Title: Adaptive cellular evolution or cellular system drift in hares

Short title: Adaptive evolution or drift in cells

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Adaptations occur at many levels, from e.g. DNA sequence of regulatory elements and cellular homeostatic systems, to organismal physiology and behaviour (Mayr, 1997). Established adaptations are maintained by purifying and stabilizing selection. Students of animal diversity tend to focus on higher order traits, anatomy, physiology, organismal function and interactions. The core cellular and metabolic systems of metazoans evolved early in their history, and are assumed to be rather similar between groups. The housekeeping functions and core metabolic functions of cells are generally considered relatively static, especially among closely related species. The extent to which evolution shapes core cellular metabolism and physiology in animals is largely unexplored. Ecological opportunities or strong positive selection can alter basal metabolic rate, activity levels and life-history traits (*e.g.* lifespan, age of maturity, offspring number) and potentially lead to divergence in core cellular and metabolic trait systems (Norin & Metcalfe, 2019; Speakman, 2005). Furthermore, systems under stabilizing selection can also change. Developmental systems of related species may produce the same phenotype or structure, but experience drift that can alter connections and even lead to turnover of cogs in the system (True & Haag,

2001). Are the cellular functions of animals highly constrained, subject to cellular system drift or affected by positive selection? This was tackled by a new study by Kateryna Gaertner and colleagues in a From the Cover manuscript in this issue of Molecular Ecology (Gaertner et al., 2022), using fibroblasts from the closely related but ecologically distinct brown and mountain hares.

The study focused on two species, brown hare (*Lepus europaeus*) and mountain hare (*Lepus timidus)* from northern Europe. They share a common ancestor about 3 million years ago, differ by ~1% in sequence of orthologs, and can hybridize in nature. They differ in several traits (figure 1), notably the mountain hare grows slower, has fewer offspring and has a lower metabolic rate during winter (Angerbjörn & Flux, 1995; Gaertner et al., 2022). Differing life history strategies might reflect or manifest as cellular or metabolic evolution or trade-offs. To assess this, Gaertner and colleagues established immortalized fibroblast cell lines from 4 individuals of each species (sampled in southern Finland) and analysed the mRNA transcriptome and multiple cellular properties. The sampled individuals differed clearly genetically, showing no indications of hybridization or introgression (Gaertner et al., 2022). Expression of several hundred orthologous genes differed by species. Gene ontology analyses indicated differences in genes related to several systems, including cell migration, cell cycle regulation and oxidative energy metabolism (including mitochondrial functions). The fibroblasts were also subject to analyses of several cellular traits. Cell divisions and wound healing was found to be faster in brown hares, consistent with their faster growth. Mitochondrial function was also studied extensively. The mtDNA copy numbers varied (but not by species) and the average mitochondrial mass was similar (as where several other parameters). However, mitochondrial membrane potential (MMP) was higher in the mountain hares. MMP reflects difference in charge across the inner membrane of mitochondria, but the relation of MMP levels to energy production or cellular stress is poorly understood. Jointly the data are consistent with divergence in cellular metabolism and traits aligning with life-history of the species such as more capacity for growth, earlier reproduction and higher metabolism in the brown hares. This study suggests immortalized fibroblasts have utility for ecological and evolutionary research, to explore divergence in cellular and metabolic traits and the cellular basis of organismal physiology.

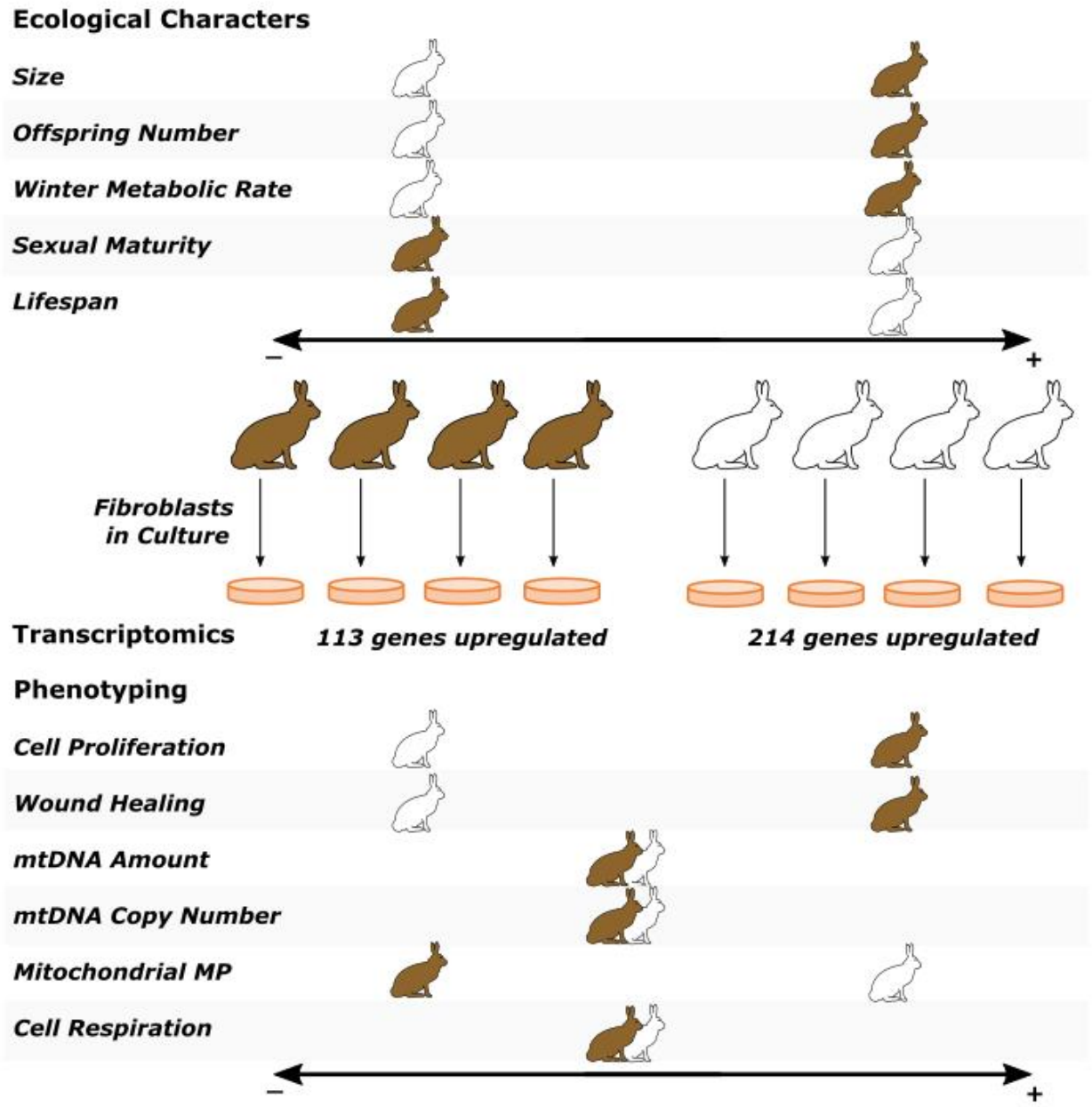

Figure 1. Summary of the experimental set up (Gaertner et al (2022) and variation in life history and cellular traits in brown and mountain hares (coloured brown and white, respectively). At the top, differences in the two species in several life history and physiological traits are summarized. Four individuals of each species were sacrificed and fibroblast cell lines established (one from each). These cells where grown under constant conditions and analyzed with transcriptome

sequencing (mRNA), and various assays for growth and wound healing potential and mitochondrial properties (below). A subset of results are shown, with the outcomes of the three that gave significant difference between species indicated by placement of hare. Outlines of hares were drawn from a photograph by Warren Garst, deposited to Wikimedia commons by Colorado State University Libraries.

https://commons.wikimedia.org/wiki/File:Arctic_hare_sitting_in_grass_-_DPLA_-_0954c78e0cd274e1fe6374ffd29c7912.jpg

This approach has both strengths and limitations, and highlights opportunities for future investigations. The fibroblast cell lines enabled the researchers to analyse cellular phenotypes that are hard to study in living animals or tissue samples (e.g. by controlling for environmental conditions and nutrition, replicating the culture and using specific assays and inhibitors). Specifically, assays on fibroblasts bridge cell biology and ecology, though whether the cells are immortalized or not influences the applicability of specific assays. This approach may be valuable for experimental validation of transcriptomic or genomic signals, or studies of sensitivity to infectious agents or zoonosis, endangered species, and multivariate analyses of cellular traits and metabolites. Fibroblasts may be particularly useful, because they can be harvested from skin biopsies. Thus, they are an ethically appealing option to sacrificing animals (not only redlisted). The main limitations of this study are the low sample size (4 cell lines per species), low replication in the transcriptome analyses, and that only one growth condition was used. With this in mind, it would be interesting to see replication of this work or approach in related systems.

Fibroblasts from multiple species have been compared, mostly to test for associations with longevity and responses to stressors (Harper, Salmon, Leiser, Galecki, & Miller, 2007; Madelaire, Klink, Israelsen, & Hindle, 2022). Multiple traits were analyzed in fibroblasts from 35 bird species, spanning several taxonomic units. The authors assessed multiple traits and found association of growth and stress responses with longevity (after correcting for phylogeny). Curiously, cells from long lived birds proliferated more rapidly than those from short lived birds. There were also indications that the cells in long lived birds were more resistant to injury (Harper et al.,

2011). Studies of mammal fibroblasts revealed that some cellular traits (resistance to protein damage and sensitivity to mitochondrial inhibitors) also associated with lifespan (Harper et al., 2007; Pickering, Lehr, Kohler, Han, & Miller, 2015). These studies highlight the utility of fibroblasts for studies of ecology, evolution and comparative physiology, see summary by Carla B. Madelaire and coauthors. Their key proposition is that fibroblasts may reflect "whole animal physiological phenotypes" sufficiently well (Madelaire et al., 2022).

Several extensions of this work can be imagined. One can ask, are two or more cell lines derived from the same individual similar or different (Jiang & Rinkevich, 2018; LeBleu & Neilson, 2020), reflecting fibroblast subtypes, stochasticity in the cell-retrieval process or natural variation among cells in the body? Also, for a particular adaptive cellular response, are fibroblasts the most suitable cell type to analyze that property? Relatively few independent samples (8 cell lines) were used in the study, and multivariate analyses thus not particularly applicable. Future studies should ideally include more biological and technical replicates, and high throughput phenotyping of cellular and metabolic traits. This could address the question whether the "cellular parameter space" has many or few dimensions? For comparison, the "shape space" of variation in cranial morphology can be summarized with remarkably few dimensions, and these axes of variation compared between species (Hallgrimsson et al., 2012). Cell cultures can be used to test hypothesis, for instance about evolutionary divergence in particular genes, metabolic pathways or cellular functions. Such experiments could validate findings from studies of genomic divergence. Finally, it would be interesting to study fibroblast function and responses to environmental factors or stressors (like temperature or starvation) or the impact of physiological, life history or behavioral related factors (e.g. molecules related to maturity, sex or social status).

Evolutionary divergence in specific traits can result from adaptive changes, drift and side-effects due to linkage or pleiotropy. Slow or no divergence can also reflect physical or metabolic constraints, or very strong stabilizing selection. Does the divergence in metabolic and cellular traits of hares (if real) indicate adaptive divergence in the mountain hares or metabolic system drift? A study of more hare species (differing in key life-history traits) and crucially outgroup(s) could address this. Other species-rich (or within species) systems with repeated evolution of similar growth and reproductive adaptations might be investigated similarly. Demonstration of repeated

adaptive evolution of similar functions in cells and metabolic systems of animals would be most exciting. Neutral evolution of core cellular (housekeeping) functions is rather unlikely due to purifying selection. The essential features of cellular machinery and their metabolic capacities are expected to be under stabilizing selection. Analyses of developmental systems have indicated that for traits under stabilizing selection, the underlying genetic architecture and regulatory logic can evolve. Regulatory elements are a good example. Their overall functions may be evolutionarily stable (turn on a gene in right place and time), but the individual functional units (transcription factor binding sites) may evolve or even turn-over (Ludwig et al., 2005). Something akin to developmental system drift (True & Haag, 2001) may occur in cellular and metabolic systems. This might manifest, in comparisons of multiple taxa, as stasis in the more fundamental traits but drift in others or the underlying machinery.

Comparative genomics and experimental studies show the diverse metabolic capacities of microbes and the evolutionarily versatility of their metabolic systems. Genomic data indicate the enzymatic potential of an organism, but the actual metabolic capacity, steady state levels and sensitivity to environmental factors has to be measured. In the future, analyses of cellular properties and responses in culture, like this interesting study of related hare species, may further challenge our assumptions about the evolutionary stability of core cellular phenotypes and physiology.

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