, a non-redundant list of target genes was used to compute the average number of 3'-UTR target sites/gene and the length distribution of the corresponding target proteins between 500 - 5,000 amino acids. The relative 'AA cost' was obtained from the weighted sum of nine 500 AA-length bins between 500 - 5,000 AA (Supplementary Tables S2).

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