Faraway, so close. The comparative method and the potential of non-model animals in mitochondrial research

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Title Page

Faraway, so close. The comparative method and the potential of non-model animals in mitochondrial research

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Abstract

Inference from model organisms has been the engine for many discoveries in life science, but indiscriminate generalisation leads to oversimplifications and misconceptions. Model organisms and inductive reasoning are irreplaceable: there is no other way to tackle the complexity of living systems. At the same time, it is not advisable to infer general patterns from a restricted number of species, which are very far from being representative of the diversity of life. Not all models are equal. Some organisms are suitable to find similarities across species, other highly specialized organisms can be used to focus on differences. In this opinion piece we discuss the dominance of the mechanistic/reductionist approach in life sciences and make a case for an enhanced application of the comparative approach to study processes in all their various forms across different organisms. We also enlist some rising animal models in mitochondrial research, to exemplify how non-model organisms can be chosen in a comparative framework. These taxa often do not possess implemented tools and dedicated methods/resources. However, due to specific features, they have the potential to address still unanswered biological questions. Finally, we discuss future perspectives and caveats of comparative method in the age of "big data".

Keywords: comparative biology; non-model organisms; mitochondria; generalisation; idiographics; nomothetics.

Model organisms

"For a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied"

—August Krogh, 1929

In August 1929, at the Thirteenth International Physiological Congress in Boston, the Danish scientist Schack August Steenberg Krogh delivered a lecture entitled "The Progress of Physiology" (Krogh 1929). Krogh touched on many significant points—still relevant to this day—but the focus of his talk was the need for physiology to undertake a comparative approach to study processes in all their various forms across different organisms. In this context, Krogh emphasized the importance of choosing the right model for a given biological problem. As an example, he cited his former teacher, Christian Bohr (father of the famous physicist Niels Bohr), that used a tortoise to study the respiratory mechanism of the lung because its anatomical features facilitated the experiments (see Wang 2011 for a review). A postdoctoral assistant attended Krogh's lecture, and was deeply inspired by it. His name was Hans Adolf Krebs, who discovered the citric acid cycle (also known as Krebs cycle or tricarboxylic acid cycle). Krebs was so inspired that 46 years later wrote a paper entitled "The August Krogh Principle: For Many Problems There is an Animal in Which It Can Be Most Conveniently Studied" (Krebs 1975). At the time of Krogh's lecture, Krebs was working in the laboratory of Otto Heinrich Warburg who had great success by choosing the right models, such as the sea urchin to study the changes in energy metabolism upon fertilization, the unicellular algae *Chlorella vulgaris* to study photosynthesis, and rats to study the metabolism of cancer (Krebs 1975). In particular, the latter studies led to the discovery of the Warburg effect (cancer cells tend to favor glycolysis over oxidative phosphorylation, even in aerobic conditions). Krebs himself applied the Krogh principle by choosing the pigeon breast muscle to study the pathways of oxidative metabolism. A causal correlation between great accomplishments in life science and well-chosen model organism is undeniable, and has been acknowledged for a long time. However, given the complexity of biological systems and the astonishing diversity of life, it is important to use models carefully, to fully exploit the advantages of the reductionist approach without falling into over-extrapolation.

What is a model organism? According to the definition by Levy and Currie (2015), a model is "a cognitive stand-in: instead of investigating the phenomenon directly, one studies an easier to handle alternative". In biology, the term is used in the context of mathematical modelling and computational simulations (theoretical models), and referred to particular organisms (model organisms). First, in its most familiar form, a model is a modified theoretical analogue that retains some features of a phenomenon

under study, while simplifying others; analyzing a simplified surrogate based on a mathematical construct it is possible to draw conclusions about a real-world target system. In the second acceptation, a model organism is a representative specimen of another organism or of a broader biological group, and inferences from such models are empirical extrapolations (Levy and Currie 2015). Jessica Bolker (2009) classified model organisms in two types: surrogate and exemplary models. This distinction is important because it entails quite different criteria regarding the choice of the model, and the different kinds of conclusions that can be drawn from studying it. On the one hand, surrogate models are mostly used in biomedical research to understand the mechanism of human diseases and to develop treatments, but the same approach is also applied to commercially relevant species and for the conservation of endangered species (e.g.: assisted reproduction). A fundamental assumption that is made when using such type of models, is that the surrogate (e.g.: the mouse) will respond to manipulation in the same way as the target (e.g.: humans), in other words we assume that the biological process we are investigating in the surrogate model is shared with the target, and we translate the findings from surrogate to target by direct substitution. Accordingly, the surrogate does not need to be phylogenetically close to the target, the only important thing is that the surrogate matches the target in regard to the investigated biological process (Bolker 2009). On the other hand, exemplary models are used in basic science as representatives of broader groups with the purpose of understanding fundamental biological mechanisms and evolutionary processes. In this case the results obtained are used to draw general conclusions based on inductive reasoning—that is making broad generalisations from specific observations—and the phylogenetic context is important, because it allows us to evaluate the representativeness of the model in a wider context with respect to particular traits (Bolker 2009).

The two approaches have been extremely successful, but it is important to issue caveats and limitations associated with both of them. As evolutionary biologists—and given the evolutionary framework of this Issue—we are focusing on exemplary model organisms. In this context, the most important concept that we need to keep in mind is that inductive reasoning is a powerful method, but can lead to false conclusions even if all the premises are true (Henderson 2019). We discuss the caveats associated with generalisation in the following section.

The problem with overgeneralisation

Generalisation is a double-edged sword. Inference from model organisms has been the engine for many discoveries in life science, but indiscriminate generalisation leads to oversimplifications and misconceptions. It is quite common—both in specialized

publications and even more so in the mass-media—to find titles of articles that omits the model organism from which the results were obtained. In this way the pairing between the model organism and the taxon that the model is representing becomes invisible (Bolker 2009). The scientific community is well-aware of such misbehaviour, as proven by the popularity of the Twitter handle @justsaysinmice run by James Heaters, a data scientist at Northeastern University in Boston, Massachusetts. All that the account does is to retweet a news story or press release containing a catchpenny headline or bold statements, and adding the words "IN MICE" to it. The account was created in April 2019, and now (just three months later) it counts nearly 66,000 followers. What initially started as a joke is now generating debates about the misuse of generalisation and inductive reasoning. To omit the subject of the study, especially (but not only) in biomedical research, generates the misconception that the finding is valid for every/most organisms, humans included. The bad practice of inflating research findings by overgeneralisation can originate either at the source—due to the increasing pressure for publishing "striking and breakthrough" research in order to get funding or make a career—or during the process of promulgation to the public. The dissemination of wrong or distorted concepts is particularly bothersome because it undermines the trust of people towards science, or, even worse, creates anti-scientific mediatic monsters. There is an ongoing crisis of mistrust in science (Kabat 2017), and misinformation is playing a key role—albeit not being the only cause—in the decline of public confidence in scientists and experts in general. We think that the scientific community should take action to reverse the trend that has been pushing science in the direction of sensationalism and "breaking news".

During his life, Thomas Hunt Morgan—one of the fathers of genetics and responsible for making *Drosophila* a model organism—worked on a considerable number of different species (Sturtevant 1959), actually following Krog's principle, nevertheless, he considered the results of research on fruit flies as applicable to all sexually reproducing organisms. Such perception regarding *Drosophila* and other model organisms is quite common among biologists and almost ubiquitous in modern textbooks (Levy and Currie 2015). Francis Crick called his theory about the information flow from DNA to proteins "Central Dogma" (Crick 1970). A dogma is a principle laid down by an authority as incontrovertibly true, which is a concept foreign to science. According to Sydney Brenner—who established *Caenorhabditis elegans* as a model organism—Crick regretted using the word "dogma", even if it was a joke, since Crick was not religious and did not believe in dogmas (Brenner and Sejnowski 2018). Also Jacques Monod, in 1954, stated that "Anything found to be true of *E. coli* must also be true of elephants".

Actually, the phrase was coined by the microbiologist Albert Jan Kluyver in 1926 (Friedmann and Friedmann 2004), in any case this is another famous example of an overstatement, it is only partially true.

The problem with generalisation was well-known to both Krogh and Krebs. Krogh considered "a general physiology which can describe the essential characteristics of matter in the living state" as an ideal goal that might need a long time to be achieved, and only by studying the same processes/structures in "myriads of organisms" (Krogh 1929; Jørgensen 2001). In 1980, Hans Krebs, together with John Krebs, wrote a paper warning about the "uncritical application" of the Krogh principle (Krebs and Krebs 1980). As they put it: "This article is a postscript to the earlier essay. It makes the point that an uncritical application of the principle may lead to fallacious generalisations, because extrapolating findings from one species to another is not invariably valid." They also pointed out that while a "unity of life" certainly exists, there is also an "infinite diversity of life", based on different combinations of a small number of basic units. Some biologists are devoted to finding common features, others are devoted to finding diversity, and often the choice between being, respectively, a "lumper" or a "splitter" depends on the personal attitude towards the same scientific issues. Needless to say, both approaches are equally important, and need to be carried out together (Gest 1995). Biology is extremely complex, but while simplification is fundamental to understand complex mechanisms, we cannot underestimate diversity (Cohen 1963; Holmes 1993; Kellogg and Shaffer 1993; Gest 1995; Jørgensen 2001; Bolker 2009; Green et al. 2018). Diversity is both the motor and the result of the evolutionary process. Evolution "overcomes" problems using whatever is available at the moment—a process described by François Jacob as "tinkering" (Jacob 2001)—thus finding multiple solutions for a given problem. An astronomically high number of genotypes can produce the same phenotype, and all these genotypes are organized in networks connected by point mutations (Wagner 2017). At the same time, the chances of overcoming a problem depend on what is available, namely from variability: contingency is an intrinsic property of evolution, so is diversity. For these reasons, it would be wise to avoid the terms "dogma" or "law" in biology.

Model organisms and inductive reasoning are irreplaceable: there is no other way to tackle the complexity of living systems. At the same time, it is not advisable to infer general patterns from a very limited number of species, which are very far from being representative of the diversity of life. How to solve this conundrum? August Krogh in 1929 pointed the way by endorsing the comparative method: "study all sorts of organisms". Some are better than others, depending on the problem under study. We must choose an organism because it has properties suitable for investigating a particular phenomenon, not as a representative for a wide taxonomic group or life in general. We must have "myriads" of models.

"Rosetta Stones" or aberrant creatures? The model-organism-based approach vs the comparative method

Model organisms are often chosen because of practical advantages. They are usually easy to find and breed, convenient to maintain in the laboratory, have a short generation time and rapid development, and respond well to experimental techniques and manipulations (Kellogg and Shaffer 1993; Bolker 2009). We will incorporate such and related features under the term "accessibility", which is what makes a model system more tractable and allows the researchers to focus on specific biological questions disentangling them from a complex background. Other than accessibility, when choosing a model system it is fundamental to assess its "representativeness"—that is, the ability to serve as a basis for inference (Bolker 2009)—with respect to the biological feature that is investigated. At the dawn of molecular biology, bacteria and bacteriophages were used as model organisms to study DNA replication, transcription, and protein synthesis. Since such processes and the molecules involved are shared by all organisms on this planet, the representativeness of these models is very high, and the choice turned out to be quite successful. In that context, bacteria and bacteriophages were "Rosetta Stones"—namely, something that acts as a key to some previously unattainable understanding (Gest 1995). Moving on to studying more complex biological processes, the researchers needed to choose other model organisms, like *C. elegans*, *D. melanogaster*, *Mus musculus*, *Rattus norvegicus*, and others (see Hunter 2008 for a detailed analysis). Of course, over time, as more knowledge accumulates, the life science community is going to study increasingly more complex traits, and this is where biodiversity kicks in, undermining the representativeness of the "traditional" model organisms. As pointed out by Krogh, the solution is to find multiple models, that is, to explore diversity to have a complete picture of the subject of study. From a conceptual point of view, on the opposite side of "Rosetta Stones", there are "aberrant creatures" (Adriaens and Herrel 2009) or "extreme organisms", that is, highly specialized organisms that show very peculiar features. Frequently, the traits that make an organism an attractive model are the result of adaptations, distinctive features that can be experimentally convenient and increase its accessibility. One good example is the work of Krebs on oxidative phosphorylation, that was carried out on pigeon breast muscles because of the robustness of the mitochondria in this kind of tissue. The adaptation to flight made breast muscle mitochondria in birds particularly resistant to stress, thus to experimental protocols (Green et al. 2018). However, adaptations reduce the representativeness of a model, so there is often a tradeoff between accessibility and representativeness (Bolker 2009), which needs to be taken into consideration when extrapolating findings from a model

species to more inclusive taxa. It is interesting to note that while "Rosetta Stones" are suitable to find similarities across species—even if genuine "Rosetta Stones" for living systems in general do not exist—extreme organisms can be used to focus on differences. Such kind of negative models can be exploited to understand, for example, plasticity and limitations of a biological process (e.g.: naked mole-rats for cancer research, and snakes for physiological adaptation to starvation, see Green et al. 2018 for a thorough discussion).

How to infer general concepts and the role of model organisms in comparative biology

When making inferences in biology, we have to pay particular attention to the kind of questions we want to address and to the taxonomic range we want to investigate: are we looking for uniqueness or for more general mechanisms? A significant contribution to the understanding of this dualism is the concept of idiographics vs. nomothetics. These terms were used by the Neo-Kantian philosopher Wilhelm Windelband to describe two distinct approaches to knowledge (Münsterberg 1899; Hurlburt and Knapp 2006). Several disciplines—historical sciences such as anthropology, paleobiology, evolutionary biology, and Evo-Devo—embody both principles. Idiographics is the description of unique and historically contingent features, while nomothetics is the discovery of regularities that can be described as general rules, and concerns the formulation of general concepts. This dualistic perspective helps us to understand the role of model organisms in biological disciplines (Jenner 2008). When we study a specific model organism, we can only deal with the idiographic range. It is not correct to generalise when studying a single taxon, since the comparison between individuals of the same species can provide insights into the biology and evolution of the species itself, but not necessarily into the biology and evolution of its relatives or other distant taxa. Generalizations are easy to propose in biology but seldom valid across all organisms (Gould 1980; Ghiselin 2005). How can we translate unique features to regularities? Namely, how can idiographics be related to nomothetic conceptual categories? We can compare and generalise only if we have a rich idiographic foundation that documents the evolutionary change of the traits we are taking into consideration in our study. This is the reason why there is a need of a judicious choice of model organisms to mine at best idiographics (Jenner 2008). What is the best choice? If we minimise character overlap between model organisms by maximising phylogenetic diversity, we consequently maximise the amount of unique idiographic details captured by the models. Consequently, we minimise the ability to draw general conclusions from them. As we pointed out above, given that model organisms are usually chosen for specific traits any general conclusion drawn from these species is rarely generalisable (Bolker and Raff 1997).

Even though analyzing a broad phylogenetic range of taxa is fundamental to assess diversity, it may not be the best choice to generate nomothetic insights (Jenner 2008). We can access nomothetic themes if we maximise character overlaps between model organisms, choosing them for specific shared traits, as part of a same class. A class is a group of biological entities (e.g.: organisms or parts of them, biochemical pathways, gene regulatory networks, etc) that share features that can be meaningfully compared. Such concept expands, or "completes", the concept of homology, and allows for comparisons beyond monophyletic taxa (Ghiselin 2005). A class can be a group of organisms that share common traits, even if independently evolved, thus distinguished from the taxonomic term "class". From here ahead, when speaking about classes, we refer to the non-taxonomic entity. This ahistorical formulation of classes provides the necessary basis to propose nomothetic insights. Idiographics are most efficiently translated into nomothetic insights when model organisms are chosen based on sharing certain traits, that is, as members of the same class (Ghiselin 2005; Jenner 2008). So, models should be chosen for possessing traits that provide an independent support for a particular concept rather than for their phylogenetic position *per se*; by doing this, the explanatory force—namely, the extension of predictive reliability—can be maximised. Of course, the availability of robust and extensive phylogenetic hypotheses is still fundamental to understand the direction of evolutionary change and the conditions under which certain traits can evolve.

To understand how we can approach generalisation, we have to consider the specific jurisdiction in which the outcome of a specific experiment can be included. We have to consider the "experimental model-organism based approach" versus the comparative approach. There is no *a priori* reason to generalise the outcome of experiments based on a single model organism, since a single counterexample actually falsifies any generalisation (Scholtz 2008). Furthermore, this kind of approach cannot give direct evolutionary inference, since the experimental changes that we can induce in the lab are just an analogy to evolutionary changes: it is not correct to compare a newly obtained phenotype in one species with naturally occurring phenotypes in that species or in others, even if similar, and deduce that a similar change has indeed happened in the course of evolution (Scholtz 2008). Similarly, it is not correct to compare performance and fitness of manipulated organisms with "wild type" organisms, since the latter are functionally balanced and probably mutations disturb this functional balance (Scholtz 2008). For this reason, a reduced fitness may not be a support for the evolutionary stability of characters: the proximate causes of functional disadvantages of change cannot directly explain the ultimate causes of evolutionary change (Scholtz 2008). Only comparison allows generalisation.

Most of the research performed with model species has been justified by their potential power for understanding human biology. To trace general themes and eventually

understand life, we have to overcome such limited view and expand research to "new" model organisms from a wider taxonomic range and in a wider set of environmental conditions. The term model organism was initially used to describe "an organism that is inherently convenient to study a particular area of biology". When saying "model organism", we often mean "an organism for which a wealth of tools and resources exist" (Russell et al. 2017). To date, several organisms are used as models particularly suited to address some research areas—thus being model organisms in the original sense, that is convenient for the study of a biological process—but these often do not possess implemented tools, and dedicated methods and resources. Russell et al. (2017) refer to these organisms as "non-model model organisms" (NMMO).

An example of the use of classes: the link between mitochondria and germline formation

A clear example of the use of classes in building reliable generalisations regards germline development (Extavour 2008) and its link with the mitochondrial inheritance process. Looking at the majority of model species studied so far, the segregation of germline from somatic lineages appears to happen early. The so-called "preformation mode"—that is, early development controlled by maternal factors stored in the egg— seemed to be the predominant mode of germline specification. Preformation was described for *C. elegans*, *Drosophila*, *Gallus gallus*, and plenty of other models. However, as data from additional animal taxa are collected, it is becoming clear that the most common and ancestral mode is epigenesis, characterized by a later segregation of germ cells due to inductive signals in the developing embryo (Extavour 2008). The first idea was the result of the biased choice of models which have in common a faster development. Actually, developmental speed is deeply connected to the use of maternal material in early development. If transcripts newly produced by the embryo and their products are necessary for development to proceed, of course the process is slowed down. If we chose models with slow development, the chance of choosing animals developing through inductive signals wound probably increase. If we consider the class of taxa sharing the preformation mode of germline specification, we may be able to identify the general features that can lead from the ancestral epigenesis to an early way of germline specification and understand how the segregation of material delivered to the progeny has evolved. This kind of approach can be applied to any kind of meaningful trait.

The relevance of germline formation goes well beyond the field of developmental biology. Investigating the times and modes of mitochondrial segregation into the germline is of basic importance: the mechanisms leading to the segregation of mitochondria into germ cells can vary depending on the type of germline specification, early or late (Milani 2015). By sampling animals with one or the other mode, in distantly

related taxa, we have the possibility to address the general principles that lead to one or the other way of specification and at the same time to find, for the two modes, the linked mechanism of mitochondrial selection (Milani 2015).

Given the increasing knowledge about the role of mitochondria in germline formation, these two topics have become tightly intermingled. The presence in many distantly related taxa of mitochondria associated with the germ plasm—material found in germ cells and containing germline determinants—suggested a general mechanism: mitochondria release material for germline specification/formation. Many studies on several animals documented the emission of mitochondrial material merging with germ plasm; this was observed in model organisms such as *Drosophila melanogaster*, *Hemicentrotus pulcherrimus*, *Xenopus laevis*, and *M. musculus* (see Isaeva and Reunov 2001; Milani and Maurizii 2019, and references therein), as well as in non-model organisms (e.g.: Reunov et al. 2019). Moreover, a mitochondrial ribosome-dependent translation in germline formation is required at least in *Drosophila* embryos (Amikura et al. 2005). However, despite this evident link between mitochondria and germ plasm, the mechanism of such interplay is still unknown.

Rising non-model model animals in mitochondrial research

The growing understanding of the diversity of life clearly advises against the routine of formulating hypotheses and inferring general patterns based on a small number of species. Historically, general conclusions inferred from studies based on a skewed and limited taxon sampling led to a restricted and incomplete knowledge of mitochondrial biology. This uneven sampling is reported in Table 1 which shows the number of complete mitochondrial genomes available in the NCBI Nucleotide database, subdivided across animal phyla. The poor (or non-existent) sampling in some groups is evident, as it is the skewed sampling in other groups. For example, human mtDNA represent the 76.7% of all the chordate sequences deposited in the database. More recent evolutionary research (see for example: Ballard and Whitlock 2004; Meiklejohn et al. 2007; Ballard and Pichaud 2014; Dowling 2014) produced clear evidence that some of the "textbook notions" about mitochondria are to be deemed as inaccurate, or plainly wrong. A major consequence of this, is an increased awareness of the central role of mitochondria in eukaryotic life that goes beyond the concept of "powerhouse" (Hill 2015; Hill et al. 2018; Sloan et al. 2018; Havird et al. 2019a,b). Accordingly, Geoffrey Hill recently coined the term "Mitonuclear Ecology" to indicate the emerging interdisciplinary field that studies fundamental concepts of evolutionary ecology such as sexual reproduction, origin of two sexes, sexual selection, adaptation, and speciation in the light of mitonuclear interactions (Hill 2015, 2019). Exploratory research on non-model

organisms broadened the variability of mitochondrial biological features well beyond the "textbook notions" (Breton et al. 2014). For this reason, it is necessary to exploit the new technologies to extend the number of species under investigation, including new models and understudied groups. Such "uncommon" biological systems can help unveiling unknown elements of mitochondrial biology and evolution. In this section, we discuss the potential of non-model animal systems in mitochondrial research and highlight some promising candidates.

There are notable examples of NMMOs systems in mitochondrial biology. We enlist some representatives in Table 2, including fish, mammals, birds, crustaceans, and bivalve molluscs. This is not intended to be a comprehensive review of the whole range of NMMO animal species under study to date, but it is just an example of how non-model organisms can be chosen in a comparative biology framework. The NMMOs highlighted here have special, often unique, traits to address questions of general importance. These organisms are studied not only because they are a kind of "aberrant creatures" useful to gather novel idiographic records, but they can also be fundamental system to address central questions about biology that have remained unanswered to this day (Russell et al. 2017).

Killifish (*Fundulus spp.*, *Nothobranchius furzeri*): environmental adaptation, ageing

Members of the genus *Fundulus* have been investigated for adaptation to different temperatures (Fangue et al. 2009; Baris et al. 2016; Chung, Bryant, et al. 2017; Chung, Morrison, et al. 2017) and different salinity (Whitehead et al. 2012). Differences within and between species (different populations of *F. heteroclitus*, and *F. grandis*) showed the involvement of mitochondria in thermal adaptation. Indeed, temperature changes affect metabolism and mitochondrial performance, playing a role in whole-animal thermal tolerance and plasticity. These studies support a general role for oxidative phosphorylation (OXPHOS) and mitochondrial oxygen kinetics in differentiating aerobic performance and in influencing species responses to environmental change (Chung, Morrison, et al. 2017). Comparisons of mitochondrial responses to thermal changes in different tissues investigated using high-resolution respirometry in subspecies adapted to different temperatures documented an extensive plasticity in mitochondrial performance following thermal acclimation in killifish, with the extent of responses differing between tissues (Chung, Bryant, et al. 2017). Also lipid remodeling in mitochondrial membranes appears to be a mechanism contributing to these changes (Chung et al. 2018). *F. heteroclitus* is also studied for cytonuclear disequilibrium that appears to act in reproductive isolation or selection against hybrids (McKenzie et al. 2016). The species is abundant in estuaries and it shows adaptations to different water salinity. Comparative experiments were performed to track the transcriptomic and

physiological responses to salinity variation, and to explore the regulatory mechanisms that may enable osmotic acclimation. Some genes showed population- and salinity-dependent patterns of expression during acclimation, including genes involved in mitochondrial function (Whitehead et al. 2012).

The killifish *Nothobranchius furzeri* is becoming a model for the study of senescence, being naturally short-lived (Hartmann et al. 2011). Shorter- and longer-lived individuals of the species differ in gene expression, especially in early life. Mitochondrial complex I expression resulted negatively correlated with lifespan, with the phenotype reversed by complex I inhibitors (reversion of aging-related regulation of gene expression and extended lifespan) (Baumgart et al. 2016). An integrative genomic and genome-editing toolkit using the *de novo*-assembled genome and the CRISPR/Cas9 technology was developed for this species. This approach will be important to target candidate genes related to ageing (López-Otín et al. 2013), including mitochondrial dysfunction (Harel et al. 2015). Aging in *N. furzeri* appears associated with a decline in mtDNA copy number, downregulation of mtDNA-associated genes and an impairment of mitochondrial function (Hartmann et al. 2011). This species appears to be an ideal model to assess the role of physiological and environmental parameters on ageing and lifespan determination.

Deer mice (*Peromyscus spp.*): environmental adaptation

At high-altitude, small mammals have to support thermogenesis and aerobic exercise in spite of the reduced oxygen availability. To address this topic, many studies have been performed involving highland and lowland *Peromyscus maniculatus* (Lui et al. 2015; Nikel et al. 2018). The integration of data from different approaches, such as measures of whole-organism thermogenic performance, measures of metabolic enzyme activities, and genomic/transcriptional profiles was used to examine the adaptive variation of this complex trait in deer mice that are native to different elevations. Different species of the same genus have been compared for such research (*Peromyscus maniculatus* and *Peromyscus leucopus*) (Cheviron et al. 2012). Highland deer mice have an enhanced thermogenic capacity under hypoxia compared with lowland conspecifics and closely related lowland species. This feature is largely due to an increased capacity to oxidize lipids associated with elevated activities of muscle metabolic enzymes that influence flux through fatty-acid oxidation and oxidative phosphorylation pathways in high-altitude deer mice, and by concomitant changes in the expression (Cheviron et al. 2012). Other than having higher respiratory capacities in high-altitude mice than in low-altitude mice, the former also showed higher mitochondrial volume densities (Mahalingam et al. 2017). The association between transcriptomic profiles and muscle phenotypes was also tested: several genes involved in energy metabolism were more expressed in highlanders, and the regulators of mitochondrial biogenesis were positively correlated

with muscle oxidative phenotype (Scott et al. 2015). In species that are distributed across steep environmental gradients, adaptive variation in physiological performance may be also due to transcriptional plasticity in underlying regulatory networks (Cheviron et al. 2014).

Naked mole-rats (*Heterocephalus glaber*): ageing

Naked mole-rats (*Heterocephalus glaber*) are the longest-living (~30 years) rodents known to date (Buffenstein 2005), thus being an important model to examine mechanisms modulating ageing. Naked mole-rats show negligible senescence, no age-related increase in mortality, and high fecundity until death (Kim et al. 2011). In addition, they are resistant to cancer—both spontaneous and experimentally induced—thus challenging the theories that link ageing, cancer, and redox homeostasis. Indeed, although characterized by significant oxidative stress—young individuals surprisingly have high levels of accrued oxidative damage (Buffenstein 2005)—the naked mole-rat proteome does not show age-related susceptibility to oxidative damage nor increased ubiquitination (Kim et al. 2011). Traditionally, the main mammalian models used for the study of ageing have been mice and rats, i.e. short-lived species (Dammann 2017). To reach a reliable generalisation and to deal with life-extending mechanisms, a long-lived species such as *H. glaber* can be precious; accordingly, in the past two decades, long-lived mammals (i.e. mole-rats and bats) became a matter of investigation. These new models drew attention to the oversimplification problem affecting existing theories about ageing in Chiroptera, and to several features considered universal components of enhanced longevity in mammals (Dammann 2017). Indeed, among mammals with high longevity considerable variation exists with respect to candidate regulatory mechanisms (Dammann 2017). Mitochondrial morphology and abundance were investigated in naked mole-rats, leading to interesting findings. In contrast to mitochondria of other mammalian cardiomyocytes, where the internal space is filled with parallel rows of cristae, in naked mole-rats they have a chaotic pattern of wave-like cristae. In addition, there are some large mitochondria exceeding normal sizes by two to three times, with cristae assembled into groups with curved and ring-like structures (Bakeeva et al. 2019). Moreover, in these animals, mitochondrial number and size increase with age. They also possess a neotenic type of chondriome accompanied by specific features of oxidative phosphorylation, and a significant decrease in the level of matrix adenine nucleotides (Holtze et al. 2016). RNA sequencing was used to compare liver gene expression profiles between naked mole-rats and wild-derived mice. Genes associated with redox processes and mitochondria were expressed at higher relative levels in naked mole-rats. Also, a protease inhibitor and a mitochondrial complex II subunit, both ageing-related genes, were found strongly over-expressed in the naked mole-rat, suggesting alterations in mitochondrial and oxidation reduction pathways in the species (Yu et al. 2011). A combination of bioinformatic algorithms with nucleotide genomic

signal processing and hierarchical cluster methods were also used to investigate differential phylogenetically-convergent genetic traits related to senescence of the long-lived naked mole-rat and various species. Two aging-related mitochondrial genes were selected based on the classification of aging-related genes in Human Ageing Genomic Resources database. Cytochrome b and Cytochrome c oxidase subunit I are both related to neurodegenerative disease (Khayal et al. 2013).

Bats (*Myotis spp.*): heteroplasmy, longevity, ageing

The "free radical theory of ageing" (Harman 1956) posits that a high metabolic rate causes mitochondrial heteroplasmy and progressive ageing (but see Milani and Ghiselli 2015).The extended longevity of bats, despite their high metabolic rate (they are the only mammals with the ability of powered flight), may provide insights into mechanisms of aging. Predictions of the free-radical theory of aging as an explanation for differences in lifespan were tested in *Myotis lucifugus* (little brown bat), *Blarina brevicauda* (Northern short-tailed shrew), and *Peromyscus leucopus* (white-footed mouse)— maximum lifespan potential: 34, 2, 8 years, respectively—by comparing whole-organism oxygen consumption, hydrogen peroxide production, and superoxide dismutase activity in several tissues. Mitochondria from *M. lucifugus* produced half to one-third the amount of hydrogen peroxide per unit of oxygen consumed compared to mitochondria from *B. brevicauda* and *P. leucopus*, respectively. These results are similar to those reported for birds that, like bats, show high metabolic rates and longevity. These results seem to provide support, at least partially, for the free radical theory of aging as an explanation to longevity of bats (Brunet-Rossinni 2004). A series of genome-wide comparative analyses between bat (*Myotis myotis*) and non-bat mammals was performed to investigate the molecular mechanisms underlying longevity. Bat-specific and differentially transcribed miRNA and mRNA that function in key longevity pathways were detected. As suggested for *M. myotis*, bats may possess unique regulatory mechanisms for resisting tumorigenesis, repairing cellular damage and preventing oxidative stress, all of which likely contribute to their extraordinary lifespan (Huang et al. 2016).

In this connection, analyses were done to see if bats exhibit increased mitochondrial heteroplasmy with age. In *M. myotis*, the majority of heteroplasmy was at a low frequency, and nucleotide changes consisted of transitions. Oxidative mutations were present in only a small number of individuals, there was no significant increase in heteroplasmy with age, and heteroplasmy was found to be dynamic (from recaptured individuals), without a uniform increase over time. All that considered, the data acquired for *M. myotis* appeared not to follow the predicted increase in heteroplasmy as posited by the free-radical theory of ageing, questioning its general validity (Jebb et al. 2018). On average, bats and birds live substantially longer than non-flying mammals of similar body size. The combination of small body size, high metabolic rates, and long lifespan

they share does not seem to support oxidative theories of ageing. However, large-scale comparative analyses on a few emerging model species have identified several mechanisms for resisting oxidative damage in mtDNA and cellular structures in both bats and birds (Munshi-South and Wilkinson 2010).

Eastern yellow robin (*Eopsaltria australis*): environmental adaptation, mitonuclear coevolution

Diversifying selection on metabolic pathways can reduce intraspecific gene flow and promote population divergence. An opportunity to explore this arises from mitonuclear discordance observed in an Australian bird *Eopsaltria australis*. Across 1,500+ km, nuclear differentiation is low, whereas two highly divergent, parapatric mitochondrial lineages show a discordant longitudinal geographic pattern and experience different climates. *E. australis* is one of several animals that shows functional mitonuclear interactions despite discordance between mitochondrial and nuclear genomes. The two mitolineages show sharp climate-correlated differences in their distributions, suggesting that the mitochondrial introgression and divergence were driven by natural selection (Sunnucks et al. 2017). Evidence of positive selection has been found in genes of OXPHOS complexes, predicted to cause differences in electrostatic subunit-subunit interactions influencing coupling efficiency of the complex (Morales et al. 2015; Sunnucks et al. 2017). This selection may reflect local environmental adaptation, a by-product of other selective processes, or genetic incompatibilities (Morales et al. 2015).

Comparison of fixation indexes in the nuclear genomes between Eastern yellow robin populations across their biogeographic range revealed the existence of two genomic islands of divergence against a background of low differentiation (Sunnucks et al. 2017). A strong genetic differentiation and sequence divergence was detected in a region of chromosome 1A mirroring the geographic pattern of mitochondrial DNA divergence. Such region is enriched for genes performing mitochondrial functions. Molecular signatures of selective sweeps in this region alongside those in the mitochondrial genome suggest a history of adaptive mitonuclear co-introgression, consistent with mitonuclear co-evolution as an important mechanism for population divergence and local adaptation (Morales et al. 2018). Studies on a large set of Australian birds were performed to investigate whether Pleistocene climate changes drove mitochondrial selection and evolution. Climate was a significant predictor of mitochondrial variation in eight out of 17 species (Lamb et al. 2018).

Tigriopus californicus: environmental adaptation, mitonuclear coevolution

The copepod *Tigriopus californicus* shows a very rapid mtDNA sequence evolution (Willett 2012), reaching extreme mtDNA divergence between populations with

exceptionally high levels of amino acid differentiation compared with other taxa (Edmands 2001; Burton et al. 2007). Hybridization between allopatric populations expose functional incompatibilities between genomes that have not coevolved and places intrinsic selective pressures on many nuclear genes involved in mitochondrial functions (Barreto and Burton 2013). Ellison and Burton (2006) measured fitness, mitochondrial function, and enzyme activity in hybrid lines of *T. californicus*, and showed that only the OXPHOS complexes composed of both nuclear and mitochondrial subunits suffered a loss of activity. Moreover, they observed a positive correlation between OXPHOS enzyme activity and mitochondrial function, and between mitochondrial function and fitness. Moreover, the analysis of transcript levels of genes involved in the OXPHOS pathway for a series of parental and inbred hybrid lines showed that only genes located on the mtDNA differed among lines. Lines bearing certain genotypic combinations of mtDNA-mitochondrial RNA polymerase showed a diminished capacity to up-regulate mitochondrial genes in response to hypo-osmotic stress. This suggests that disruption of coadaptation between nuclear and mitochondrial genes contributes to the phenomenon of hybrid breakdown (Ellison and Burton 2006, 2008). *T. californicus* nuclear genome shows an elevated protein evolutionary rates and putative positive selection in genes predicted to interact with mtDNA, mtRNAs, and the corresponding proteins encoded. Thus, the rapid mitochondrial evolution appears to drive compensatory nuclear evolution within populations, thereby providing a potentially important mechanism for reproductive isolation (Barreto et al. 2018).

As reported above for certain non-model vertebrates, variation in thermal tolerance plays a key role in determining the biogeographic distribution of organisms, and variation in mitochondrial function likely contributes to variation in thermal tolerance. Populations of *T. californicus* were also studied in this concern, showing a latitudinal thermal gradient along the coast of California (USA), suggesting thermal adaptation. The obtained data supported a population-specific rates of ATP synthesis at chronic temperatures with a role for mitochondria in setting thermal range limits, and indicate that divergence in mitochondrial function is likely a component of adaptation across latitudinal thermal gradients (Harada et al. 2019).

T. californicus differs from standard animal models used in genetics since it lacks heteromorphic chromosomes but has a polygenic sex-determination mode. Reciprocal F2 hybrids between two strongly differentiated populations were used for quantitative trait loci mapping. Although many studies on standard speciation models found the strongest genetic incompatibilities to be nuclear-nuclear (i.e.: X chromosome-autosome), in *T. californicus* the strongest deleterious interaction was mito-nuclear. This system thus is important to study the genetics of reproductive isolation in comparison to standard model systems (Foley et al. 2013).

Bivalve molluscs: heteroplasmy, mitochondrial inheritance, mitonuclear interactions, genomic conflicts

More than a hundred species of bivalve molluscs are known for their unique mode of mitochondrial transmission, known since 1994 as doubly uniparental inheritance (DUI) (see Zouros 2013 for a review). DUI species present two mitochondrial lineages, one transmitted by females (F-type), one by males (M-type), this achieved by having homoplasmic gametes: eggs for the F-type, spermatozoa for the M-type. Adult animals are heteroplasmic to various degrees, with interspecific mtDNA nucleotide divergence reaching exceptionally high values (Ghiselli et al. 2019 and references therein). The unusual mechanism of inheritance and the natural heteroplasmy provide an interesting point of view about mitochondrial biology in general. For example, the "division of labour hypothesis" (Allen 1996) posits that since mtDNA is maternally inherited, female gametes would prevent oxidative stress and mtDNA damage by repressing OXPHOS, thus being quiescent genetic templates. Interestingly, DUI species have swimming spermatozoa transmitting their mtDNA for hundreds million years, with no apparent consequence for species survival (Milani and Ghiselli 2015; Ghiselli et al. 2018). The presence of a stable and natural heteroplasmy of highly divergent mtDNAs makes DUI animals ideal systems to investigate genomic conflicts (Milani et al. 2016), and how an individual, a tissue, a cell, an organelle can deal with multiple mitochondrial variants (Ghiselli et al. 2019). So far, the DUI system was investigated mainly at the mtDNA level (Zouros 2013), and with RNA-Seq (Ghiselli et al. 2012, 2013; Capt et al. 2018; Iannello et al. 2019), even if more recently mitochondrial phenotypes of different kinds has been analyzed. For example, differences between F- and M-type in OXPHOS activity—inferred by bioinformatics tools (Skibinski et al. 2017), or by direct test on oxygen consumption and bioenergetics (Bettinazzi et al. 2019; Bettinazzi et al. this Issue)—were recently examined. Such divergent phenotypes may be produced by amino acid substitutions at specific sites of OXPHOS subunits differentiating the F-type from the M-type (Skibinski et al. 2017). According to these new findings, the membrane potential may be involved in mitochondrial segregation into the germline (Milani 2015), and/or with sperm-specific adaptations (Ghiselli et al. 2013; Bettinazzi et al. 2019; Bettinazzi et al. this Issue). These studies evaluated mitochondrial functions associated with sex-linked mtDNAs in DUI species—among which *Arctica islandica*, the oldest animal known to date (Butler et al. 2013)—, compared DUI species with bivalve species showing the common strict maternal inheritance of mitochondria, and compared homoplasmic with heteroplasmic tissues. The results suggest that the inheritance of sex-linked mitochondria not only could better fulfill different energetic requirements of sperm and eggs, but could also be associated with success in facing heteroplasmy and ageing (Bettinazzi et al. 2019; Bettinazzi et al. this Issue).

The comparative method in the "-omics" era: future perspectives

In the last 15 years biology changed radically. New technologies have reshaped the way data are collected and, most importantly, the amount of data that it is possible to obtain at an increasingly lower cost. Above all, High Throughput Sequencing (HTS) granted virtually all labs around the world access to volumes of data unthinkable before. As a result, "big data" became the foundation of most of the research in life sciences, and "data mining" became one of the most utilized methods. While such change represents a breakthrough innovation that greatly improved the potential of solving increasingly complex problems, it also has downsides. The deluge of data generated gave rise to what Sydney Brenner called "factory science" in his 2007 Vanderbilt Discovery Lecture entitled "The Next 100 Years of Biology" (video available at: http://hdl.handle.net/1803/1088), citing his words: "I like to call it low-input, high-throughput, no-output biology". This departure from hypothesis-driven research towards model-independent data mining was prophetically discussed in two papers by John Allen in 2001 (Allen 2001a, 2001b). Allen used Karl Popper's metaphor of "the bucket and the searchlight" (Popper 1975) indicating two approaches to knowledge: the big-data approach, represented by the bucket, as opposed to the hypothesis-driven research, represented by the searchlight. It is true that HTS has made more systems accessible, but maybe it has unified the approach, extinguishing other methodological approaches in its wake. Are we moving from "a few model systems" to "a few model technologies", and if so, which bottleneck is more consequential? We do not wish to demonize HTS and new technologies which will likely be the pillars of new discoveries in the next few years. That said, we need to use such augmented technical power wisely.

HTS has surely made research on non-model species more accessible, but there are still limitations, and oftentimes the optimism about the revealing potential of genomics/transcriptomics data is excessive. We briefly list the major difficulties the researchers have to face when applying the comparative method.

Sequencing disparity

New sequencing technologies were supposed to facilitate the study of organismal biodiversity, by making non-model species more accessible. This is not happening. David et al. (2019) analyzed species representation of nonhuman eukaryotes in NCBI Sequence Read Archive, and found out that while species richness has been increasing, species evenness is decreasing. They reported a growing bias in favour of a minority of species: the top 1% most studied organisms represent a larger proportion of

the experiments. In the next future it will be necessary to reverse such trend, trying to explore diversity much more extensively. Initiatives such as the Global Invertebrate Genomics Alliance (GIGA) aims to promote standards that facilitate comparative approaches to genomics and collaborations across the scientific community (Voolstra et al. 2017). It might sound ridiculous to split animals in vertebrates (including a subphylum of Chordata) and invertebrates (including all the other animal phyla), but given the disparity of genomics resources available—mostly vertebrates—it has a practical use. During the Biogenomics 2017 Conference in Washington D.C. it was announced the Earth Biogenome Project, which aims to sequence, catalog, and characterize the genomes of all eukaryotic biodiversity over a period of 10 years (Lewin et al. 2018). This project might seem overly ambitious, but 10 years is a long time for science and technology, and this impressive collaborative effort will likely reduce sequence disparity and enhance our ability to do comparative analyses.

What to compare and how?

When choosing a model or extrapolating findings to other organisms, it is extremely important to know the phylogenetic context (Kellogg and Shaffer 1993; Bolker 2009). Not only knowing the phylogenetic relationships among organisms provides information about relatedness and divergence time, but it is fundamental to assess character evolution, polarity, and homology: all essential data for the comparative method. For most of the animal taxa there is a lack of phylogenetic framework—even for taxa closely related with model organisms—and this results in a problematic assessment of biodiversity and evolutionary relationships. Not being able to reconstruct the evolutionary history of the organisms undermines the comparative method.

Another fundamental resource we need when performing comparative analyses is gene annotation, which is the assignment of an identity and a function. Annotation works by translating the information available for model or well-known species, to the species subject of study (Primmer et al. 2013). Such translation is mostly based on sequence similarity—with all the obvious limitations—and nowadays it is usually performed through fully automated procedures. The massive amount of genomics data generated and the consequent lack of human curation is causing a decrease in annotation quality (Salzberg 2019). Annotation is a difficult and time-consuming task, but unfortunately the reward for such effort is almost non-existent: it would be difficult to publish a curated genome annotation in a relevant journal. In general, producing data is much more rewarding than curating them; in Sydney Brenner's words: "We presently have huge amounts of data that are largely unassimilated. I have discovered that while there are rewards for collecting data and distributing data, there is little, if any, support for organizing it." (

Strictly related with annotation, there is another big challenge—perhaps the biggest one —for comparative biologists, that is the concept of homology. Homology refers to the historical continuity of characters and it is a fundamental concept in biology, nevertheless, it is "conceptually highly elusive" (Wagner 2007). The main difficulty with homology is basically the topic of this Issue: the loose connection between genotype and phenotype. To make meaningful evolutionary studies we need to compare genes that share ancestry and that retained the same function through evolutionary time. The so-called "ortholog conjecture" (Nehrt et al. 2011) states that orthologs—homologous genes originated by a speciation event—retain the same function in the course of evolution, while paralogs—homologous genes originated by a duplication event—tend to evolve new functions. Currently, the typical procedure in comparative analyses is strictly based on the "ortholog conjecture", and consists in comparing putatively orthologous sequences identified by sequence similarity (Koonin 2005). As usual, the reality is much more complicated. The multi-domain architecture of most eukaryotic proteins, gene duplication, recombination events, exon shuffling, gene fusion and fission, are only some of the several processes that go under the spot-on definition of "molecular tinkering" (Graur 2016), which together with hybridization and introgression make the notion of homology at the molecular level an "elusive concept" (for a thorough discussion on homology and related concepts, see Wagner 2014). A possible solution to this problem is to compare gene regulatory networks instead of individual genes. Such "character identity networks" as defined by Günter Wagner (2007) would enable more meaningful comparisons.

The importance of "basic research"

The representativeness of a model cannot be established in advance. Steel (2008) defined the "extrapolator's circle", a paradox of model organisms and comparative research: "establishing the suitability of the model would require already possessing detailed knowledge of the of the causal relationship in the target, in which case extrapolation would be unnecessary". The choice of models is an iterative process: we make starting assumptions, test them against our knowledge, and then adjust as necessary, given our new discoveries (Bolker 2009). The key aspect of a model organism is whether a community of scientists is working with it. Implementing the comparative method using as much models as possible basically means doing "curiosity driven" science (also known as basic research). Right now, basic research is underfunded, and mostly overlooked. We hope that, albeit more than 90 years later, an increasing number of biologists will start following the words of, among others, August Krog and Hans Krebs. That would bring more balance between the currently predominant

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Table 1: Availability of complete mitochondrial genomes on NCBI Nucleotide Database.
The table shows the number of living species for each animal taxon, as reported by the 2019 Annual Checklist of the "Catalogue of Life" project (https://www.catalogueoflife.org/col/info/totals), and the number of complete mitochondrial genomes available on the NCBI Nucleotide database (data collected on July 7, 2019). In the "notes" column, we indicated strong biases towards a restricted number of species. Between brackets the percentage of mitochondrial genomes of the cited species, with respect to the total number of available genomes.

Taxon	Current number of living species in CoL	# Complete mt genomes	Notes
Acanthocephala	1,330	7	
Annelida	14,399	100	
Arthropoda	1,082,297	4,427	*Anopheles spp.* 393; *Drosophila spp.* 206; *Magicicada spp.* 125; *Daphnia spp.* 90; *Apis mellifera* 67; *Bombyx mori* 61. Tot = 942 (21.3%)
Brachiopoda	396	5	
Bryozoa	5,434	10	
Cephalorhyncha	237	3	
Chaetognatha	132	11	
Chordata	69,913	63,801	*Homo sapiens* 48,026; *Pan spp.* 239; *Menidia menidia* 190; *Camelus* spp. 179; *Mus* spp. 162; *Rattus norvegicus* 118. Tot = 48,914 (76.7%). *Homo sapiens* = 75.3%
Cnidaria	11,151	237	*Acropora spp.* 72 (30.4%)
Ctenophora	200	5	
Cycliophora	2	0	
Dicyemida	122	0	
Echinodermata	6,828	96	
Entoprocta	171	2	
Gastrotricha	852	1	
Gnathostomulida	100	2	
Hemichordata	139	6	
Micrognathozoa	1	0	
Mollusca	65,442	791	*Architeuthis spp.* 38; *Mytilus spp.* 38; *Potamogyrus spp.* 23; *Octopus spp.* 21; *Unio spp.* 20; *Anodonta spp.* 12. Tot = 152 (19.2%)
Myxozoa	245	4	

Nematoda	3,455	258	*Anisakis spp.* 17; *Caenorhabditis spp.* 17; *Ascaris spp.* 12; *Angiostrongylus spp.* 11. Tot = 57 (22%)
Nematomorpha	361	0	
Nemertea	1,371	19	
Onychophora	167	5	
Orthonectida	25	0	
Phoronida	19	1	
Placozoa	2	6	*Polyplacotoma mediterranea* 1; *Trichoplax aderens* 1; "Placozoan sp." 4.
Platyhelminthes	18,616	436	*Schistosoma spp.* 149; *Echinococcus spp.* 113; *Taenia spp.* 21. Tot = 283 (64.9%)
Porifera	9,092	66	
Rotifera	2,014	3	
Sipuncula	205	7	
Tardigrada	1,018	4	
Xenacoelomorpha	456	12	

Table 2: Non-model model animals in mitochondrial studies.

TAXON	SPECIAL FEATURES	INVESTIGATED TRAITS	REFERENCES
Class Actinopterygii			
Fundulus heteroclitus (Atlantic killifish)	Inhabits a steep latitudinal thermal gradient, with variation in mitochondrial properties	Role of mitochondria and mitochondrial oxidative phosphorylation in variation in the pace of life	Baris et al. 2016 Chung et al. 2017a Chung et al. 2017b Chung et al. 2018 Fangue et al. 2009 McKenzie et al. 2016 Whitehead et al. 2012
Nothobranchius furzeri (African turquoise killifish)	Currently the shortest-lived vertebrate bred in captivity It recapitulates typical age-dependent phenotypes and pathologies	Mitochondrial dysfunction Ageing	Baumgart et al. 2016 Harel et al. 2015 Hartmann et al. 2011
Class Mammalia			
Peromyscus maniculatus (North American deer mouse)	Inhabits high altitude has evolved an enhanced aerobic capacity in hypoxia	Mitochondrial basis for adaptive variation in aerobic performance	Cheviron et al. 2012 Cheviron et al. 2014 Lui et al. 2015 Mahalingam et al. 2017 Nikel et al. 2018 Scott et al. 2015 Scott et al. 2018
Heterocephalus glaber (naked mole-rat)	High longevity Cancer resistant Rarely suffering from age-related diseases	Ageing Age-Dependent Structural and Functional Changes of Mitochondria	Bakeeva et al. 2019 Buffenstein 2005 Dammann 2017 Holtze et al. 2016 Khayal et al. 2013 Kim et al. 2011 Yu et al. 2011
Myotis myotis *Myotis brandtii* *Myotis lucifugus* (bats)	The only mammals capable of powered flight, with an extremely high metabolic rate, but associated with extended longevity	Longevity/Ageing Heteroplasmy Challenging the Free Radical Theory of Ageing	Ball et al. 2018 Brunet-Rossinni 2004 Brunet-Rossinni & Wilkinson 2009 Dammann 2017

			References
			Huang et al. 2016
			Jebb et al. 2018
			Munshi-South & Wilkinson 2010
			Seim et al. 2013
Class Aves			
Eopsaltria australis (Eastern yellow robin)	Low nuclear differentiation with two highly divergent, parapatric mitochondrial lineages with sharp climate-correlated differences in their distributions	Climate-driven mitochondrial selection; Mitonuclear coevolution	Lamb et al. 2018; Morales et al. 2015; Morales et al. 2018; Sunnucks et al. 2017
Class Hexanauplia			
Tigriopus californicus	Populations with extreme mitochondrial DNA divergence	Thermal tolerance and its relationship to mitochondrial functions; Mitonuclear coevolution	Barreto & Burton 2013; Barreto et al. 2018; Burton et al. 2007; Edmands 2001; Ellison & Burton 2006; Ellison & Burton 2008; Foley et al. 2013; Harada et al. 2019; Willett 2012
Class Bivalvia			
Species with doubly uniparental inheritance (DUI) of mitochondria	Natural and evolutionary stable mitochondrial heteroplasmy; Transmission of sperm mitochondria across generations; The longest-lived animal to date has DUI (*Arctica islandica*: the specimen "Ming" was 507 years old; Butler et al. 2013)	Mitochondrial biology and inheritance; Heteroplasmy; Challenging the Free Radical Theory of Ageing; Longevity/Ageing; Mitonuclear interactions; Genomic conflicts	Bettinazzi et al. 2019; Bettinazzi et al. (this Issue); Capt et al. 2018; Ghiselli et al. 2012; Ghiselli et al. 2013; Ghiselli et al. 2018; Ghiselli et al. 2019; Iannello et al. 2019; Milani 2015; Milani & Ghiselli 2015; Milani et al. 2016; Skibinski et al. 2017; Zouros 2013