Trends in Ecology & Evolution

What ancient DNA can tell us about Holocene fauna

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Review

Using new tools to solve an old problem: the evolution of endothermy in vertebrates

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During the past 30 years, the evolution of endothermy has been a topic of keen interest to palaeontologists and evolutionary physiologists. While palaeontologists have found abundant Permian and Triassic fossils, suggesting important clues regarding the timing of origin of endothermy, physiologists have proposed several plausible hypotheses of how the metabolic elevation leading to endothermy could have occurred. More recently, molecular biologists have developed powerful tools to infer past adaptive processes, and gene expression mechanisms that describe the organization of genomes into phenotypes. Here, we argue that the evolution of endothermy could now be elucidated based on a joint, and perhaps unprecedented, effort of researchers from the fields of genomics, physiology and evolution.

What is endothermy?

Many multicellular organisms produce and store heat in their bodies in such a way that they can maintain their body temperature above 30 °C over a broad range of ambient temperatures. This phenomenon, called 'endothermy', has appeared independently in several groups of living organisms, including some plants, insects, fish, amphibians, reptiles, birds and mammals [1–6]. The endothermy that mammals and birds exhibit, however, is distinct from the transient endothermic status that other organisms show because the source of heat is generated continuously at rest rather than by muscular contraction (with the exception of hibernating birds and mammals, see below) in internal organs such as brain, heart, liver, kidneys and gut [7,8]. The origin and evolution of 'visceral endothermy' (hereafter, endothermy) in mammals and birds represents one of the most popular and puzzling topics in evolutionary physiology [9–11]. Endothermy is considered to be one of the causes of the ecological success of birds and mammals, especially with regard to their broad geographic distribution and habitat use [8,12–14]. In fact, having a body temperature higher than the average ambient temperature represents an advantage for thermoregulatory systems because physiological systems are more efficient at heating than cooling bodies [15]. Also, a higher, constant body temperature provides a more efficient and stable environment for enzyme reactions, enables the development of a complex nervous system and, in general, allows

Glossary

Basal metabolic rate (BMR): the VO_2 that is measured at the thermo-neutral zone, in an adult (i.e. non-growing), post-absorptive (i.e. not digesting or absorbing food), non-reproductive (i.e. not expending energy in any kind of courtship behaviour, gestation or lactation) and resting animal. In other words, it is intended that the BMR represents the minimum cost of maintenance in endothermic animals.

Futile cycles: the outcome when two metabolic pathways act at the same time in opposite directions without overall effect other than to dissipate energy in the form of heat.

Leaky membranes: a term applied to the inner mitochondrial membrane of some thermogenic tissues that are comparatively permeable to ions such as Na^+ and K^+ and protons. Although endotherms are characterized by having leaky membranes in their visceral organs, leaky membranes exist in many specialized organs for heat production in several animal and plant species.

Neognathae: according to [79], this clade comprises all living species of birds, except the palaeognaths.

Non-synonymous substitution: a nucleotide substitution of one base for another that changes the encoded amino acid and, hence, the function of the protein.

Oxygen consumption rate (VO_2): the amount of oxygen that an animal removes from the air per unit of time, which is linearly equivalent to the rate of energy expenditure. This is one of the most precise and straightforward measures of aerobic metabolism in animals. Oxygen consumption is obtained from the method of 'indirect calorimetry', which is based on recording the changes in oxygen concentration within a chamber where the animal is located, while controlling the flow of air that passes through the chamber (also known as 'metabolic chamber').

Palaeognathae: according to the last avian phylogeny [79], palaeognaths correspond to the sister group of modern birds (Neognathae), comprising struthioniform (ostriches and casuaries) and tinamiform (quails) birds.

Positive selection: natural selection that promotes the fixation of advantageous mutations. This term is equivalent to molecular adaptation and molecular adaptive evolution.

Purifying selection: natural selection that removes alleles that are deleterious. This term is equivalent to negative selection.

Red skeletal muscles fibres: in contrast with white fibres, which are specialized with muscle anaerobic metabolism, red fibres are mostly associated with aerobic metabolism as they have oxygen-storing capacities (i.e. myoglobin, which gives them their red appearance) more mitochondria and blood vessels. Red muscles are smaller in diameter and contract more slowly than do white fibres.

Synonymous substitution: a nucleotide substitution of one base for another that does not change the encoded amino acid and, hence, maintains the function of the protein.

Thermo-neutral zone (TNZ): in a graph relating VO_2 and ambient temperature of an endotherm, the TNZ represents the region where both variables are independent and body temperature is maintained

at a constant temperature because the rate of metabolic heat production equals or surpasses the rate of heat loss from the body (Figure 1, main text).

Uncoupling proteins: a mitochondrial protein from the inner membrane that dissipates the proton gradient before it can be used to provide the energy for oxidative phosphorylation. They are present in all kinds of animal but are especially important in endotherm vertebrates.

Visceral organs: an operational definition for internal organs other than muscles, bones, connective tissue and blood vessels. In addition to the organs within the thoracic and abdominal cavities, this definition includes the brain (in vertebrates). All these organs produce heat in resting conditions and contribute to the BMR in endotherms.

for a higher reproductive rate, compared with equivalently sized non-endotherms [13,16,17]. Additionally, the high capacity for aerobic metabolism seen in endotherms enables them to sustain high levels of activity, with evident ecological benefits such as increased capability to search for food and to track the appropriate climate conditions (i.e. migration) [14]. However, these long-term benefits contrast with the important short-term costs of energy requirements. In fact, an ectotherm tetrapod, such as a reptile, consumes one-fifth of the food that a mammal of the same size needs in a day [18]. Hence, although endothermy is recognized as an important vertebrate adaptation, the following question arises: what was the target of natural selection that ultimately triggered the increase in aerobic metabolism and body temperature?

Comparative and evolutionary physiologists have long recognized that endotherms differ fundamentally from ectotherms in the shape and direction of the curve relating ambient temperature and rate of metabolism [a quantity usually measured as the amount of oxygen consumed per unit of time, VO_2 (see Glossary); Figure 1]. Whereas in ectotherms, VO_2 increases with increasing ambient temperature, in endotherms this relationship is more complex, because such organisms use metabolic heat production to regulate their body temperature below the zone of thermoneutrality (Figure 1). Under some conditions, VO_2 in the thermoneutral zone is defined as an exclusive feature of endothermic metabolism, commonly known as the basal metabolic rate (BMR; Figure 1). High BMR is considered to be the cause of high body temperature in endotherms and represents an irreducible energy cost of existence [7,9,18]. The unique aspect of avian and mammalian physiology represented by the existence of BMR resides in the fact that the heat that maintains body temperature during the resting state of an animal comes mainly from the visceral organs (heart, kidneys, liver and intestines) [19,20]. These organs have a higher metabolism per gram of tissue compared with other organs and, thus, contribute disproportionately to warming up the body [21,22]. One of the proximal mechanisms that explains this enhanced thermogenic capacity is related to an evolutionary increase in the leakiness of cellular membranes: mitochondrial membranes are leaky to protons and plasma membranes are leaky to Na^+ and K^+ ions and protons [8,20]. In addition, endotherm visceral organs have approximately 2–3 times the mitochondrial membrane volume, and 1.5 times the mitochondrial density of ectotherms [5–8]. In mitochondria, ion gradients across internal membranes are constantly dissipated at a greater rate in endotherms than in
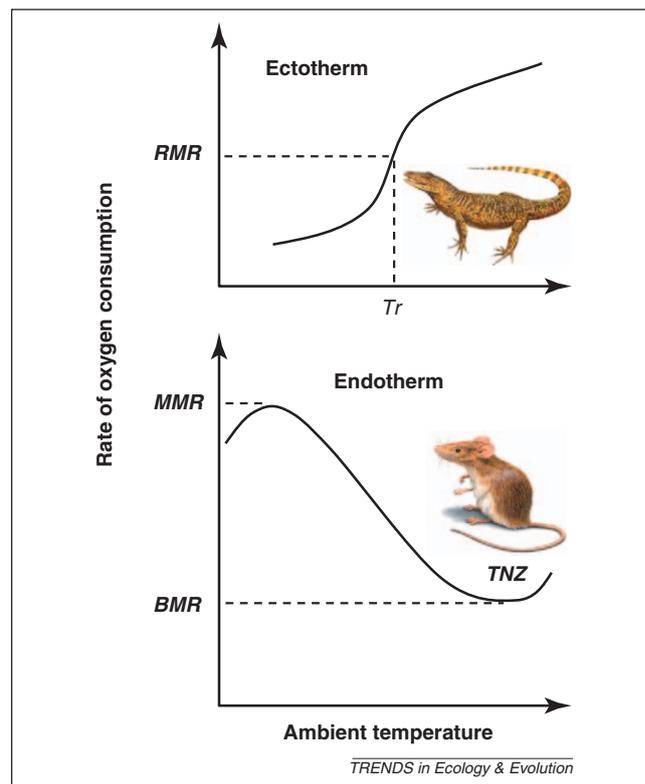

Figure 1. The relationship between rate of oxygen consumption (metabolic rate) and ambient temperature in an ectotherm (**a**) and in an endotherm vertebrate (**b**). In (**a**), RMR is defined as the oxygen consumption measured under resting conditions at routine temperature (Tr; which is why RMR is also known as the 'routine metabolic rate' [17]). When metabolic rate is measured under more precise conditions (i.e. in adults, in the absence of digestion and stress) RMR is also known as standard metabolic rate, a typical variable that characterizes the minimum costs of maintenance in ectotherms. In endotherms, a very precise variable characterizing minimum costs of existence can be defined: BMR. Basal metabolic rate should be measured in the TNZ and under conditions that exclude any energy expenditure other than maintenance costs: activity, digestion, growth or reproductive efforts (e.g. lactating) [80]. In endotherms, all of the former variables are defined, whereas BMR is not defined in ectotherms.

ectotherms. As a consequence, heat is released instead of work, in the same way as in futile cycles [7,8].

Timing of the endothermy evolution

Modern mammals are therapsids, a specialized lineage of synapsids that originated from several lineages of pelycosaurs during the lower Permian, approximately 300 million years ago (mya) [23,24] (Figure 2a). Modern birds, by contrast, diversified from a lineage of theropod dinosaurs approximately 160 mya, during the Jurassic [25] (Figure 2b). Hence, there is a general consensus about the lineage origins of birds and mammals. Less clear is whether endothermy could be associated with the very definition of these groups, which would imply that endothermy originated simultaneously with the appearance of the first birds and mammals. Although physiological processes do not leave much fossil evidence, palaeontologists have identified structures associated either directly or indirectly with endothermic metabolism that do leave fossil evidence; for instance, nasal turbinates and fibrolamellar bone. Nasal turbinates (maxilloturbinates) are convoluted cartilaginous structures projecting from the nasal cavity walls. They are found in most modern amniotes, with
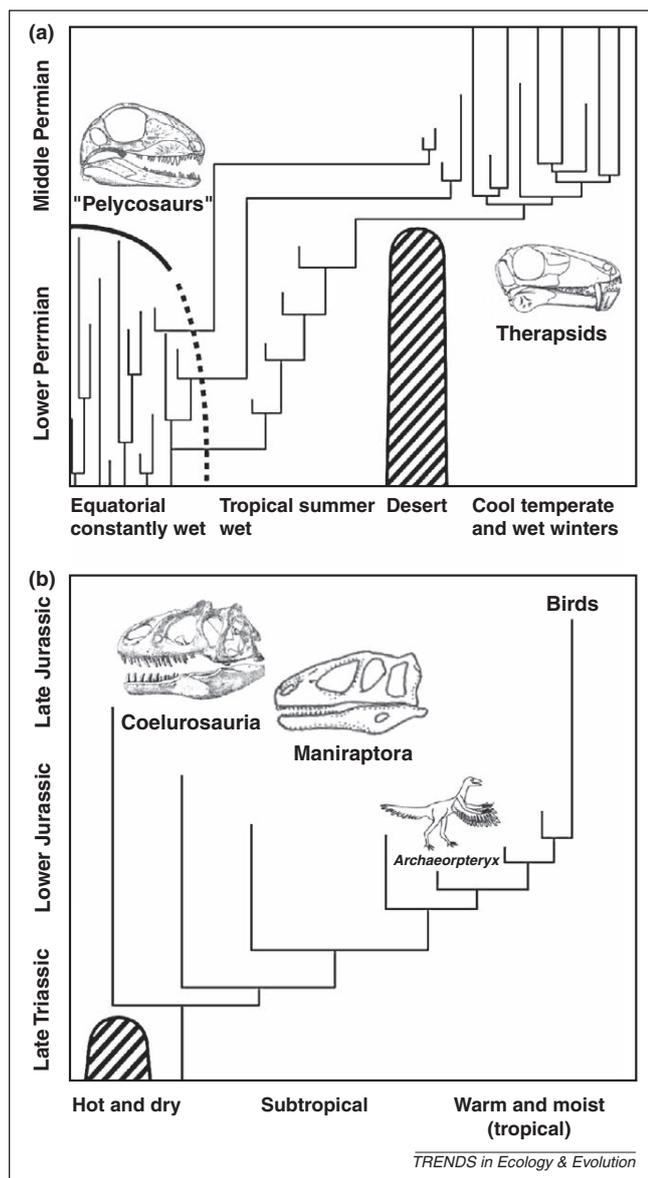
Figure 2. A schematic diagram depicting the evolution of therapsids **(a)** and theropod dinosaurs **(b)** under the climatic scenario of each period. Whereas therapsids had a polyphyletic origin from pelycosaurs during the lower Permian, theropods are a monophyletic clade originating from thecodonts during the Triassic. Modified, with permission, from [4] **(a)**.

Modern reptiles and other ectothermic species characterized by low and interrupted growth rates exhibit lamellar-zonal bones. By contrast, fast-growing animals are characterized by fibrolamellar bone, which presents less dense regions without arrested growth zones, and is highly vascularized [4,5,27]. Another line of evidence for the thermophysiological status of extinct vertebrates is the concentration of O^{18} in the phosphate of teeth, because, in vertebrates, the amount of O^{18} deposited in the teeth is proportional to body temperature [28,29].

Nasal turbinates have been identified early in the synapsid lineage during the Late Permian and Early Triassic periods (250–200 mya) [14]. According to this evidence, it has been suggested that an elevated metabolic rate was already present approximately 30–40 million years before the existence of true mammals [4,14]. Early birds and theropod dinosaurs, however, do not appear to present respiratory turbinates, which led Hillenius and Ruben [14] to conclude that birds acquired endothermy late in the theropod lineage, during the Cretaceous. However, this is a matter of debate, because some authors have claimed, based on crocodilian endothermic features, that endothermy in birds dates back to the Triassic (Box 1), at the origin of the archosaur diversification [10,29,30] (Figure 3).

Proposed adaptive mechanisms: the aerobic capacity model

During the past 30 years, comparative and evolutionary physiologists have developed a range of ideas about the evolution of endothermy and have provided several explanatory models, which were lucidly summarized by Hayes and Garland [31]. These authors proposed a research agenda based on applying evolutionary quantitative genetics [32] and multivariate selection theory [33] to test the aerobic capacity model for the evolution of endothermy in extant species (Box 2).

According to [9], two forms of the aerobic capacity model have been proposed: the 'strong' and the 'weak' forms. The strong form proposes that a positive genetic correlation between resting metabolic rate (RMR) and maximum metabolic rate (MMR) must have been present both in proto-endotherms (i.e. "pelycosaurs" and theropods) and in modern endotherms (i.e. birds and mammals). In other words, 'the positive genetic correlation is an essential feature of the design of all vertebrate lineages that led to endothermy' [9]; see also [11,34]. This form assumes that genetic variances and covariances have persisted over long periods of evolutionary time and, hence, the model can be tested using standard quantitative genetics in present-day endotherms. Therefore, evidence of a negative genetic correlation in any endotherm might falsify the fundamental assumption of the model [9]. By contrast, the weak form of the aerobic capacity model is based on classic genetic models of evolution [35], which predict that the outcome of a strong and constant directional selection is fixation of alleles for the target trait and, consequently, reduced genetic variance and covariance between the target trait (such as MMR) and any other trait (e.g. RMR) [32,36]. Thus, the weak form of the aerobic capacity model predicts that a positive genetic correlation was present in proto-endotherms but 'it was lost in some or all lineages subsequently' [9]. Therefore, according to this

considerable complexity in birds and especially in mammals [14]. Comparative physiologists long ago recognized respiratory turbinates as adaptations against dehydration in animals with high metabolic levels, because they reduce the water content of exhaled air by condensation (because of a lower temperature of the nasal zone, compared with the rest of the body) [17,26]. According to [14], with the exception of specialized endotherms, such as marine mammals, nasal turbinates represent a key piece of evidence for endothermic metabolism, because they leave identifiable marks on fossils.

Bone histology has also been proposed as important evidence for inferring endothermic metabolism [27]. This is because slow-growing bones are characterized by what is known as 'lamellar-zonal' bone: poorly vascularized and dense bone tissue, punctuated by lines of arrested growth.

Box 1. Was the *Archaeopteryx* an endotherm?

According to Hillenius and Ruben [14], endothermy appeared late in ornithurine birds, during the Cretaceous period. Based on the absence of nasal turbinates in dinosaurs and the primitive structure of the rib cage, these authors concluded that endothermy in *Archaeopteryx* was unlikely. The problem with nasal turbinates as evidence of a high rate of metabolism is that they provide positive evidence but not the contrary (i.e. failing to find them, does not prove ectothermy [81]); and, unfortunately, they appear to be hard to find (Sebastian Apesteguia, unpublished observations).

By contrast, palaeontologists have provided several findings that, when taken together, challenge these conclusions and put the origin of avian endothermy before the diversification of Theropoda (i.e. during the Jurassic; Figure I). These findings include: (i) a variety of full-feathered (or covered by protofeathers [82]), small,

non-avian theropods [25] and also ornithischians, thus indicating a possible feathered dinosauriform common ancestor; (ii) fibrola-mellar bone histology without growth rings (indicating arrested growth during cold seasons) in species from polar zones [39,83]; (iii) fossils of individuals incubating eggs [84]; (iv) oxygen isotopic composition of bones (a 'palaeo-thermometer', [39]), suggesting body temperature control as in mammals and birds and (v) biophysical and bioenergetic modeling, coinciding in the necessity for an internal source of heat and insulation to maintain body temperature, in small species from high latitudes [18,38]. In fact, several authors maintain that many other archosaurs were endothermic, including crocodilians [30] and marine species [29]. Thus, an emerging consensus indicates that that the *Archaeopteryx* was endothermic.
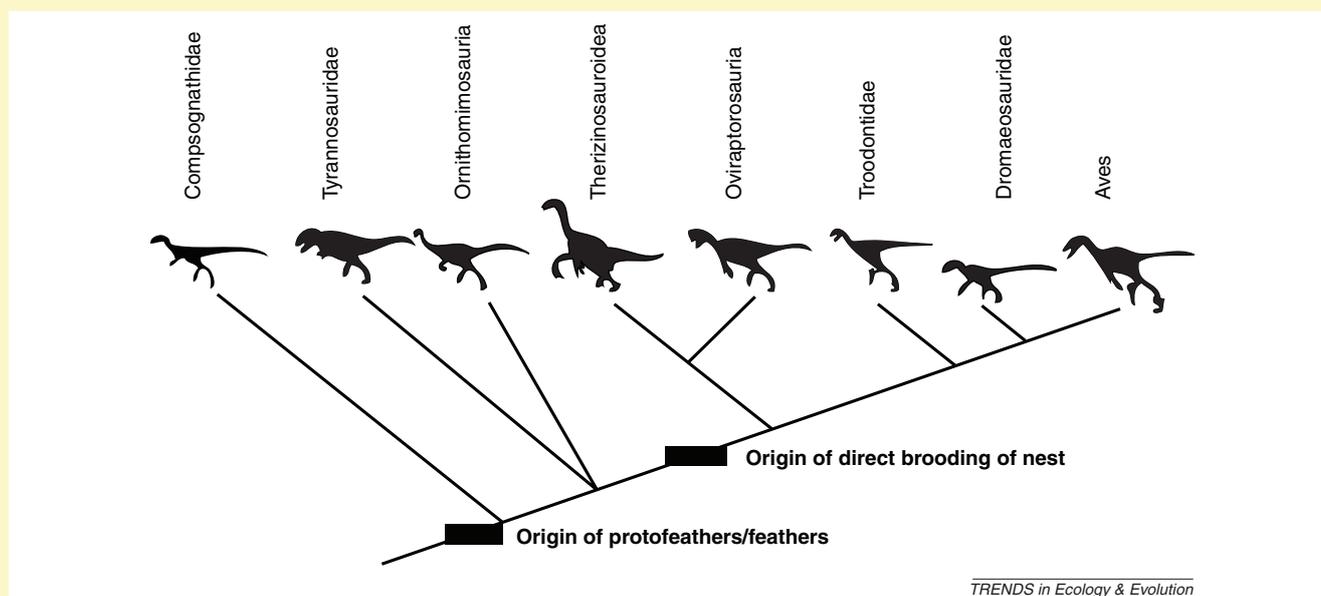

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Figure I. Diagram of theropod evolution, indicating where the evidence of brooding and feathers (or proto-feathers) has been found. According to the current fossil evidence, and assuming a single origin of feathers, all coelosaurs might be descendants of a feathered ancestor. Similarly, discovery of oviraptosaur and troodontid brooding nests implies that the common ancestor of maniraptorans was a brooder. Reproduced, with permission, from [41].

author, classic approaches of phenotype decomposition (i.e. quantitative genetics [33,36,37]) do not provide a means for testing the weak form of the aerobic capacity model. Other approaches, we believe, could do so.

It appears that the metabolic elevation leading to endothermy occurred over a rather long period of time. In mammals, the process lasted from the 'indisputably ectothermic pelycosaur' (*sensu* [4]) to the 'equally indisputable' early mammals; encompassing the whole Permian period (Figure 2a). As discussed above, in the case of birds, this is less clear: an 'indisputably ectothermic thecodont' (the common ancestor of the dinosaur lineage) lived during the late Triassic [5,38,39]. In addition, an 'indisputably endothermic theropod' (i.e. *Archaeopteryx*; Box 1) appeared to live during the late Jurassic [5,25,28,39–41] (Figure 2b). Hence, fossil evidence suggests that the evolution of endothermy in birds, as well as in mammals, occurred during different periods of time, in both cases a time lapse of approximately 150 million years. The consensus tree for amniote evolution (Figure 3) [42–44] indicates that mammals and birds shared a common ancestor during the lower Permian, 324 mya; which is 100 million years before the

appearance of mammals. Mammal endothermy might have originated within this period, whereas, in birds, this might have occurred 50 million years later (red lines in Figure 3).

Testing the aerobic capacity model in the last common ancestors of birds and mammals: genomic signatures of natural selection

A powerful method for inferring past periods of adaptive evolution is based on comparing orthologous genes of extant species in a phylogenetic framework, specifically by calculating the ratio of the rate of non-synonymous substitutions (d_N) to the rate of synonymous substitutions (d_S) in different lineages of the tree (d_N/d_S; Box 3). Use of this procedure has enabled researchers to establish that a group of genes associated with aerobic metabolism experienced positive selection during the evolution of flight in the last common ancestor of bats, allowing for the adaptation of the energy requirements required for flight [45]. A similar approach was used to identify adaptive evolution on genes related to aerobic metabolism in the evolutionary history of primates, this being associated with the expan-
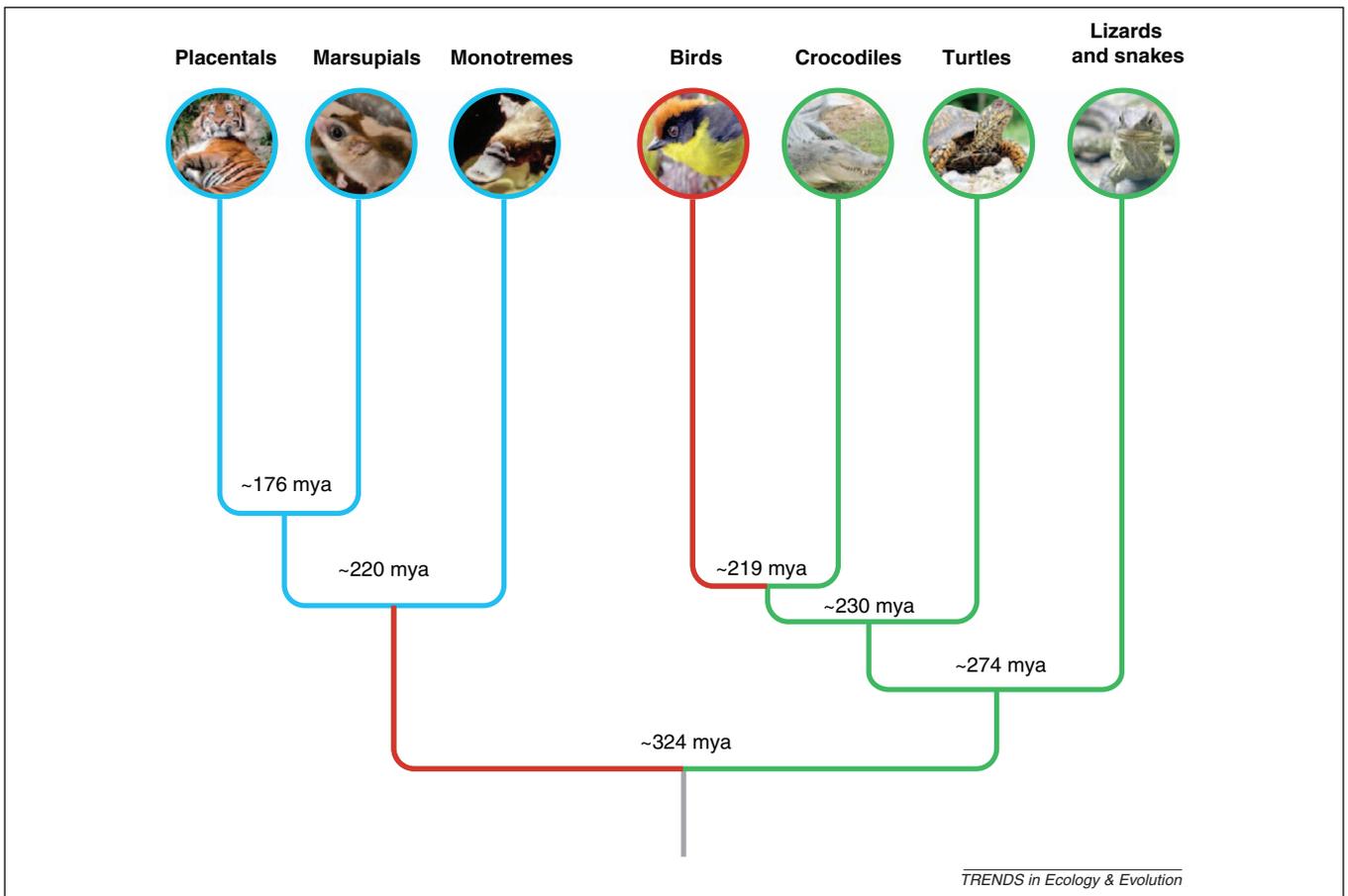
Figure 3. Phylogenetic relationships among the major groups of amniotes based on a loose consensus of recent studies (after [43,44]). Numbers above each node represent an approximate time of divergence.

sion of the energy-consuming neocortex during the radiation of anthropoid apes [46,47].

The uncoupling proteins and those proteins involved in the electron transport chain in birds and mammals have been well characterized and comprise approximately 13 mitochondrial and 76 nuclear genes [48]. This makes it possible to identify the signature of positive selection after comparing these known genes in a phylogeny that includes

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Box 2. Testing the aerobic model of the evolution of endothermy

Currently, the most accepted mechanistic hypothesis explaining the gradual elevation of RMR in pelycosaur reptiles and theropod dinosaurs, giving rise to mammals and birds with elevated body temperatures and BMR (the hallmark of endothermic metabolism, see Figure 1, main text), is the aerobic model for the evolution of endothermy [9,85,86]. In the short term, the evolution of an endothermic lifestyle only brings about additional costs, and the advantages of being endothermic only appear as a consequence of an already established high and constant body temperature. Hence, it is unlikely that BMR (or RMR in reptiles) has been the target of directional selection.

The original formulation of the aerobic capacity model proposed that the target of selection was high levels of sustained locomotor activity supported by aerobic metabolism (and, therefore, limited by the MMR), because this has several benefits now recognized in the ecology and life histories of birds and mammals [13,14]. The first version [85] was coined in terms of the general benefits of aerobic activity in adult animals, whereas the version by Koteja [86] strengthens the benefits of high activity capacities in terms of a cascade of changes beginning with offspring food provisioning. Recently, [87] further modified the model by highlighting the importance of temperature for muscle contraction and, hence, activity capacity. In a renaissance of the thermoregulatory model [15], this model combines thermal benefits with the relationship between MMR and RMR in explaining the metabolic elevation that characterized endothermic animals and BMR. In all cases, the key assumption of the aerobic capacity model is the existence of a mechanistic link between RMR and MMR, which appears to be supported by the fact that, in vertebrates, part of the metabolic machinery responsible for MMR is common to RMR (or BMR in endotherms) [51]. In fact, most aspects of systemic physiology (i.e. ventilation, pulmonary diffusion, cardiac output and capillary-to-cytosol-diffusion), which in part determine BMR, have been shown to be affected when MMR is maximized in trained, acclimated and/or in climate-adapted species [51]. As a consequence, MMR is approximately five to ten times RMR [88,89], although considerable variation exists [90]. Hence, the aerobic capacity model proposed that selection on MMR provoked the concomitant increase of RMR (leading to BMR in current endotherms), as a correlated response.

Hayes and Garland [31] connected the original model with the framework of quantitative genetics (see [33,36,78]), and proposed that the main empirical test for the model should be based on the demonstration of a positive genetic covariance between RMR and MMR. Hence, several physiologists have been involved in the quest for this genetic covariance in modern endotherms, especially in mammals (reviewed in [9,11,91–93]).

Box 3. Revealing signatures of selection in genomes across species and phylogenies

Natural selection and genetic drift are the two most important processes underlying changes at the molecular level and, as natural selection is ultimately responsible for evolutionary innovations, it is the process that has attracted most of the attention from the scientific community. For instance, by comparing orthologous genes in extant species, it is possible to identify base substitutions with varying effects on the protein that they encode [94]. Here, the identification of substitutions that alter the amino acid that they encode (non-synonymous) and those that do not alter amino acids (synonymous) enables researchers to study the signatures of natural selection at the molecular level.

Assuming that all synonymous substitutions are neutral (a realistic assumption in many cases [95]), the value of this ratio gives unambiguous indications of the operating selective regime. If non-synonymous substitutions are neutral [96] (they do not represent an advantage or disadvantage to the organisms), they will be fixed at the same rate as synonymous substitutions, and the value of the d_N/d_S ratio should be 1 ($d_N = d_S$ and $\omega = 1$). By contrast, if non-synonymous substitutions are deleterious, purifying selection will reduce their fixation rate and the value of the d_N/d_S ratio will be less than 1 ($d_N < d_S$ and $\omega < 1$). Finally, if the non-synonymous substitutions represent an advantage, their rate of fixation will be increased by positive darwinian selection, and the value of the ratio will be greater than 1 ($d_N > d_S$ and $\omega > 1$).

Using this procedure, it is possible to identify periods of adaptive change in specific periods of time at a given point of the phylogeny [93] (including extinct species, by inferring hypothetical, ancestral sequences). The process of adaptive evolution at the molecular level is characterized by short periods of accelerated rate of non-synonymous substitutions in comparison to synonymous substitutions ($d_N > d_S$ and $\omega > 1$), in which amino acid changes remodel the molecule until a new optimum is achieved. Afterwards, rates of amino acid replacement decrease, as the majority of additional changes would be detrimental ($d_N < d_S$ and $\omega < 1$; Figure I) [97,98]. If this was the case for genes related to the aerobic metabolism leading to the

evolution of endotherms, the prediction from the aerobic model (Box 2) is to find a d_N/d_S ratio above 1.0 for BMR and MMR genes in the last common ancestor of endotherms (separately for mammals and birds). Similarly, d_N/d_S values below 1.0 should be found in any other position in the amniote tree.

Amino acids replacements that are remodeling the molecule are being selected

Protein sequence is conserved as now most amino acid replacements are deleterious

Positive selection (dN/dS > 1)

Neutrality (dN/dS = 1)

Purifying selection (dN/dS < 1)
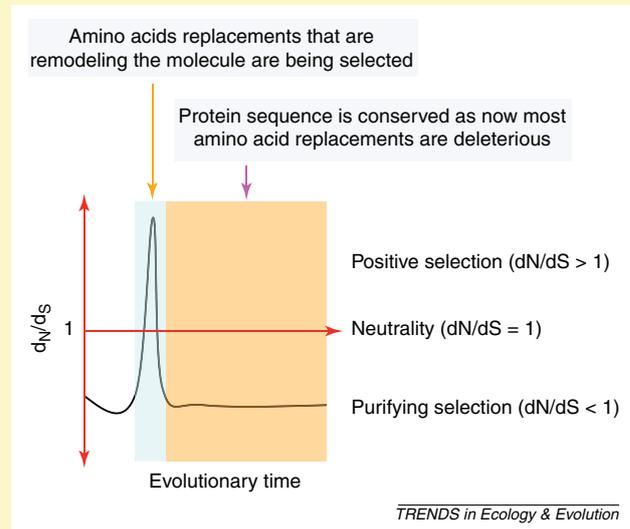
Evolutionary time

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Figure I. Textbook example (see [99]) of how molecular signatures of natural selection are usually studied using sequence variation among living taxa. The process of adaptive evolution at the molecular level is characterized by a relatively short period of time with an accelerated rate of non-synonymous substitution. At this stage, the amino acid replacements that result in improved protein function are favoured (light-blue area). Once the molecule has reached the new optimum, natural selection slows down the rate of non-synonymous substitution (orange area), as subsequent amino acid changes are mostly detrimental.

reptiles, mammals and birds. There is consensus regarding the fact that MMR is, in part, explained by features and mechanisms different from those determining BMR [49] (Box 2): whereas BMR depends on the aerobic metabolism of leaky membranes in visceral organs [8,50], MMR depends mostly on red fibres in skeletal muscle, particularly associated with actomyosin ATPase and Ca^{2+} ATPase [51]. It should therefore be possible in principle to identify genes exclusive to BMR or to MMR ('RMR genes' and 'MMR genes' for simplicity). Also, from a comparative perspective, the signature of positive selection in genes related to the origin of endothermy would have occurred in the last common ancestor of both endothermal clades (Figure 3). This prediction can be tested in a phylogenetic framework taking advantage of all genomic data currently available [52,53]. In phylogenetic-based comparisons that include reptiles, birds and mammals, several outcomes are possible, in terms of the signatures of natural selection (Box 3). First, d_N/d_S values above 1.0 could be obtained for BMR genes and for MMR genes in the last common ancestor of both endothermic groups (birds and mammals). This should be crucial support for the aerobic capacity model as it might indicate adaptive metabolic expansion in both traits, which occurred during the origin of birds and mammals. Second, d_N/d_S values above 1.0 could be identified for RMR genes but not for MMR genes in the last common ancestor of both endothermic groups (birds and mammals). This could falsify the aerobic capacity model as it might indicate that the metabolic expansion in RMR was not

associated with the elevation in MMR. Third, both ancestral branches could yield d_N/d_S ratios below 1.0, an outcome that can falsify any hypothesis assuming the level of aerobic metabolism is a target for natural selection (Figure 4a).

Studying the patterns of endothermic gene expression between ectotherms and endotherms

Since the recent revolution in functional genomics [54,55], it has become clear that many phenotypic differences within and between populations and species are due to differences in gene expression [56–58]. According to Seebacher and co-workers [50,59,60], this might be the case for aerobic metabolism in vertebrates. In vertebrates, metabolic pathways leading to heat production appear to be qualitatively similar, and the mechanistic differences between them are a matter of degree of expression of the same genes (the 'evolutionary conservatism' hypothesis [59]). A prediction of this hypothesis is to expect a higher divergence of gene expression for these genes (both in the number of regulated genes and in the relative level of expression) in endotherms compared with ectotherms (Figure 4b). Failing to find such a difference, or finding the inverse, might falsify this hypothesis. This is a prediction with important consequences for the evolution of endothermy because it implies that the evolution of the aerobic metabolism leading to endothermy proceeded through changes in only a few regulatory genes rather than through changes in many structural genes (the latter
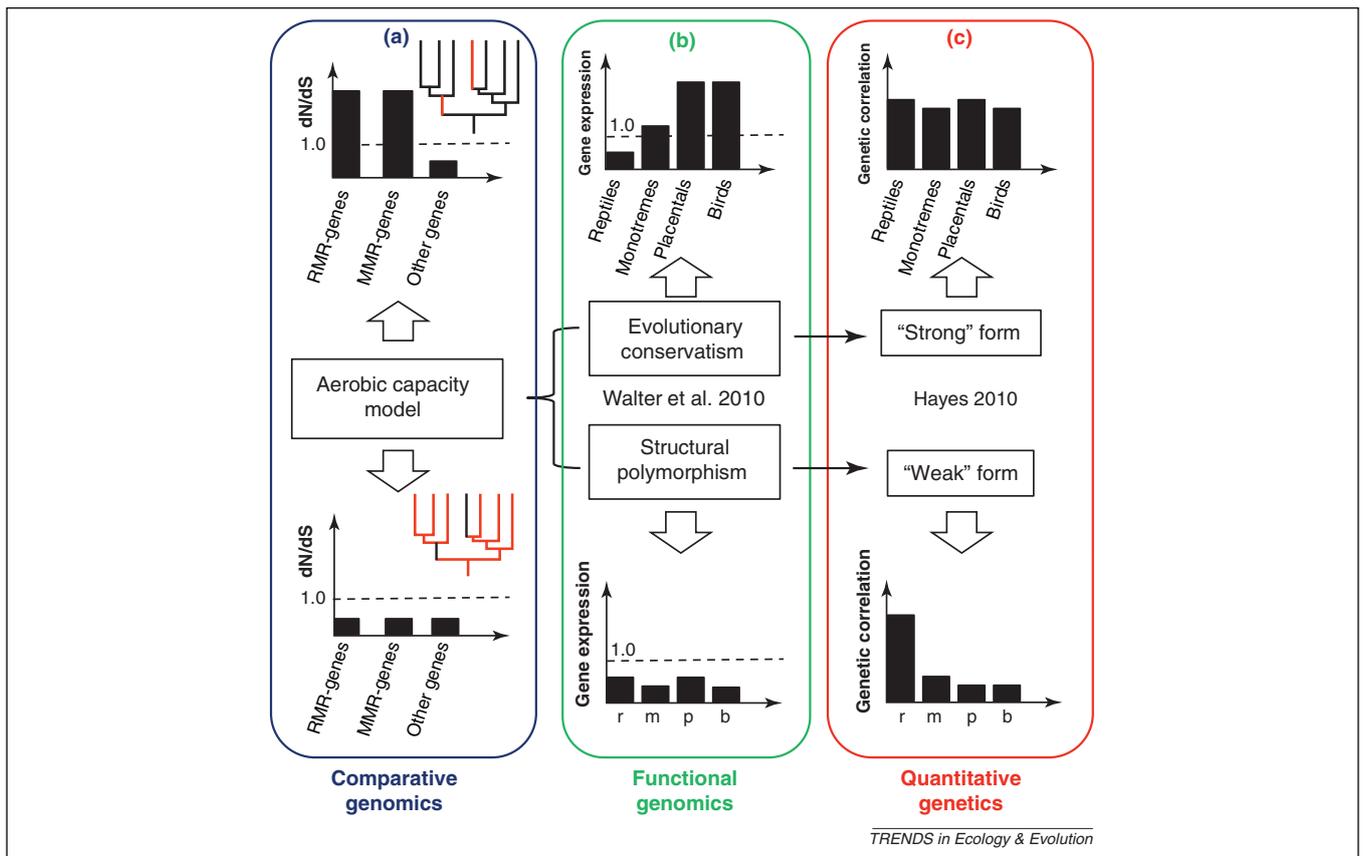
Figure 4.   Schematic representation of a three-way approach to test the tempo and mode of evolution of endothermy in the phylogeny of vertebrates. Rectangles represent main hypotheses (derived from the aerobic capacity model), vertical arrows indicate predictions, bar graphs represent predicted experimental outcomes (schematic) and coloured ovals show main disciplinary approaches. **(a)** shows predictions of the aerobic capacity model testable within the framework of comparative genomics, which enables calculation of the d_N/d_S ratio in homologous sequences of genes related to MMR and RMR. This ratio equals 1.0 under neutral evolution, is below 1.0 after purifying selection [**(a)** bottom] and above 1.0 after positive selection [**(a)**, top, see Box 3 for details]. Only species for which whole genomes are available can be analysed. According to the timing of endothermy evolution suggested by the fossil record, signatures of positive selection should be detected in the common ancestor of mammals and birds [red zones in the upper graph, **(a)**]. Other comparisons [red zones in the bottom graph, **(a)**], might yield values of d_N/d_S below 1.0. **(b)** shows the predictions of two possible modes of action of genes related to metabolic pathways. The graphs show the levels of gene expression. Standard gene expression unities are relative to a control condition where the gene is expressed normally, compared with a treatment where the gene is over- or underexpressed. Hence, values above 1.0 would represent overexpressed genes of RMR and values below 1.0 would represent underexpressed genes of RMR; see main text for details. Evolutionary conservatism represents the hypothesis that metabolic pathways responsible for aerobic metabolism are qualitatively similar in vertebrates, and differences between ecto- and endotherms are mainly to the result of differences in gene expression [58]. According to this idea, the expression of genes related to aerobic metabolism should be gradually larger in modern endotherms compared with reptiles or proto-endotherms [**(b)** top]. By contrast, the classic view of population genetics assumes that phenotypic differences in populations are to the result of differences in the allele composition of many genes of small effect [structural polymorphism **(b)** bottom]. In this case, gene expression should show no consistent trend across taxa [**(b)** bottom]. The latter two hypotheses generate different consequences in terms of the effect of natural selection on genetic variance and the presence of a positive genetic correlation between RMR and MMR, which is the crucial assumption of the aerobic capacity model. The classic genetic model of population genetics (i.e. structural polymorphism) predicts that directional selection results in fixation of genes conferring a phenotypic advantage and, consequently, reduced genetic variation and covariation between traits. After many generations of directional selection, the genetic correlation between RMR and MMR should be reduced to near-zero values and, therefore, it should be much lower, if present at all, in extant endotherms than in species resembling their ancestors, such as reptiles [**(c)**, bottom]. This scenario has been named the 'weak' version of the aerobic capacity model [9]. By contrast, if the phenotypic differences are to the result of differences in gene expression (the evolutionary conservatism hypothesis), the genetic variation can be increased and maintained even after strong selection. In this case, the genetic correlation between RMR and MMR can be large and present in all vertebrates [**(c)**, top]. This scenario is also known as the 'strong' form of the aerobic capacity model [9]. The bar graphs are schematic drawings to show the general predictions of each model in some selected groups of vertebrates; they do not include all possible comparisons suggested in the main text, such as the expected differences between palaeognath and neognath birds. In mammals only the expected most contrasting results are shown, such as the comparison between monotremes and placental mammals (i.e. marsupials are not included).

is known as 'structural polymorphism' [55,61,62]). This also has a crucial consequence for the maintenance of genetic variation in populations. According to several authors, when phenotypic variation is explained by differences in gene expression rather than by structural polymorphism [55], the maintenance of heritable variation is promoted [63–65]. It follows that a positive verification of the evolutionary conservatism hypothesis might also give support to the strong version of the aerobic capacity model (Figure 4b,c). By contrast, falsifying the evolutionary conservatism hypothesis can lead to the idea that metabolic pathways are dependent on structural polymorphism, as classic genetic models suggest. Therefore, even if the ge-

netic correlation between RMR and MMR was present in proto-endotherms, it would be reduced to near-zero values in the subsequent lineages, as assumed in the 'weak' version of the aerobic capacity model [9].

Comparing gene expressions across taxa has been shown to be problematic because different species should differ in many other factors, in addition to being ecto- or endothermic. This is why only a few studies have compared gene expressions among taxa, and mostly in closely related species [56,57]. In addition, gene expression is considerably labile so that even quite small changes in environmental conditions could elicit different gene regulation [50,55–57]. A potential solution to this problem is to

consider the effects of thyroid hormones on aerobic metabolism. It is known that aerobic metabolism in visceral organs is controlled by thyroid hormone [21,60,66]. In fact, RMR can be down- or upregulated by inducing hypo- or hyperthyroidism [66,67]. Most gene expression studies contrast a 'normal' with an 'experimental' condition, and compare the patterns of expression. In the case of endothermy, it might be necessary to induce higher or lower levels of resting metabolism artificially as treatment and control conditions, for which hormone manipulation offers a good alternative [21,60,66,68]. Following this line of thought, it is not hard to conceive of a large scale experiment where a sample of similarly sized reptiles, different representatives of mammals (i.e. monotremes, marsupials and placentals) and birds (palaeognathes and neognathes) are submitted to a common garden experiment (homogeneous temperature, food and environmental conditions) to compare gene expression patterns after artificially elevating resting metabolism, and then comparing with unmanipulated controls. Additionally, hypothyroid-induced animals could be included to examine other regulated genes [67,69]. A preliminary survey using some crucial metabolic transcription factors indicate that these appear to be a good starting point from which to test divergence in gene expression among taxa, and this approach can be complemented with some functional tests (e.g. mitochondrial enzyme activities) [50,60,70]. The expected outcome of the experiment might be that gene expression (both the number of genes and the relative expression of each) will increase in treated animals compared with controls. Less obvious, however, are the predictions across sampled taxa. If the hypothesis of evolutionary conservatism is correct, then one can expect to find that the within-species differences in gene expression are lowest in reptiles and highest in placental mammals and neognath birds, and intermediate differences might be found in monotremes, marsupials and palaeognath birds (Figure 4B).

Analysing the sources of phenotypic variance in reptiles, birds and mammals

Comparative physiologists have known for some time that the observed variations in physiological attributes, such as body temperature and RMR, of present-day tetrapods are generally good proxies for these attributes in their ancestors, excluding specialized species (e.g. penguins or naked mole rats). For instance, body temperature is progressively higher and better controlled (i.e. exhibits less variation) in placental mammals and passerine birds, compared with monotremes and ratites, respectively [16,71,72]. This pattern is also supported by more sophisticated comparisons, as well as in other traits [16,72–75]. In fact, recent sequencing of the platypus genome revealed that many apparently reptilian phenotypic attributes (e.g. oviparity, morphology of spermatozoa and retina) are in fact related to reptilian genes, which are absent in more recent mammalian lineages [76]. The rationale that physiologists have implicitly used in all experimental tests of hypotheses about past physiologies, including endothermy evolution, is to assume that present representatives generally retain the design features of their ancestors. This is what physiologists had in mind when performing experiments on

crocodiles [38], lizards [77] and echidnas [16] to understand the physiological processes of extinct vertebrates. If directional selection promoting higher capacities in aerobic work (i.e. MMR or any other related physiological capacity) began to act persistently (the weak version of the aerobic capacity model [9]) on ancient populations of pelycosaurs and theropods (Figure 2), generating an evolutionary response [33,36,78], then genes of MMR should have gradually fixed, thereby reducing genetic variation. This could also have reduced to zero the genetic covariance between RMR (or BMR in full endotherms) and MMR. Two predictions can be drawn from this reasoning, in terms of the amniote phylogeny depicted in Figure 3. First, at one extreme, turtles, squamate reptiles and crocodilians should exhibit large genetic variances and covariances between RMR and MMR. Second, at the opposite extreme, placental mammals and neognath birds should exhibit low or nonsignificant genetic variances and covariances between BMR and MMR (Figure 4c).

By contrast, the strong version of the aerobic capacity model predicts that the genetic correlation between RMR and MMR was maintained in the subsequent lineages of endotherms and, hence, it can be found in living birds and mammals. The maintenance of genetic variation in the genes for RMR and MMR could be facilitated by the fact that phenotypic differences are mainly to the result of differences in gene expression patterns rather than of allele composition (the evolutionary conservatism hypothesis [50,59,60]). Hence, the prediction is obvious: the genetic correlation will be present in ancient as well as in modern lineages of vertebrates. Consequently, the strong form of the aerobic capacity model could be falsified by finding that modern endotherms exhibit smaller genetic correlation compared with reptiles (Figure 4c).

Concluding remarks

The proposed experimental framework for studying the evolution of endothermy by further tests of the aerobic capacity model is depicted in Figure 4. The general idea is to tackle the problem through a multidisciplinary approach, using tools that were not completely available for Hayes and Garland in 1995, and previous authors. We hope these ideas will be useful for new generations of evolutionary physiologists.

References

1 Penick, D.N. (2004) Endothermy of ambystomid salamanders: new perspectives on the evolution of endothermy in vertebrates. *Integr. Comp. Biol.* 44, 620

2 Watanabe, M.E. (2005) Generating heat: new twists in the evolution of endothermy. *Bioscience* 55, 470–475

3 Patio, S. *et al.* (2000) Endothermy by flowers of *Rhizanthes lowii* (Rafflesiaceae). *Oecologia* 124, 149–155

4 Kemp, T.S. (2006) The origin of mammalian endothermy: a paradigm for the evolution of complex biological structure. *Zool. J. Linn. Soc.* 147, 473–488

5 Schweitzer, M.H. and Marshall, C.L. (2001) A molecular model for the evolution of endothermy in the Theropod–Bird lineage. *J. Exp. Zool.* 291, 317–338

6 Dickson, K.A. and Graham, J.B. (2004) Evolution and consequences of endothermy in fishes. *Physiol. Biochem. Zool.* 77, 998–1018

7 Hulbert, A.J. and Else, P.L. (2004) Basal metabolic rate: history, composition, regulation and usefulness. *Physiol. Biochem. Zool.* 77, 869–876

8 Else, P.L. *et al.* (2004) The evolution of endothermy: role for membranes and molecular activity. *Physiol. Biochem. Zool.* 77, 950–958

9 Hayes, J.P. (2010) Metabolic rates, genetic constraints, and the evolution of endothermy. *J. Evol. Biol.* 23, 1868–1877

10 Koshiba-Takeuchi, K. *et al.* (2009) Reptilian heart development and the molecular basis of cardiac chamber evolution. *Nature* 461, 95–99

11 Swallow, J.G. *et al.* (2009) Selection experiments and experimental evolution of performance and physiology. In *Experimental Evolution* (Garland, T. and Rose, M.R., eds), pp. 301–351, University of California Press

12 Portner, H.O. (2004) Climate variability and the energetic pathways of evolution: the origin of endothermy in mammals and birds. *Physiol. Biochem. Zool.* 77, 959–981

13 Koteja, P. (2004) The evolution of concepts on the evolution of endothermy in birds and mammals. *Physiol. Biochem. Zool.* 77, 1043–1050

14 Hillenius, W.J. and Ruben, J.A. (2004) The evolution of endothermy in terrestrial vertebrates: who? When? Why? *Physiol. Biochem. Zool.* 77, 1019–1042

15 Crompton, A.W. *et al.* (1978) Evolution of homeothermy in mammals. *Nature* 272, 333–336

16 Grigg, G.C. *et al.* (2004) The evolution of endothermy and its diversity in mammals and birds. *Physiol. Biochem. Zool.* 77, 982–997

17 Willmer, P. *et al.* (2005) *Environmental Physiology of Animals*, Blackwell

18 McNab, B.K. (2009) Resources and energetics determined dinosaur maximal size. *Proc. Natl. Acad. Sci. U.S.A.* 106, 12184–12188

19 Else, P.L. and Hulbert, A.J. (1987) Evolution of mammalian endothermic metabolism: 'leaky' membranes as a source of heat. *Am. J. Physiol.* 253, R1–R7

20 Hulbert, A.J. (1990) The cellular basis of endothermic metabolism: a role for 'leaky' membranes. *News Physiol. Sci.* 5, 25–28

21 Hulbert, A.J. and Else, P.L. (1981) Comparison of the 'mammal machine' and the 'reptile machine': energy use and thyroid production. *Am. J. Physiol.* 241, R350–R356

22 Else, P.L. and Hulbert, A.J. (1981) Comparison of the 'mammal machine' and the 'reptile machine': energy production. *Am. J. Physiol.* 240, R3–R9

23 Kemp, T.S. (2006) The origin and early radiation of the therapsid mammal-like reptiles: a palaeobiological hypothesis. *J. Evol. Biol.* 19, 1231–1247

24 Kemp, T.S. (2009) Phylogenetic interrelationships and pattern of evolution of the therapsids: testing for polytomy. *Palaeontologica Africana* 44, 1–12

25 Norell, M.A. and Xu, X. (2005) Feathered dinosaurs. *Annu. Rev. Earth Planet. Sci.* 33, 277–299

26 Ruben, J.A. (1995) The evolution of endothermy in mammals and birds: from physiology to fossils. *Annu. Rev. Physiol.* 57, 69–76

27 Montes, L. *et al.* (2007) Relationships between bone growth rate, body mass and resting metabolic rate in growing amniotes: a phylogenetic approach. *Biol. J. Linnean Soc.* 92, 63–76

28 Pontzer, H. *et al.* (2009) Biomechanics of running indicates endothermy in bipedal dinosaurs. *PLoS ONE* 4, A127–A135

29 Bernard, A. *et al.* (2010) Regulation of body temperature by some mesozoic marine reptiles. *Science* 328, 1379–1382

30 Seymour, R.S. *et al.* (2004) *Evidence for endothermic ancestors of crocodiles at the stem of archosaur evolution* 1051–1067

31 Hayes, J.P. and Garland, T. (1995) The evolution of endothermy: testing the aerobic capacity model. *Evolution* 49, 836–847

32 Lynch, M. and Walsh, B. (1998) *Genetics and Analysis of Quantitative Traits*, Sinauer

33 Arnold, S.J. *et al.* (2008) Understanding the evolution and stability of the G-matrix. *Evolution* 62, 2451–2461

34 Wone, B. *et al.* (2009) Genetic variances and covariances of aerobic metabolic rates in laboratory mice. *Proc. R. Soc. B* 276, 3695–3704

35 Roff, D.A. (2010) *Modeling Evolution: An Introduction to Numerical Methods*, Oxford University Press

36 Roff, D.A. (1997) *Evolutionary Quantitative Genetics*, Chapman & Hall

37 Falconer, D.S. and Mackay, T.F.C. (1997) *Introduction to Quantitative Genetics*, Longman

38 Seebacher, F. (2003) Dinosaur body temperatures: the occurrence of endothermy and ectothermy. *Paleobiology* 29, 105–122

39 Barrick, R. (2000) The thermodynamics of dinosaurs. In *Dinosaurs* (Paul, G.S., ed.), pp. 310–322, St. Martins Griffin

40 Fountaine, T.M.R. *et al.* (2005) The quality of the fossil record of Mesozoic birds. *Proc. R. Soc. B* 272, 289–294

41 Holtz, T.R. (2000) Classification and evolution of the dinosaur groups. In *Dinosaurs* (Paul, G.S., ed.), pp. 140–168, St. Martin Griffin

42 Hedges, S.B. and Kumar, S. (2009) *The Time Tree of Life*, Oxford University Press

43 van Rheede, T. *et al.* (2006) The platypus is in its place: nuclear genes and indels confirm the sister group relation of monotremes and therians. *Mol. Biol. Evol.* 23, 587–597

44 Meyer, A. and Zardoya, R. (2003) Recent advances in the (molecular) phylogeny of vertebrates. *Annu. Rev. Ecol. Evol. Syst.* 34, 311–338

45 Shen, Y.Y. *et al.* (2010) Adaptive evolution of energy metabolism genes and the origin of flight in bats. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8666–8671

46 Grossman, L.I. *et al.* (2001) Molecular evolution of aerobic energy metabolism in primates. *Mol. Phylog. Evol.* 18, 26–36

47 Nielsen, R. *et al.* (2007) Recent and ongoing selection in the human genome. *Nat. Rev. Genet.* 8, 857–868

48 Grossman, L.I. *et al.* (2004) Accelerated evolution of the electron transport chain in anthropoid primates. *Trends Genet.* 20, 578–585

49 Weibel, E.R. *et al.* (2004) Allometric scaling of maximal metabolic rate in mammals: muscle aerobic capacity as determinant factor. *Respir. Physiol. Neurobiol.* 140, 115–132

50 Walter, I. and Seebacher, F. (2009) Endothermy in birds: underlying molecular mechanisms. *J. Exp. Biol.* 212, 2328–2336

51 Hochachka, P.W. and Burelle, Y. (2004) Control of maximum metabolic rate in humans: dependence on performance phenotypes. *Mol. Cell. Biochem.* 256, 95–103

52 Waterston, R.H. *et al.* (2002) Initial sequencing and comparative analysis of the mouse genome. *Nature* 420, 520–562

53 Rubin, C.J. *et al.* (2010) Whole-genome resequencing reveals loci under selection during chicken domestication. *Nature* 464, 587–593

54 Stapley, J. *et al.* (2010) Adaptation genomics: the next generation. *Trends Ecol. Evol.* 25, 705–712

55 Whitehead, A. and Crawford, D.L. (2006) Variation within and among species in gene expression: raw material for evolution. *Mol. Ecol.* 15, 1197–1211

56 Abzhanov, A. *et al.* (2006) The calmodulin pathway and evolution of elongated beak morphology in Darwin's finches. *Nature* 442, 563–567

57 Oleksiak, M.F. *et al.* (2002) Variation in gene expression within and among natural populations. *Nat. Genet.* 32, 261–266

58 Fay, J.C. and Wittkopp, P.J. (2008) Evaluating the role of natural selection in the evolution of gene regulation. *Heredity* 100, 191–199

59 Seebacher, F. *et al.* (2006) Transition from ectothermy to endothermy: the development of metabolic capacity in a bird (*Gallus gallus*). *Proc. R. Soc. B* 273, 565–570

60 Walter, I. *et al.* (2010) AMP-activated protein kinase controls metabolism and heat production during embryonic development in birds. *J. Exp. Biol.* 213, 3167–3176

61 Wray, G.A. *et al.* (2003) The evolution of transcriptional regulation in eukaryotes. *Mol. Biol. Evol.* 20, 1377–1419

62 Wagner, G.P. and Lynch, V.J. (2008) The gene regulatory logic of transcription factor evolution. *Trends Ecol. Evol.* 23, 377–385

63 Bergman, A. and Siegal, M.L. (2003) Evolutionary capacitance as a general feature of complex gene networks. *Nature* 424, 549–552

64 Masel, J. (2005) Evolutionary capacitance may be favored by natural selection. *Genetics* 170, 1359–1371

65 Landry, C.R. *et al.* (2007) Genetic properties influencing the evolvability of gene expression. *Science* 317, 118–121

66 Farmer, C.G. (2000) Parental care: the key to understanding endothermy and other convergent features in birds and mammals. *Am. Nat.* 155, 326–334

67 Yang, Y. and Gordon, C.J. (1997) Regulated hypothermia in the hypothyroid rat induced by administration of propylthiouracil. *Am. J. Physiol.* 272, R1390–R1395

68 Niewiaroski, P.H. *et al.* (2000) Phenotypic effects of leptin in an ectotherm: a new tool to study the evolution of life histories and endothermy? *J. Exp. Biol.* 203, 295–300

69 Gordon, C.J. (1997) Behavioral and autonomic thermoregulation in the rat following propylthiouracil-induced hypothyroidism. *Pharmacol. Biochem. Behav.* 58, 231–236

70 Walter, I. *et al.* (2009) AMP-activated protein kinase: function as cellular energy gauge during the development of endothermy in birds (*Gallus gallus*)? *Comp. Biochem. Physiol. A* 153A, S171

71 Schmidt-Nielsen, K. (1995) *Animal Physiology: Adaptation and Environment*, Cambridge University Press

72 Maloney, S.K. (2008) Thermoregulation in ratites: a review. *Aust. J. Exp. Agric.* 48, 1293–1301

73 Withers, P.C. *et al.* (1987) Metabolic, water and thermal relations of the Chilean tinamou. *Condor* 89, 424–426

74 Mzilikazi, N. and Lovegrove, B.G. (2005) Daily torpor during the active phase in free-ranging rock elephant shrews (*Elephantus myurus*). *J. Zool.* 267, 103–111

75 McNab (1996) Metabolism and temperature regulation of kiwis (Apterygidae). *Auk* 113, 687–692

76 Warren, W.C. *et al.* (2008) Genome analysis of the platypus reveals unique signatures of evolution. *Nature* 453, 175–183

77 Bennett, A.F. *et al.* (2000) An experimental test of the thermoregulatory hypothesis for the evolution of endothermy. *Evolution* 54, 1768–1773

78 Hackett, S.J. *et al.* (2008) A phylogenomic study of birds reveals their evolutionary history. *Science* 320, 1763–1768

79 Bennett, A.F. and Ruben, J.A. (1979) Endothermy and activity in vertebrates. *Science* 206, 649–653

80 Piskurek, O. *et al.* (2009) The evolution of two partner LINE/SINE families and a full-length chromodomain-containing Ty3/Gypsy LTR element in the first reptilian genome of *Anolis carolinensis*. *Gene* 441, 111–118

81 Turner, A.H. *et al.* (2007) Feather quill knobs in the dinosaur Velociraptor. *Science* 317, 1721

82 Curry, C. (2000) Growth rates among the dinosaurs. In *Dinosaurs* (Paul, G.S., ed.), pp. 297–309, St. Martin's Griffin

83 Norell, M.A. *et al.* (1995) A nesting dinosaur. *Nature* 378, 774–776

84 Nielsen, R. (2005) Molecular signatures of natural selection. *Annu. Rev. Genet.* 39, 197–218

85 Koteja, P. (2000) Energy assimilation, parental care and the evolution of endothermy. *Proc. R. Soc. Lond. B* 267, 479–484

86 Clarke, A. and Pörtner, H.O. (2010) Temperature, metabolic power and the evolution of endothermy. *Biol. Rev.* 85, 703–727

87 Bishop, C.M. (1999) The maximum oxygen consumption and aerobic scope of birds and mammals: getting to the heart of the matter. *Proc. R. Soc. Lond. B* 266, 2275–2281

88 Hinds, D.S. (1995) Maximum metabolism and the aerobic factorial scope or endotherms. *J. Exp. Biol.* 182, 41–56

89 Hammond, K.A. and Diamond, J. (1997) Maximal sustained energy budgets in humans and animals. *Nature* 386, 457–462

90 Gebczynski, A.K. and Konarzewski, M. (2009) Metabolic correlates of selection on aerobic capacity in laboratory mice: a test of the model for the evolution of endothermy. *J. Exp. Biol.* 212, 2872–2878

91 Gebczynski, A.K. and Konarzewski, M. (2009) Locomotor activity of mice divergently selected for basal metabolic rate: a test of hypotheses on the evolution of endothermy. *J. Evol. Biol.* 22, 1212–1220

92 Sadowska, E.T. *et al.* (2005) Genetic correlations between basal and maximum metabolic rates in a wild rodent: consequences for evolution of endothermy. *Evolution* 59, 672–681

93 Seymour, R.S. (2004) Getting warmer, getting colder: reconstructing crocodylomorph physiology - Reply to Hillenius and Ruben. *Physiol. Biochem. Zool.* 77, 1073–1075

94 Nei, M. *et al.* (2010) The neutral theory of molecular evolution in the genomic era. *Annu. Rev. Genomics Human Genet.* 11, 265–289

95 Kimura, M. (1968) Evolutionary rate at the molecular level. *Nature* 217, 624–626

96 Goodman, M. (1982) Positive selection causes purifying selection. *Nature* 295, 630

97 Goodman, M. (1981) Decoding the pattern of protein evolution. *Prog. Biophys. Mol. Biol.* 38, 105–164

98 Futuyma, D. (2009) *Evolutionary Biology*, Sinauer

99 McNab, B.K. (2002) *The Physiological Ecology of Vertebrates. A View from Energetics*, Comstock