Research Article

Rapid Adaptation of Cellular Metabolic Rate to the microRNA Complements of Mammals

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SUMMARY

While terrestrial mammals range over six orders of magnitude in size, metabolic rate/cell *(sMR*) varies only ~5-fold, constrained by matching of the rate of protein synthesis (20% *sMR*) to cell size. Based on energy efficiency, we hypothesized that global repression of translation by microRNAs would vary directly with *sMR*, an expectation that was met by the number of microRNA families (*miRNA.Fam*). The dependence of *sMR* on *miRNA.Fam* fit an Ornstein-Uhlenbeck (OU) process of stabilizing selection and OU simulations with an adaptive shift between Atlantogenata and Boreoeutheria accounted for 95% of the variation in *sMR.75* ($rMR/M^{0.75}$). The branchwise rates of evolution of *sMR.75*, *miRNA.Fam* and body temperature increased ~10-fold between the divergence of Atlantogeneta and Boreoeutheria, independent of mass. The estimated energy saved by predicted interactions between conserved microRNAs and their conserved 3'-UTR targets also acccorded well with the evolution of *sMR.75*.

INTRODUCTION

The resting metabolic rate (*rMR*) and body mass of mammals have evolved in tandem according to a conserved allometry (Kleiber, 1932; Brody and Proctor, 1932; Savage et al, 2004; White et al, 2006; McNab, 2008; McNab, 2009; Naya et al, 2013; Gillooly et al, 2017; Ballesteros et al, 2018; McNab, 2019) that has limited the range of rMR/M^{exp}, or specific *MR* (*sMR*) (White et al, 2019 and Supplementary Figure S1). Since cellular *MR* accounts for 90% of *sMR* (Rolfe and Brown, 1997), the 10^6-fold range in the mass of terrestrial mammals represents, to a first approximation, variation in the total number of cells with highly conserved cell size and cellular *MR*.

An important constraint on cellular *MR* is the cell cycle-related coupling of macromolecular synthesis and cell size in mammalian cells, as in yeast cells (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). This adaptation 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). Absent the evolution of compensatory mechanisms, the fitness benefits associated with the evolution, in late-branching placental mammals, of faster cellular *MR*s, higher body temperatures and permanent homeothermy would have been offset by increasingly noisy and error-prone translation. Assuming selection against energy inefficiency, we postulated that the global attenuation of intrinsic translation noise by microRNA activity (Bartel and Chen, 2004; Hornstein and Shomron, 2006; Schmiedel et al, 2015; Bartel,

2018) would vary directly with cellular *MR*. This expectation was met by the number of conserved microRNA families (*miRNA.Fam*) and their conserved 3'-UTR target sites.

Unlike the impact of other regulators of gene expression, the gloal repression of translation by total combinatorial microRNA activity (hereafter "*global microRNA activity*") has an obligatory effect on *sMR*, by decreasing the overall rate of translation (Krek et al, 2005; Baek et al, 2008; Selbach et al, 2008; Friedman et al, 2009). Changes in the rate of translation disproportionally affect *sMR*, since protein synthesis, which drives 20% of ATP turnover (Buttgereit and Brand, 1995), is the single least efficient and most costly cellular metabolic process (Rolfe and Brown, 1997). 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 *rMR*) due to protein overproduction. 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).

Indeed, the rates of translation of low- and high-abundance transcripts in mammalian cells appear to have evolved under an energy constraint and conform to the predicted outcome of global microRNA activity (Vogel et al, 2010; Schwanhäusser et al, 2011). MicroRNAs preferentially repress low-abundance mRNAs which, owing to suboptimal ratios of translation/transcription, are more costly to translate than are highly abundant mRNAs (Bartel and Chen, 2004; Schmiedel et al, 2015). The latter are also enriched in the transcripts of housekeeping genes, and encode proteins that tend to be shorter and more stable (Eisenberg and Levanon, 2003; Schwanhäusser et al, 2011).

In previous studies, the evolutionary constraint on the ratio of *MR* to mass could be inferred from the analysis of the co-variation of the individual traits fit to models separately (Venditti et al, 2011; Baker et al, 2015; Avaria-Llautureo et al, 2019; White et al, 2019). Here we implement allometric scaling by maximum likelihood in order to test *sMR* directly in models of dependence on *miRNA.Fam*, body size and body temperature (T_b). Specifically, we asked whether the similar pattern of variation between *sMR* and *miRNA.Fam* better reflected phylogenetic distance (Brownian motion model) or the outcome of stabilizing selection (Ornstein-Uhlenbeck model) (Bartoszek et al, 2012), and at which stage of mammalian evolution an adaptive shift (Bastide et al, 2018) would have been most likely to simulate the parallel increases in *sMR* and *miRNA.Fam*. We inferred branch-wise rates of evolution of *sMR.75*, log mass, *miRNA.Fam*, and T_b to test for selection (Baker et al, 2016) and compared regression models to test for trait independence. Since the global attenuation of intrinsic translation noise also depends on target site density and sensitivity to combinatorial microRNA activity, we compared the variation in *sMR* to the relative energy saving per cell estimated from all of the conserved target sites of conserved microRNAs.

RESULTS

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 1A). The variation in *miRNA.Fam* was unrelated to genome size or number of coding genes (Supplementary Table S2), but was 87% correlated with *sMR.80* ($rMR/M^{0.80}$) across 16 non-avian vertebrates (P = 2.20e-16, Spearman rank correlation) (Figure 1B). 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). These differences correspond to gains of 0.92% per million years in pre-placental mammals *versus* 2.24% per million years among placental mammals (Fromm et al, 2020; Álvarez-Carretero et al, 2022). 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),

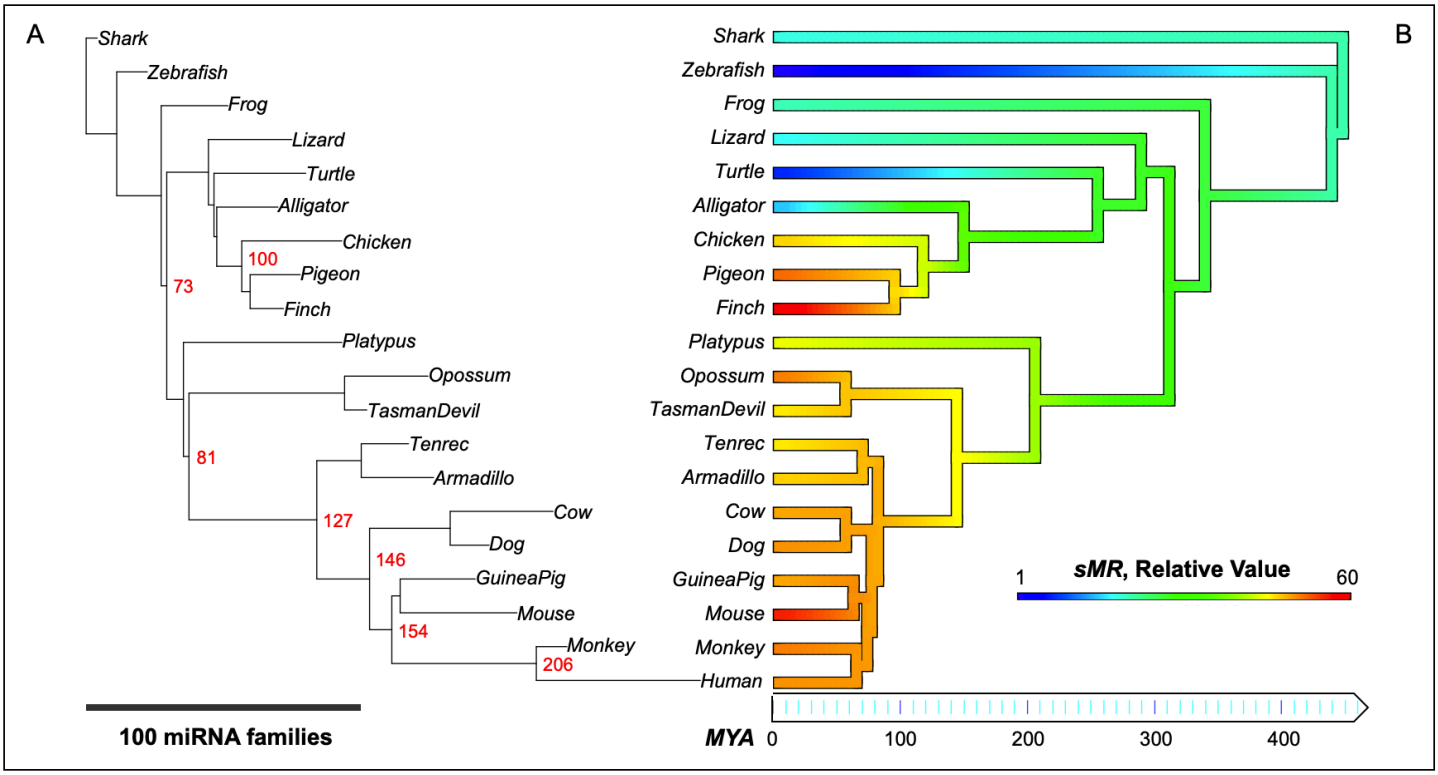

Figure 1. Evolution of the number of microRNA families and cellular metabolic rate (*sMR*).
A. Distance tree of microRNA families. Branch lengths represent the total number of families. Node values represent the number of shared microRNA families in each clade.
B. The log ratios of *sMR*s ($rMR/M^{0.80}$) were scaled to the original range of *sMR* and mapped onto the vertebrate phylogeny. Divergence times were drawn from Álvarez-Carretero et al 2022) for mammals, and Irisarri et al (2017) for earlier-branching vertebrates.

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 residual analysis of the regression of log *rMR* on log *M* (see, for example, Kleiber, 1932; McNab, 2008; McNab, 2009; Naya et al, 2013; Ballesteros et al, 2018; McNab, 2019). We first established that our sample of 20 vertebrate species in MirGeneDB was broadly representative of vertebrates with respect 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 0.8 for both ectotherms and endotherms, normally distributed residuals, and a smaller residual error than the log-based method for either endotherms or ectotherms (R^2 = 0.9996, P = 6.36e-32) (Figure 1B).

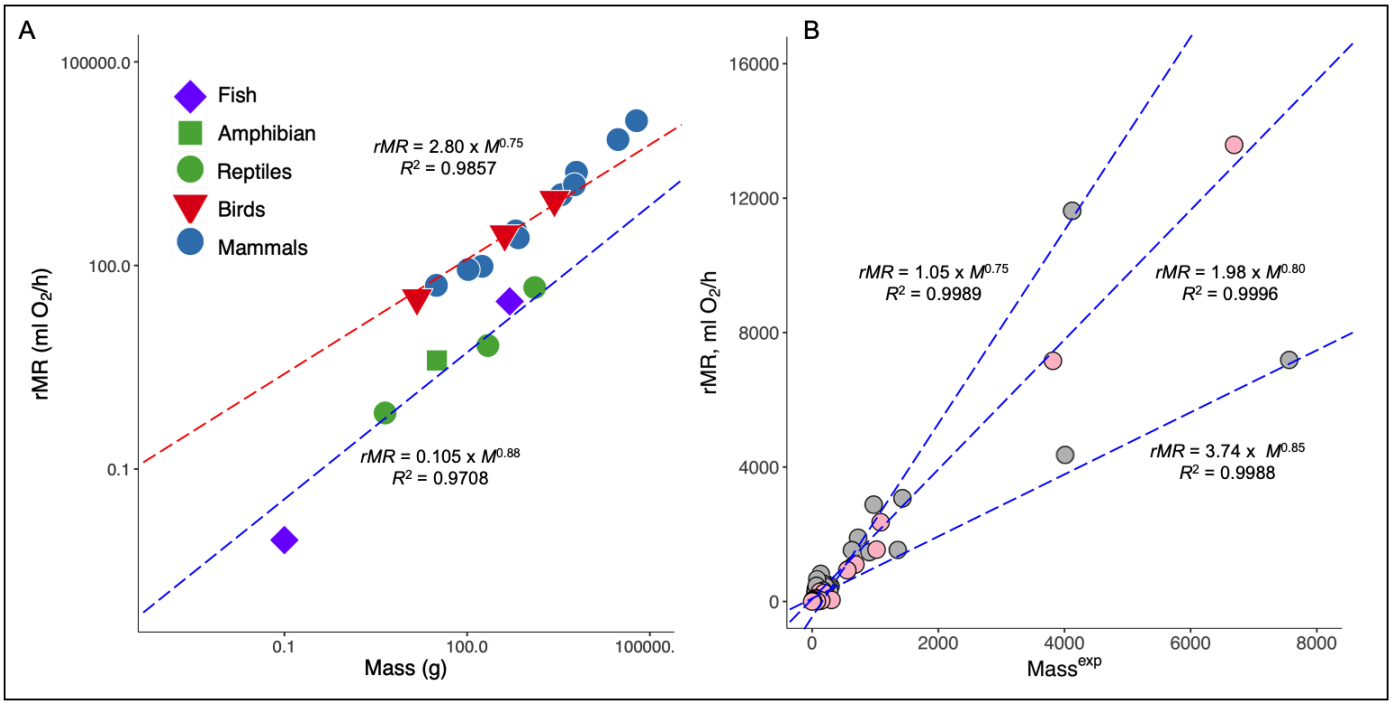

Figure 2. Comparison of the allometric scaling of the *sMR*s of 20 vertebrates by (A) the conventional power law method, or (B) maximum likelihood scaling according to the optimal fit ($M^{0.80}$: pink circles) and two suboptimal fits ($M^{0.75}$, $M^{0.85}$: gray circles).

As the basis for modeling *sMR*, variable allometric scaling across the arithmetic range of *rMR* is more sensitive to the ratio of surface area/mass than the log-based method. For example, using log scaling, there was no significant difference between the slopes of log *rMR*/log *M* among three groups in this study (ectotherms, earlier-branching mammals, and later-branching mammals (Boreoeutheria): 0.88, 0.5 and 0.74, respectively; P = 0.2164) or between the two groups of mammals (P = 0.9064, ANCOVA). However, the optimal slopes of these groups with respect to *miRNA.Fam* ($rMR/M^{0.88}$, $rMR/M^{0.78}$ and

rMR/$M^{0.70}$, respectively) were significantly different: P = 6.27e-07 for all three groups, and P = 2.96e-04 between the two mammalian groups (ANCOVA) (Supplementary Table S2; Supplementary Figure S2).

Most importantly, even though the relationship of *rMR* to M^{exp} is heteroscedastic (Figure 2B), when the scaling of M^{exp} is optimized in order to match the distribution of the ratio *rMR*/M^{exp} to that of a third covariate, such as such as *miRNA.Fam*, the variation is homoscedastic (Figure 3 and Supplementary Figure S3), and therefore suitable for comparing the *sMR* dependence on covariates in species ranging in size over several orders of magnitude (Benson et al, 2022).

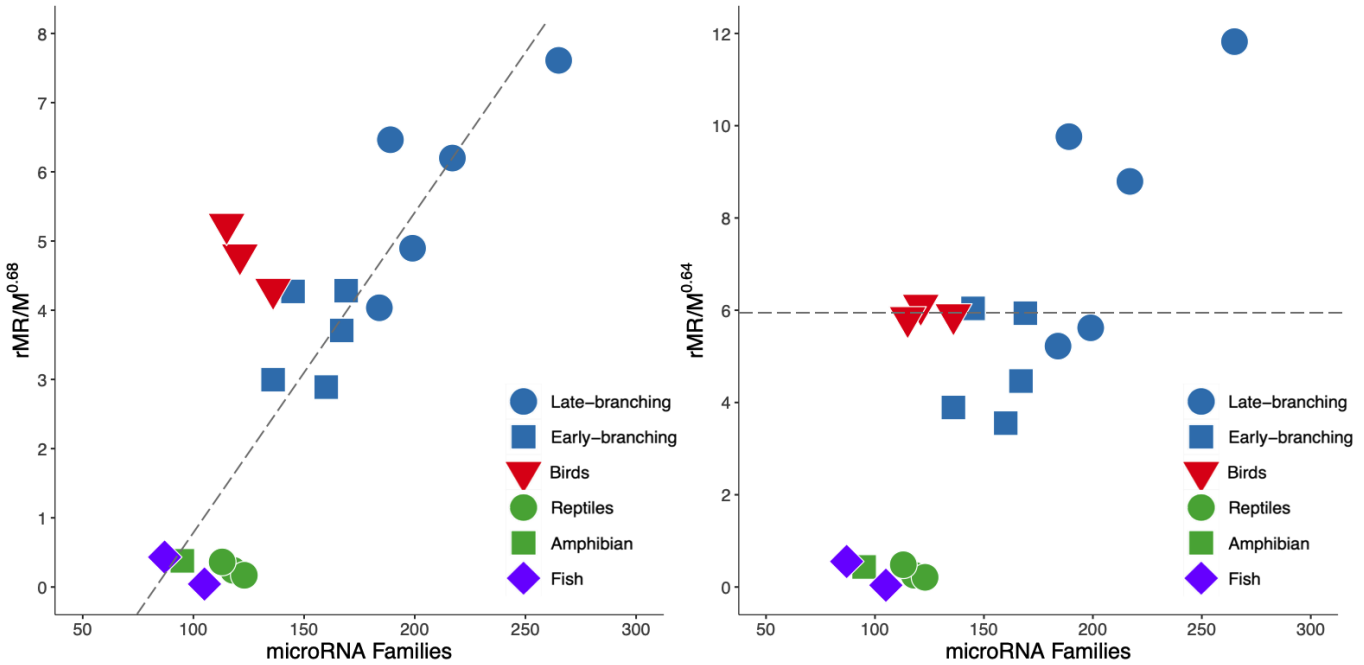

Figure 3. Phylogenetic regression of *sMR.68* (*rMR*/$M^{0.68}$) on *miRNA.Fam*.

A. The number of microRNA families in non-avian tetrapods accounted for 84% of the variation in *sMR*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).

Tetrapod sMRs are correlated with microRNA families independent of phylogenetic distance

Phylogenetic linear regression (assuming uniform rates of evolution) indicated that the distribution of *miRNA.Fam* across 14 non-avian tetrapods accounted for 84% of the variation in optimally scaled *sMR* (*rMR*/$M^{0.68}$, P = 2.26e-06) (Figure 3A), independent of phylogenetic distance (Pagel's *lambda* = 0.00). The variation with respect to the number of microRNA genes was weaker and secondary to phylogeny (Pagel's *lambda* = 1.0). Similar results were obtained when only mammals were considered, with a shift in the optimal allometric exponent from 0.68 to 0.71 (Supplementary Table S2).

The previous analysis excluded the three bird species in our sample (chicken and two flying birds) since the absolute number of avian microRNA families decreases in phylogenetic order (Figure 1A) (Supplementary Table S1). In large datasets, the log-based allometric exponent for *rMR* in birds is markedly lower than that found in mammals, *e.g.* $M^{0.64}$ *versus* $M^{0.73}$ (Fristoe et al, 2015; Ballesteros et al, 2018). When scaled to $M^{0.64}$, the slope of the regression of the bird *sMRs* on the number of miRNA families was 0.00 (Figure 3B), and turned positive for any allometric exponent < 0.64. That is, when scaled with an allometric exponent < 0.64, bird *sMR*s do vary positively with *miRNA.Fam*. Thus, as in the case of mammals, the optimal allometric scaling of *sMR* with respect to *miRNA.Fam* in birds may be displaced toward a greater dependence on surface area *i.e.* lower allometric exponent, relative to the slope of log *rMR*/log mass.

The evolution of cellular MR in relation to miRNA.Fam **conforms to the 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 *sMRs* diverge as a function of phylogenetic distance, *versus* an Ornstein-Uhlenbeck (OU) process, according to which *sMR*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 applied two complementary methods in order to test these models: first, we compared how well each process fit the relationship of *sMR* 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 *sMR* values with those generated by BM and OU simulations that included an adaptive shift at different stages of mammalian evolution.

For any *sMR* scaling in the range $M^{0.66}$ - $M^{0.80}$, the BM model for the evolution of *sMR* with respect to *miRNA.Fam* was inferior to the OU model (Figure 4A): at $M^{0.75}$, the ∆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 S3). The inferred rate of adaptation of *sMR* 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 4B). Specific *MR*s exhibited a similar ∆AICc optimum with respect to the number of microRNA genes (*mirGen*), albeit with smaller differences in the ∆AICc between the OU and BM models (Supplementary Figure S5). However no such inflection was observed in models of *sMR* variation in relation to genome size (*Gnm*) or number of coding genes (*Cdg*), and AICc values declined monotonically (Supplementary Figure S4).

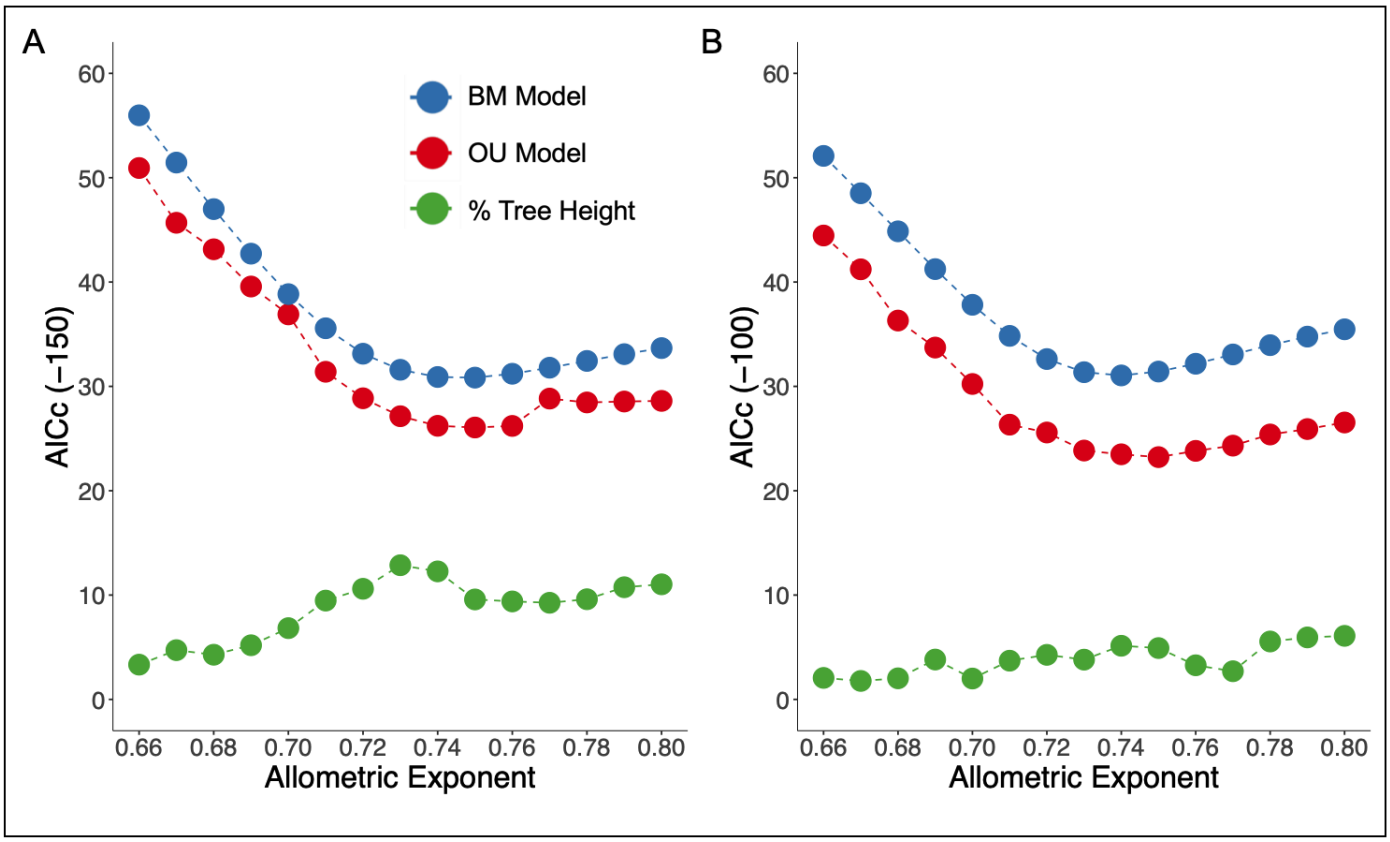

Figure 4. Comparison of BM and OU models for the variation of s*MR* with respect to *miRNA.Fam* across 14 tetrapods (A) or 10 mammals (B). Curves represent the corrected values of the Akaike information criterion (AICc) for each model, and the rate of adaptation (% tree height) for the OU models. Note the difference in range of the ordinate axes.

In order to compare models incorporating an adaptive shift, we simulated the evolution of *sMR* with respect to *miRNA.Fam* in 14 tetrapods by either a BM or OU process, and assuming two adaptive shifts: the first corresponding to the divergence of mammals, and the second shift corresponding to the divergence of either Placentalia, Boreoeutheria, or Euarchontoglires (Figure 5A) (Bastide et al, 2018).

OU and BM models simulated the biphasic evolution of *sMR* in mammals most precisely when this was scaled to $M^{0.75}$ (Supplementary Figure S5). An OU model with an adaptive shift at the divergence of Boreoeutheria accounted for 95% of the variation in *sMR.75* (P = 4.10e-09), compared to 88% for the corresponding BM model (Figure 5B). 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 5C).

In sum, for both OU and BM models of the variation in *sMR* 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 *sMR* scaling in BM and OU models of *sMR* dependence on *miRNA.Fam*

corresponds to the ⅔ power law model that fits avian resting metabolic rates (Fristoe et al, 2015; Ballesteros et al, 2018).

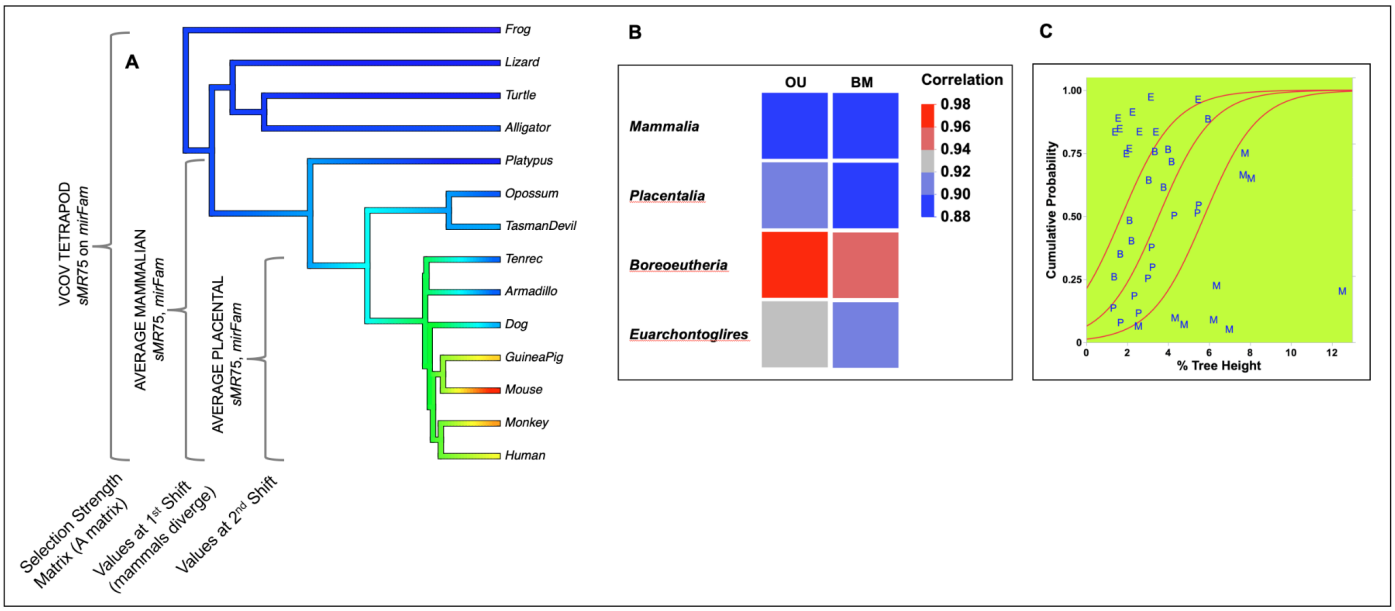

Figure 5. Design and analysis of simulated adaptive shifts in the evolution of *sMR* in relation to the predictor *miRNA.Fam.*

A. The variance/covariance matrix for the regression of *sMR.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 *sMR.75* values scaled to the original, 36-fold range. B. Correlations with the actual *sMR.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 acceleration in the rates of evolution of *sMR.75, miRNA.Fam and body temperature.*

Support for the adaptive shift model is based on the apparent jump in number of microRNA families between two species of Atlantogenata (Armadillo: 169; Tenrec: 160) and two species of Laurasiatheria (Dog: 189; Cow: 212), with parallel increases in cellular *MR*s and body temperatures (Supplementary Table S1). We anticipated a corresponding jump in the branch-wise rates of evolution of *miRNA.Fam* and *sMR.75*, in excess of any change in the rate of evolution of mass (Baker et al, 2015). We generated models using the Bayesian variable rates algorithm (Venditti et al, 2011; Baker et al, 2016) in order to estimate the relative rates of evolution of these variables (Figure 6). The variable rate trees representing *sMR.75*, *miRNA.Fam*, log *M* were strongly supported (Bayes factor > 5) based on

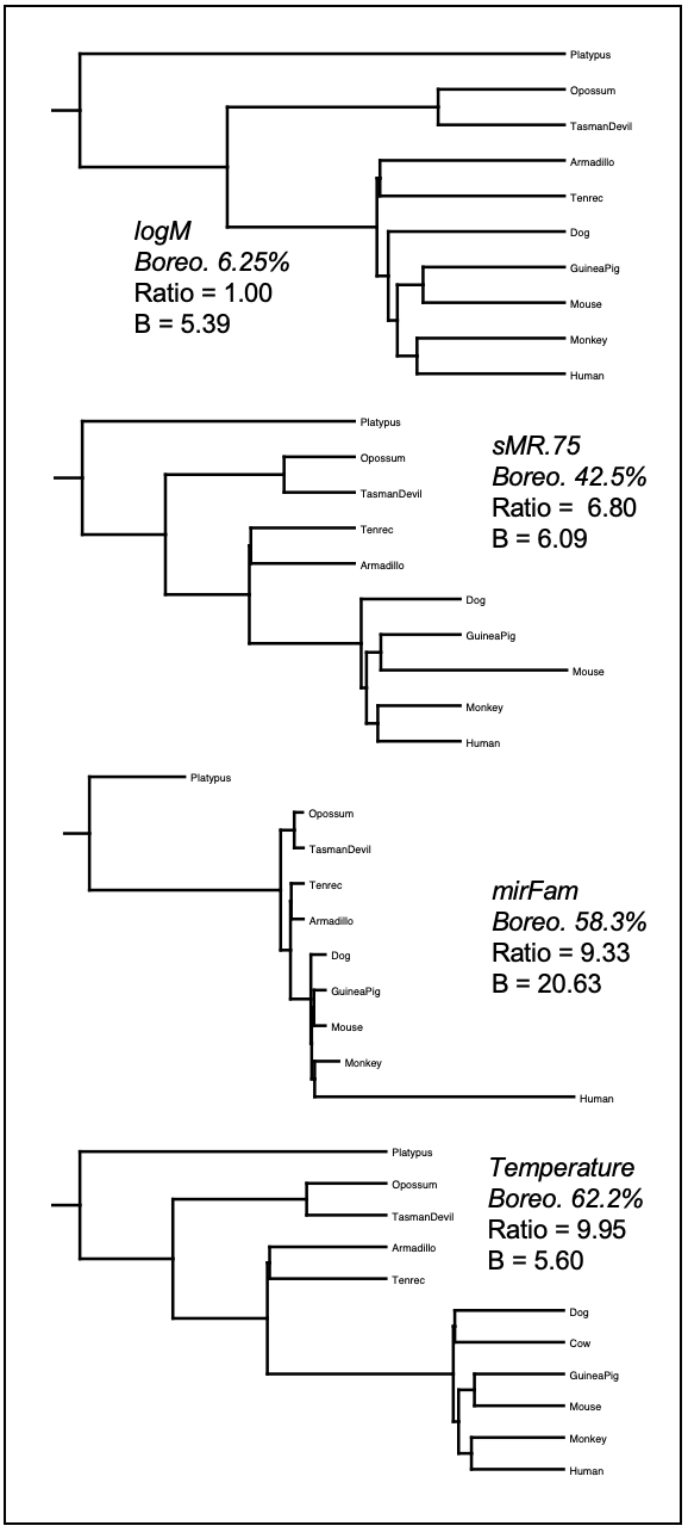

Figure 6. The ultrametric phylogeny of mammals was rescaled according to the rate of evolution of each of four traits.

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 these 100 trees with the median log likelihoods of the trees with the smallest sum of changes of scale, *i.e.* trees that differed the least from the constant rate model. The cow was included in the variable rate tree for temperature in order to to be able to infer the temperatures at the internal nodes (below).

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: (Raftery, 1996; Baker et al, 2016). In the variable rate trees for *sMR.75*, *miRNA.Fam* and T_b, the branch lengths representing the descent of primates from theria were 99% correlated (Supplementary Table S4), and the relative length of the branch separating early-branching (Atlantogenata) from late-branching placentals (Boreoeutheria) corresponded to 8- to 10-fold increases in the rate of trait evolution compared to the that in the variable rate tree for mass (Figure 6).

Our hypothesis 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). We adopted two complementary approaches in order to determine whether the dependence of *sMR.75* on *miRNA.Fam* was secondary to body size or body temperature. Conventional (non-phylogenetic) analysis of covariance revealed that the dependence of *sMR.75* on *miRNA.Fam* (adjusted R^2 = 0.6232, P = 4.03e-03) was still significant after accounting for its dependence on size (log mass) (P = 2.67e-02), but not when controlling for body temperature (P = 0.2133). We also compared Bayesian models for the dependence of *sMR.75* on *miRNA.Fam* using the best 100/10,000 variable rate trees for *sMR.75*. When mass was included in the model, the overall relationship strength (R^2 = 0.63) was unchanged but the regression coefficient for mass was negative in the majority of the models and the corresponding Bayes factor (Variable rate model likelihood - Constant rate model likelihood) was lower. On the other hand, when the model included body temperature, the majority of models had a positive regression coefficient for temperature and the overall fit improved (R^2 = 0.67), as did the Bayes factor (Supplementary Table S5).

Given that in large mammalian trees, increases in mammalian basal *MR* were not generally associated with increases in T_b, and that T_b decreased in more branches than increased (Avaria-Llautureo et al (2019), we considered the possiblity that the difference in T_b between the two early-branching (average T_b = 33.25°C) and six late-branching placentals (average T_b = 37.75°C) reflected opposite trajectories from a common, warmer-bodied ancestor. Uing the 100 (1%) best variable rate trees for body temperature, Bayesian reconstruction of the temperatures at the ancestral nodes did not detect cooling of body temperature in any descendent clade. Instead, this difference could be attributed to a jump in body temperature in the most recent common ancestor of Boreoeutheria (Figure 7).

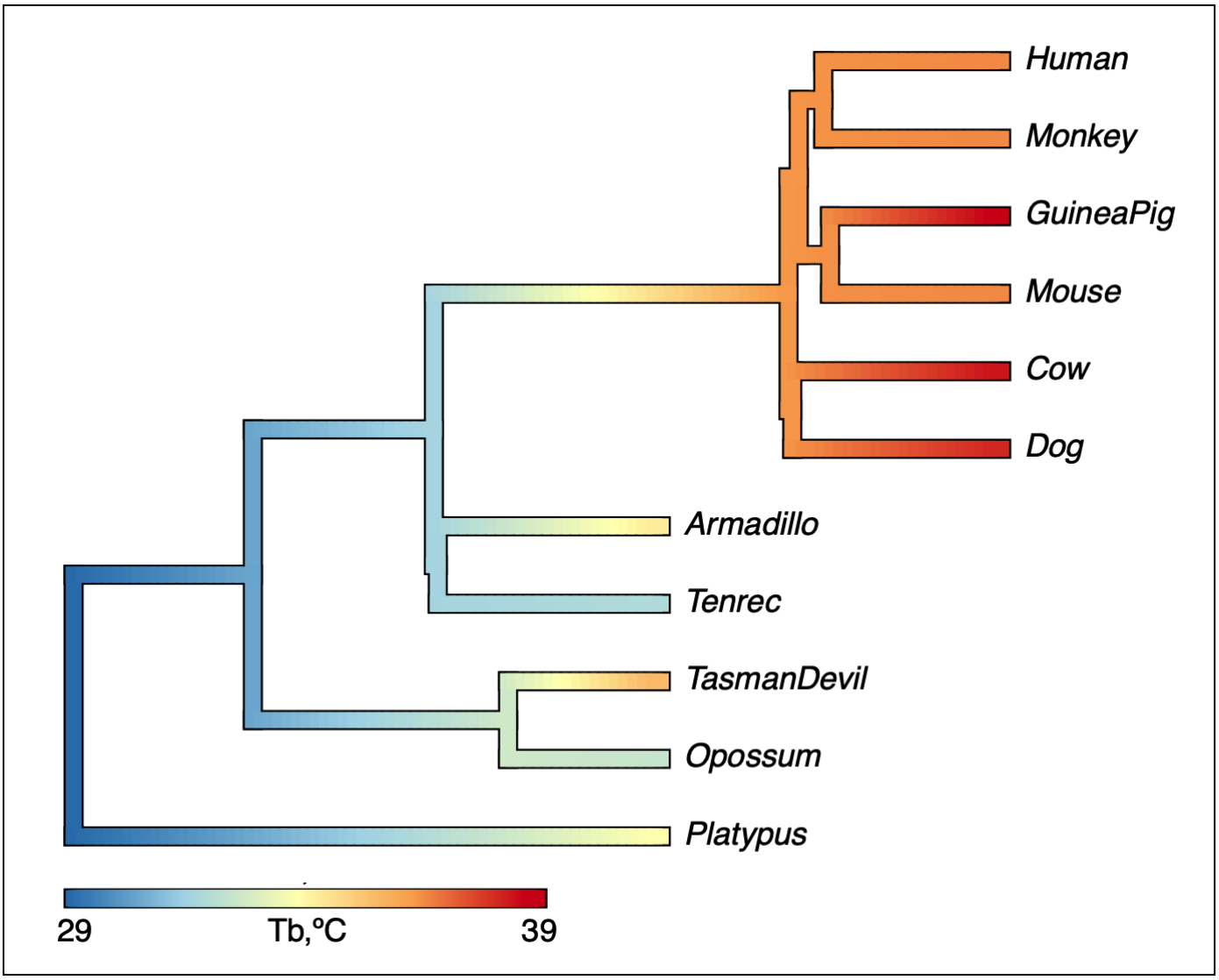

Figure 7. Bayesian reconstruction of ancestral body temperatures. The reference phylogeny was rescaled according to the rates of T_b evolution and ancestral node values were inferred in a Brownian motion context after fixing of the optimal ancestral mammalian T_b (29°C). The values at each node represent the median estimates for the 64/6,400 trees with the greatest likelihood (Brownian motion variance = 0.26°C).

Our hypothesis 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). We adopted two complementary approaches to determine whether the dependence of *sMR.75* on *miRNA.Fam* was secondary to body size or body temperature. Conventional (non-phylogenetic) analysis of covariance revealed that the dependence of *sMR.75* on *miRNA.Fam* (adjusted R^2 = 0.6232, P = 4.03e-03) was still significant after accounting for its dependence on size (log mass) (P = 2.67e-02), but not when controlling for body temperature (P = 0.2133). We also compared Bayesian models for the dependence of *sMR.75* on *miRNA.Fam* using the best 100/10,000 variable rate trees for *sMR.75*. When mass was included in the model, the overall relationship strength (R^2 = 0.63) was unchanged but the regression

coefficient for mass was negative in the majority of the models and the corresponding Bayes factor (Variable rate model likelihood - Constant rate model likelihood) was lower. On the other hand, when the model included body temperature, the majority of models had a positive regression coefficient for temperature and the overall fit improved (R^2 = 0.67), as did the Bayes factor (Supplementary Table S5).

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 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 S6). 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 such a discovery would not shed light on the central question of this study, the nature of the broad correlation across mammals between cellular metabolic rate, the size of the microRNA repertoire, and the predicted number of microRNA-target interactions.

Our hypothesis assumes that global repression of translation by the combinatorial activity of microRNAs is proportional to the number of microRNA-target interactions and their sensitivity to repression. Since the TargetScan database lacks data from Atlantogenata, we could only compare late-branching 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 (Supplementary Tables S7), we evaluated the following model to predict the cellular energy saving from the total number of 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 18% of *sMR*, we predicted that the ~ 30% increase in *sMR.*75 between marsupials and late-branching placental mammals would be associated with an increase in overall microRNA-mediated repression of translation of about 167%. Figure 8 depicts the ratios of the value for each factor in the model to the corresponding value in the opossum. Compared 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 7). 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 distribution of target proteins 500 - 5,000 AA in length, the predicted differences in overall translation cost were 180%, 192% and 269% (Figure 8). Since the C_n (combinatorial repression) strengths, which are based on the most effective microRNA from each family, are likely to be overestimates, these results are in reasonable agreement with our prediction (Agarwal et al, 2015; McGeary et al, 2019).

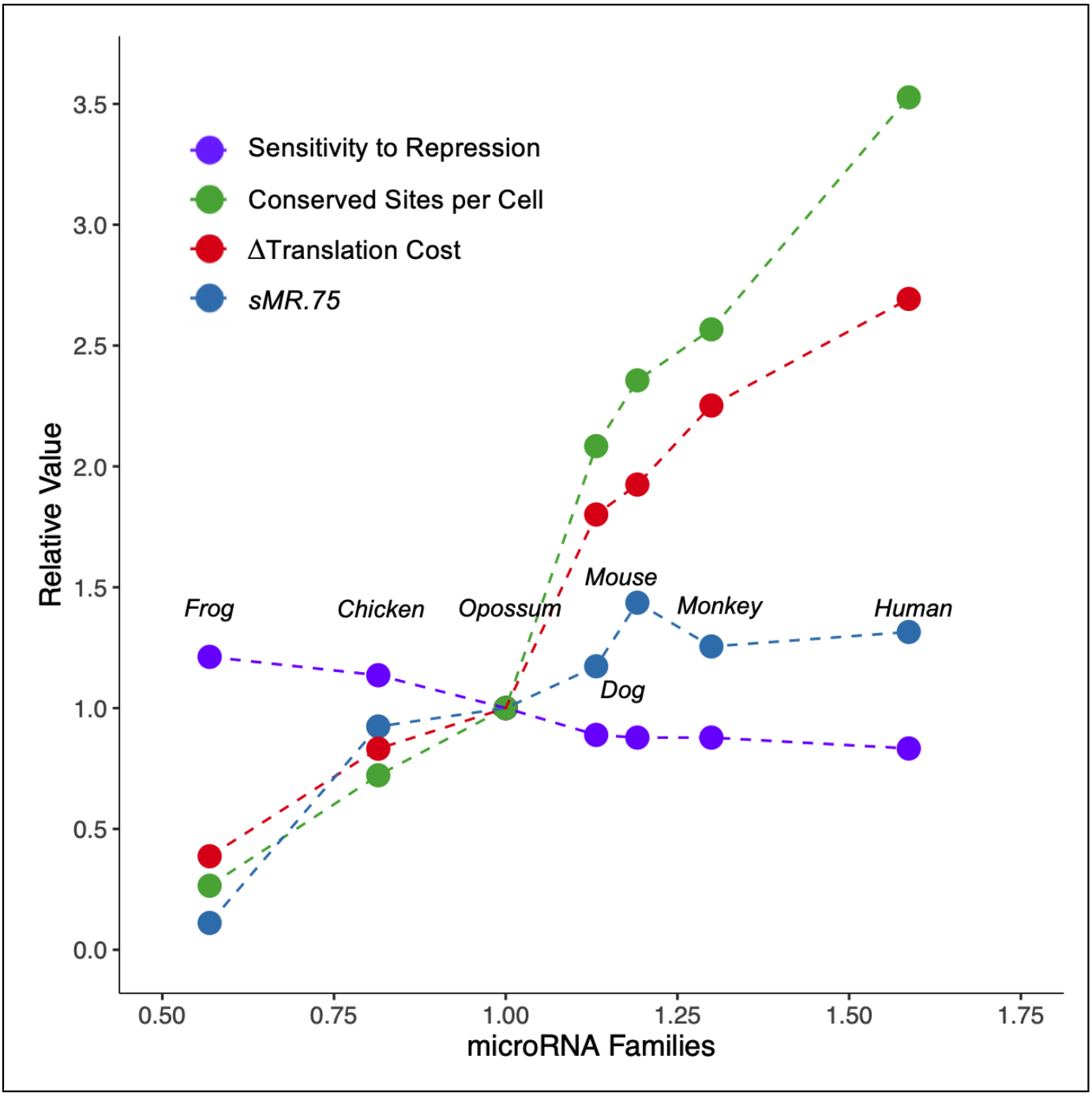

Figure 8. Evolution of factors in a model for energy cost saving by global microRNA activity.

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 directly analyse the dependence of *sMR* on microRNA complement size, complementary to the 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 M^{exp} made it possible to detect differences in the surface area dependence of *sMR*-covariate relationships even between small groups of animals. The method itself, fitting the distribution of the rMR/M^{exp} ratio to that of 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 novel bioinformatics tools (https://github.com/sinanugur/MirMachine).

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 S7). 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, 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 near to ambient (Clarke et al, 2010; Supplementary Figure S4), 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: 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), two different 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 *sMR* 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 *sMR* on *miRNA.Fam* across tetrapods or mammals were $M^{0.68}$ and $M^{0.71}$, respectively (Supplementary Table S2). However, the AICc information criteria for both BM and OU models reached their minimal values when *sMR* was scaled to $M^{0.75}$, where BM and OU processes most precisely simulated the biphasic evolution of *sMR* (Supplementary Figure S5). This example illustrates an aspect of the allometric scaling of *MR* that is generally overlooked when comparing allometric exponents obtained by the power law method, namely that differences in allometric slopes generally represent shifts in the rank order of the ratios between *sMR* and a third covariate. For example, in the sample of 10 mammals included in this study, the dog has the highest ratio of *sMR*/*miRNA.Fam* when *sMR* is scaled to $M^{0.70}$, third highest at $M^{0.75}$ and fourth lowest at $M^{0.80}$.

Three-quarter power scaling corresponds closely to the conserved slope of log *rMR*/log mass reported for mammals in countless reports since Kleiber (1932), Brody and Proctor (1932) (see Introduction for additional references). This observation suggests that the relationship between *sMR* and the microRNA complement evolved under constraints similar to those governing the highly conserved relationship between *MR* and mass, and supports the assumption that *miRNA.Fam* is a useful proxy for global microRNA activity. Furthermore, the concurrent increases in the branch-wise rates of evolution of *sMR*, *miRNA.Fam* and T_b support the notion that these relationships have been maintained by selection, and thus represent manifestations of a multivariate selection on *rMR* and *M* (White et al, 2019).

Analogous models with respect to microRNA genes exhibited the same $M^{0.75}$ optimum and a similar rate of adaptation for the OU process, but no optimum was observed for *sMR* variation in relation to genome size or the number of coding genes (Supplementary Figure S5). Thus, *sMR* variation appears to be specifically linked to the number of microRNA families. Given that the mechanism by which microRNAs conserve metabolic energy is well established, the assocation with *sMR.75* most likely reflects the metabolic impact of global microRNA activity.

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

The advent of placentation allowed some orders to exploit high quality resources through an increase in energy expenditure and reproductive output, adaptations denied to monotremes and marsupials with access to the same resources (McNab, 2008). Among Boreoeutheria some clades, like Eulipotyphla and Chiroptera, retained the lower body temperatures (≤ 36°C) characteristic of early-branching placentals (Xenarthra and Afrotheria), while others evolved higher T_b's, such as the Carnivora, Artiodactyla, primates and most rodents (Supplementary Figure S6). This divergence in body temperature 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 (Ruf and Geiser, 2015; Fjelldahl et al, 2022). After accounting for mass, climate and geography, early-branching orders exhibit more variable T_b (Boyles et al, 2013), a higher incidence of heterothermy (Ruf and Geiser, 2015), a lower proportion of skeletal muscle mass, and lower metabolic rates (McNab, 2019), compared to the majority of late-branching placental mammals, as well as birds, where T_b > 36°C and permanent homeothermy predominate .

Optimally scaled MRs may also predict the microRNA complement in birds

In linear phylogenetic regressions, the optimal *sMR* scaling with respect to *miRNA.Fam* in tetrapods ($rMR/M^{0.68}$) or mammals ($rMR/M^{0.71}$) represents a higher than average *MR* per unit mass or volume, as have been predicted for field metabolic rates (Speakman and Król, 2010) and a thermoregulatory core (Scholander et al, 1950; Porter and Kearney, 2009). The greater surface area dependence of *sMR* with

respect to *miRNA.Fam* 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 were significantly related to *miRNA.Fam*, 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 in the optimal scaling of *sMR* scaling in birds, where *sMR*s are positively related to *miRNA.Fam* only when the allometric exponent is lower than the exponent (0.64) reported for the overall *rMR* of birds (Fristoe et al, 2015).

Furthermore, 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 *c.* 2 Gb to *c.* 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, 2014a; 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 in birds.

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 et al, 2017). For example, shorter introns enable faster mRNA splicing (Heyn et al, 2014), while compact genomes and nuclei, if found in smaller cells with higher ratios of surface area/mass, could 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 very similar to 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 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 S7). Therefore we considered only the conserved targets of these reference microRNA families (see Methods).

In order to estimate target site sensitivity to combinatorial microRNA binding in each species, we estimated C_n, the cumulative combinatorial repression per site, 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 was 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 overall s*MR*.75. While the majority of mammalian-specific target sites appear to be evolving neutrally (Simkin et al, 2020), an increase in the number of such interactions, even if they are phenotypically silent, may have provided an immediate energy-saving benefit.

A model for the evolution of cellular MR in relation to the number of microRNA families

The demonstration that the *sMR* 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, corresponding to the present range of species in MirGeneDB (Fromm et al, 2021). Figure 9 illustrates estimated temperature gradients (similar to those of Avaria-Llautureo et al, 2019) and optimal linear phylogenetic regression of *sMR* on *miRNA.Fam* in five earlier- and five later-branching mammals (Supplementary Table S2). A separate optimum is shown for birds, corresponding to the clustering of bird T_b's around 39.8°C (2.66% coefficient of variation, n = 211) and an optimal allometric exponent shifted below the slope (0.64) of log *rMR*/log mass in birds (Fristoe et al, 2015). A scale of Carnot efficiency is provided, assuming an ambient temperature of 20°C. For example, there is a theoretical difference of approximately 1% in thermodynamic efficiency between early- and late-branching mammals, potentially offsetting some of the cost of sustaining higher body temperatures.

The inference of distinct adaptive peaks is based on the currently available mammalian microRNA repertoires in MirGeneDB, which represent 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, may eventually bridge these hypothetical optima, include examples of decreases in body temperature (Avaria-Llautureo et al, 2019), and better conform to a gradual model of genetic drift (Pagel et al, 2022).

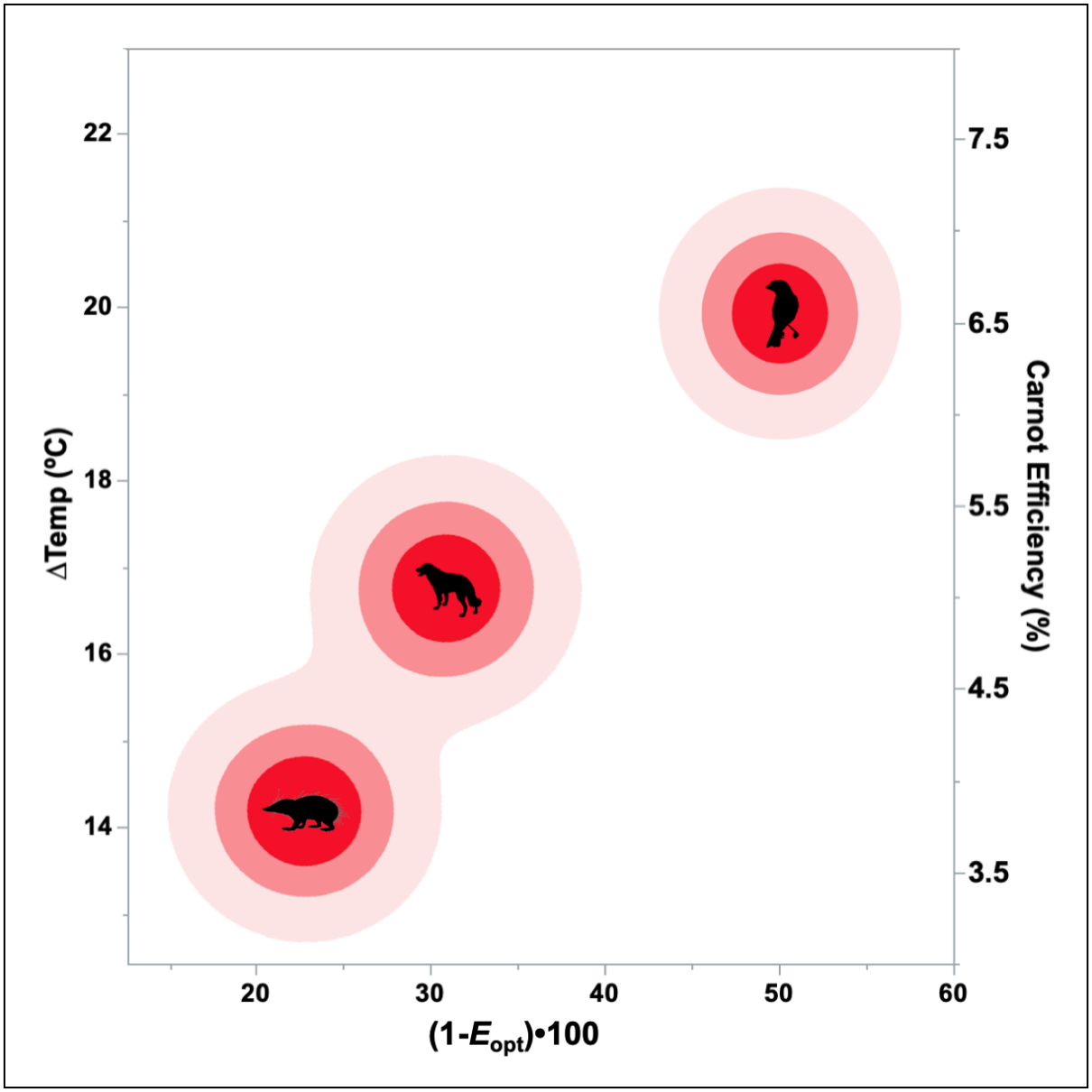

Figure 9. Contour plot of a hypothetical fitness landscape for optimal *sMR* scaling with respect to *miRNA.Fam versus* a core-surface temperature gradient.
E_{opt} **:** optimal allometric exponent. Δ**Temp** = T_b - 20°C. Silhouettes represent early-branching and late-branching placental mammals and birds (Phylophic © 2023: Tenrec, Yan Wong; Dog, Tracy A. Heath; Bird, T. Michael Keesey).

On the other hand, 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 (Avaria-Llautureo et al, 2019), consistent with an adaptive barrier to the emergence of higher T_b's. 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 (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. In the mechanism proposed by Cassidy et al (2019), the effectiveness of transient signalling by a transcription factors 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 analogy, in addition to maintaining metabolic efficiency by limiting protein overproduction, increased repression by microRNAs may have been necessary to maintain the orderly unfolding of developmental programs in warmer, late-branching mammals. Bats are mammals that alternate between activity with thermoregulation to maintain homoeothermy or torpor, a state in which they are essentially ectotherms (Fjelldahl et al, 2022). According to the mechanism proposed above, signaling that is microRNA-dependent during homeothermy may be disrupted in the transition to torpor unless there is a temperature-related decrease in global microRNA activity, which is normally limited by the rate of Ago2-loading (Gebert and MacRae, 2019; Reichhof 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.* Legendre and Davesne, 2020), rather than on the mechanism of thermostatic control (Bernhardt et al, 2020). In this context, the emergence of permanent homeothermy among later-branching mammals can be understood as the endpoint of 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 reserves among somatic maintenance, reproductive reserves, and parental care (Koteja, 2000; Maino et al, 2014; Swanson et al, 2017; Bernhardt et al, 2020). Analysis of the relationships among *sMR*, *miRNA.Fam* and T_b in mammals yielded evidence consistent with multivariate selection for stable and efficient thermoregulation. First, relative to the evolution of mass, all three traits exhibited ~10-fold increases in evolutionary rate between the divergence of Atlantogenata and Boreoeutheria, independent 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).

Second, we found a downward shift, relative to $M^{0.75}$, in the optimal scaling of *sMR* with respect to *miRNA.Fam* in all 10 mammals ($M^{0.71}$) or five late-branching mammals (Boreoeutheria) ($M^{0.70}$), but not in five earlier-branching mammals (including Atlantogenata) ($M^{0.78}$) (Supplementary Table S2). Speakman

and Król (2010) have argued that evolutionary fitness must be more sensitive to field metabolic rate (*fMR*), *i.e.* the realized rate of energy expenditure over a period of days, than to basal metabolic rate. Controlling for phylogeny, they found that the slope of the phylogenetic regression for *fMR* across 123 mammals was $M^{0.68}$, significantly lower than $M^{0.75}$, and similar to the allometric scaling predicted by their model of maximal heat dissipation (Speakman and Król, 2010). By extension, the downward shift in Boreoeutheria in the surface area/volume dependence of *sMR* with respect to *miRNA.Fam* suggests a an association with thermal tolerance and evolutionary fitness.

Third, a lower than average exponent for the allometric scaling of *MR i.e.* rMR/M^{exp}, represents a higher than average, or "amplified" *sMR*. Any effective system for homeostatic control depends on amplification of the difference between output and setpoint (Singh, 2011; del Vecchio et al, 2016), in this case $rMR/M^{0.75}$. Thus, the fact that the distribution of *miRNA.Fam* matches the distribution of amplified, rather than setpoint *sMR*s, is consistent in theory with an adaptation for temperature homeostasis.

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).

Relatively little attention has been paid to the nature of global microRNA activity (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). 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. The evolution of cellular *MR* in mammals has been characterized by rapid adaptation in relation to the number of microRNA families which, in turn, has tracked the evolution of body temperature. Whatever the ultimate cause of the steady acquisition of microRNA families in vertebrates (Peterson et al, 2021), these observations support a role for global microRNA activity in maintaining metabolic efficiency, potentially facilitating an adaptive shift among late-branching placental mammals 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.

METHODS

microRNA Families, Genes and Targets, and other genomic variables

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 are those shared by two or more species: the species used herein are phylogenetically distinct enough to make this definition valid and workable. The number of conserved mammalian mRNA targets was extracted from the TargetScan8.0 database (Agarwal et al, 2015; McGeary et al, 2019). Values for the other genetic variables (genome size and number of coding genes) were obtained from the NCBI genome assembly database (as of March, 2022).

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). A value of 1.0 indicates that the observed variation in phenotype conforms to a Brownian motion model of trait evolution, mirroring phylogenetic distance, while a value of 0.0 implies phylogeny-independent trait evolution, equivalent to a star phylogeny (Felsenstein, 1985; Pagel, 1999; Revell, 2010). All R^2 values were adjusted for the number of predictors.

Multivariate correlations, ANCOVA and ordinal logistic regression were performed in JMP®16. For analysis of the fold variation by quintile in cellular *MR*, Chiroptera and 21 outliers (*sMR*68, Cauchy robust fit) were removed from the original dataset of Clarke et al (2010), leaving 532 species. Like other ruminants, the cow was considered an outlier with respect to *MR* (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 optimal *sMR* 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 S3).

The Phylogenetic EM package (Bastide et al, 2018) was used to simulate *sMR* and *miRNA.Fam* distributions using variance/covariance matrix for *sMR.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 *sMR.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) (see Figure 5A). Ten simulations were generated and averaged for each shift or pair of shifts.

Bayesian inference of the branch-wise rates of evolution

For each individual 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 mass, *sMR.75*, *miRNA.Fam* or T_b (Venditti et al, 2011). Bayes factors were obtained by comparing the 100 trees with the highest log likelihoods and the 100 trees with the smallest non-zero sum of scale changes. 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 tree for each trait.

In order to evaluate the dependence of *sMR.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). Regression models using 100/10,000 most likely variable rate trees were compared with models using the 100/10,000 trees with the smallest non-zero sum of scale changes.

Using an 11-mammal tree (including cow), after optimizing the value (29°C) for the most recent common mammalian ancestor, body temperatures at the internal nodes were inferred from the directional variation model run for 8.0 million iterations with 20% burnin and sampled every 1000 trees.

Characterization of predicted interactions between conserved microRNAs and conserved 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 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 target proteins between 500 - 5,000 amino acids long. The relative 'AA' cost was obtained from the weighted sum of nine 500 AA-length bins between 500 - 5,000 AA (Supplementary Table S7).

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