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Authors for correspondence:
Ryan J. Weaver
e-mail: ryan.weaver@utexas.edu
Justin C. Havird
e-mail: JHavird@utexas.edu

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Physiology

High mitochondrial mutation rates in *Silene* are associated with nuclear-mediated changes in mitochondrial physiology

Ryan J. Weaver[1], Gina Carrion[1], Rachel Nix[2], Gerald P. Maeda[1], Samantha Rabinowitz[1], Erik N. K. Iverson[1], Kiley Thueson[1] and Justin C. Havird[1]

[1]Department of Integrative Biology, University of Texas at Austin, Austin, TX 78712, USA
[2]Hankamer School of Business, Baylor University, Waco, TX 76798, USA

RJW, 0000-0002-6160-4735; JCH, 0000-0002-8692-6503

Mitochondrial (mt) respiration depends on proteins encoded both by the mitochondrial and nuclear genomes. Variation in mt-DNA mutation rates exists across eukaryotes, although the functional consequences of elevated mt mutation rates in some lineages remain underexplored. In the angiosperm genus *Silene*, closely related, ecologically similar species have either 'fast' or 'slow' mt-DNA mutation rates. Here, we investigated the functional consequences of elevated mt-DNA mutation rates on mt respiration profiles of *Silene* mitochondria. Overall levels of respiration were similar among Species. Fast species had lower respiration efficiency than slow species and relied up to 48% more on nuclear-encoded respiratory enzymes alternative oxidase (AOX) and accessory dehydrogenases (DHex), which participate in stress responses in plants. However, not all fast species showed these trends. Respiratory profiles of some enzymes were correlated, most notably AOX and DHex. We conclude that subtle differences in mt physiology among *Silene* lineages with dramatically different mt mutation rates may underly similar phenotypes at higher levels of biological organization, betraying the consequences of mt mutations.

1. Introduction

The mitochondria (mt) of most eukaryotes contain their own genome which encodes essential proteins that form the functional core of the mitochondrial electron transport system (ETS, figure 1*a*). The primary function of the ETS is to generate ATP via oxidative phosphorylation (OXPHOS) which provides a critical resource for myriad cellular functions. Proteins encoded by mt-DNA alone, however, are insufficient for OXPHOS. Many nuclear (N) encoded proteins are targeted to mitochondria and interact with mt-encoded protein subunits to form functional chimeric ETS complexes (figure 1*a*, e.g. CI, CIII, CIV and CV). The resulting mito–nuclear interactions are proposed to be critical for OXPHOS and other mitochondrial functions, and to play broad roles in evolution [1–3].

The ETS also contains strictly N-encoded proteins that act as alternative entry (alternative NAD(P)H dehydrogenases; DHex) and exit (alternative oxidase; AOX) pathways for electrons (figure 1*a*) that are activated under certain cellular conditions to maintain OXPHOS [4]. AOX and DHex activation mitigates changes in cellular redox conditions which, if left unchecked, can lead to oxidative stress and damage to cellular components, including nucleic

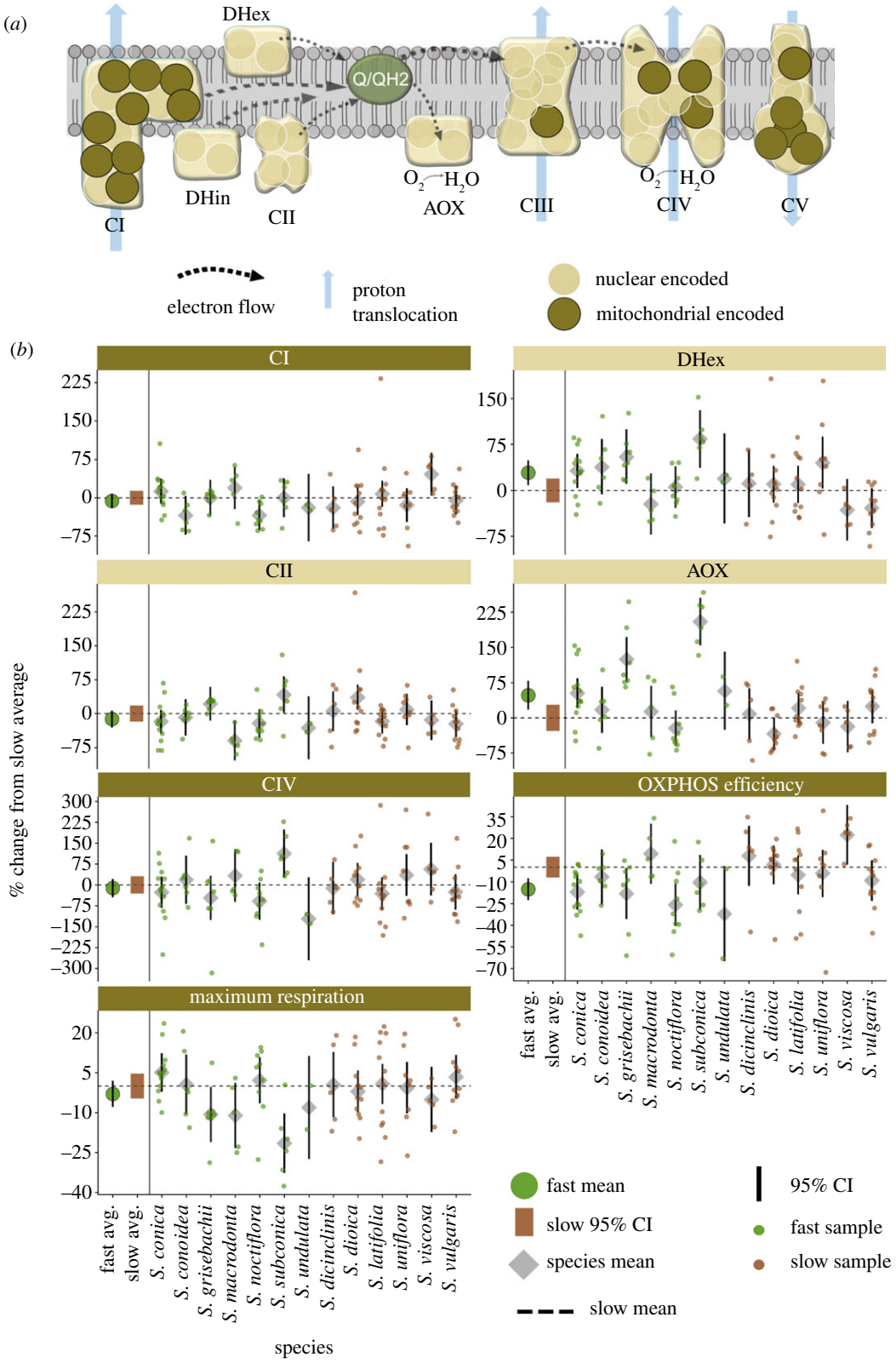

Figure 1. (*a*) Overview of relevant aspects of plant mitochondrial electron transport chain components. Electrons enter through complex I, (CI), CII or the alternative NADH dehydrogenases (DHex/in) and follow either the alternative oxidase pathway (AOX) or the cytochrome pathway CIII – CIV to reduce oxygen to water. CI, CIII and CIV translocate protons to establish the protonmotive force used by CV for ATP production. (*b*) Differences in mitochondrial (mt) flux control factors (FCF) and maximum respiration between *Silene* species with slow or fast evolving mt-DNA. Means with 95% confidence intervals (CI) that do not overlap the slow mean are statistically different at $\alpha = 0.05$.

acids [5–7]. Such changes in redox conditions occur, for example, when the cytochrome pathway (CIII–CIV) is impeded by endogenous or exogenous stressors. Accordingly, DHex and AOX have been hypothesized to play general roles in cellular stress responses, especially in plants [4,8–12].

While mt mutation rates are elevated compared to nuclear DNA in most bilaterian animals [13], this trend is reversed in most angiosperm lineages [14]. However, some closely related species within the angiosperm genus *Silene* have experienced a relatively recent, rapid increase in mt evolution.

Silene species therefore have dichotomous mt mutation rates, despite overall similar morphology and ecology [15]. 'Fast' species have mt mutation rates on a par with mammals (i.e. mt-DNA evolves faster than N-DNA), while 'slow' species show rates similar to typical angiosperms (i.e. mt-DNA evolves slower than N-DNA). The accumulation of slightly deleterious mt mutations in particular is predicted to disrupt mitochondrial function, owing to the uniparental inheritance of mt-DNA and resulting Hill–Robertson like effects [16,17]. Yet how the recent acceleration in mt mutation accumulation in *Silene* has affected mt physiology is unknown.

To illustrate the evolutionary rate differences in *Silene*, in the mt-encoded gene *COX1*, 32 amino acid substitutions have accumulated in the fast species *S. conica* since it shared a common ancestor with *Arabidopsis*, while only seven have accumulated in the slow species *S. latifolia* [15]. Previous work suggests that these substitutions are driven by increased mutation rates and not demographic processes such as a bottleneck in population size—although more definitive tests are needed. Fast species show increases in both nonsynonymous (d_N) and synonymous (d_S) substitutions in mt-encoded genes, but not an elevated d_N/d_S ratio (a hallmark of relaxed selection) [18,19]. Similarly, only N-mt genes show increased d_N/d_S in fast species, whereas all N genes are expected to show increased d_N/d_S after a genetic bottleneck [18,19].

In this study, we assessed the functional consequences of mutation accumulation on mitochondrial respiration in fast and slow *Silene* species. We calculated flux control factors (FCFs), which describe the capacity of an ETS complex to contribute to mitochondrial respiration (see electronic supplementary material, [20]). If rapid increases in mt mutation rates cause deleterious effects, we predicted that chimeric ETS complexes from fast species would show a lower contribution to respiration than those from slow species. Additionally, we predicted that fast species would show increased reliance on strictly N-encoded accessory complexes associated with stress responses.

2. Material and methods

(a) Plant care and representative species

We grew 100 plants representing seven 'fast' and six 'slow' *Silene* species in an environmental chamber under fixed light, humidity and watering schedules to minimize variation from environmental effects, similar to [21]. Multiple accessions were used for many species (i.e. species were represented by multiple collections when possible). See, electronic supplementary material table S1 for sample sizes, species, accessions and origins.

(b) Mitochondrial isolation and respirometry

To account for slight variation in growth rates among individuals, we developmentally matched our samples by harvesting plants with mature leaves prior to flowering and included representatives from both speeds on each sampling day. We sampled 1 g of cauline/rosette leaves from each plant for mitochondrial isolation, following [21]. Briefly, leaves were minced, ground in ice-cold mt isolation buffer [22] and intact mitochondria were isolated using differential centrifugation.

To quantify mt respiration we used an established protocol [21] to measure seven specific aspects of respiratory control of the ETS and overall OXPHOS function using high-resolution respirometry (see electronic supplementary material for details). Briefly, we measured the rate of O_2 consumption from isolated mitochondria from each sample in the presence of specific ETS electron-donors and inhibitors using the Oroboros O2 K system (Innsbruck, Austria). From these consumption rates, we calculated six flux control factors (FCFs): chimeric core ETS complexes CI and CIV, the entirely N-encoded core ETC complex CII, the N-encoded alternative complexes DHex and AOX, and overall OXPHOS efficiency. OXPHOS efficiency in our protocol is similar to the widely used respiratory control ratio and is calculated as the ratio of respiration rate before and after the addition of ADP [23]. We also recorded the maximum respiration rate observed prior to the addition of ETS inhibitors.

(c) Statistical analyses

We used linear mixed-effects models (LMMs) to compare differences in FCFs between fast and slow mutation rates and among species. To control for multiple observations within a species, we included accession as a random factor (see electronic supplementary material for details). We found heteroscedasticity in AOX FCFs between fast and slow rates, so we estimated standard errors separately for each group. We log-transformed the CIV FCF and maximum respiration to meet the assumption of normality. Because FCFs have no meaningful units, we present the results as per cent change from the slow taxa with 95% confidence intervals (95% CI). We also modelled correlations among FCFs and whether correlations differed in slow versus fast species by fitting LMMs with the same random factor as above. We performed these analyses and visualization in R 4.0.0. [24]

3. Results

For the entirely N-encoded accessory ETS complexes AOX and DHex, we found that average FCF values for fast *Silene* species were 48.3% (±30.9, 95% CI) and 29.1% (±20.3) greater than slow species, respectively (figure 1*b*. AOX; d.f. = 71, $p = 0.019$. DHex: d.f. = 70, $p = 0.038$). However, FCF values of CII, which is also strictly nuclear encoded but considered a part of the core ETS, were slightly lower in fast species, although not statistically different (figure 1*b*, electronic supplementary material table S2, d.f. = 72, $p = 0.35$). OXPHOS efficiency of fast species was 15.1% (±7.4) lower than slow species (figure 1*b*. d.f. = 71, $p = 0.005$). We found that chimeric ETS complexes, CI and CIV, and maximum respiration capacity tended to be slightly lower in fast species, although not statistically different (figure 1*b*, electronic supplementary material table S2. $p > 0.38$ for all comparisons).

The magnitude of some FCF values was variable among fast species (figure 1*b*, electronic supplementary material table S3, S4) such that the overall trends observed between fast versus slow species are not uniformly distributed across the sampling of species in this study. The greatest differences in FCFs among fast species were in AOX ($p = 0.001$), CII ($p = 0.01$), CIV ($p = 0.02$) and maximum respiration ($p = 0.002$) (electronic supplementary material table S4). *Silene subconica*, and to a lesser extent, *S. grisebachii* and *S. conica* typically showed greater differences from the slow average than the other fast species (figure 1*b*).

Among the seven respiratory measures calculated, we found that several were correlated, and that the magnitude of that correlation was similar between fast and slow species in most cases (figure 2*a–f*, electronic supplementary material figure S1). However, the strength of the relationship between AOX and DHex depended on mt mutation rate (interaction $p < 0.001$). OXPHOS efficiency was lower when AOX flux

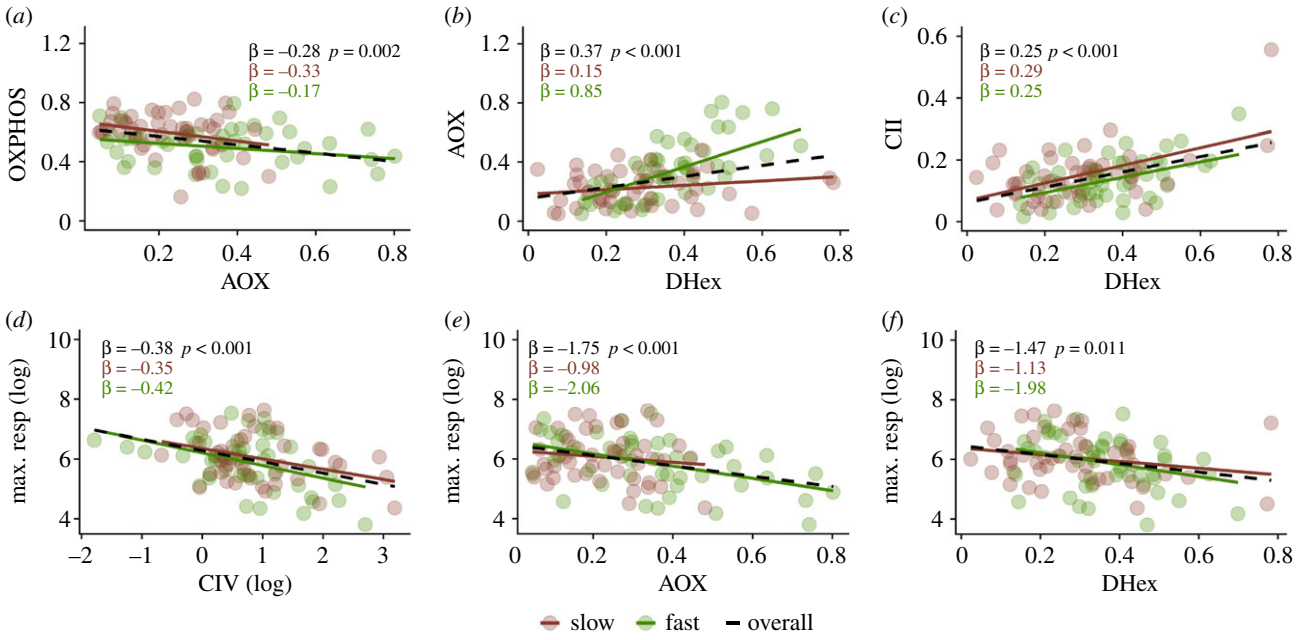

Figure 2. Relationships among FCF types in *Silene* with different mitochondrial mutation rates. Dashed lines show the relationship between the two variables overall from LMM. The brown circles and line show the individual samples and model estimated slope for the slow species and the green circles and line show the same for the fast species.

was higher in both fast and slow species ($p = 0.002$, interaction $p = 0.44$). CII values increased with DHex in both groups ($p < 0.0001$; interaction $p = 0.72$). Higher CIV, AOX, and DHex FCFs were associated with lower maximum respiration rates in both groups (figure 2*d–f*; $p < 0.01$ for all; interaction $p > 0.37$ for all).

4. Discussion

Here we found subtle, yet measurable differences in mt physiology between closely related species with dramatically different mt mutation rates. Overall, mitochondria from fast species performed similarly to slow species, but showed a higher capacity of nuclear-encoded accessory respiratory proteins to contribute to mitochondrial respiration (figure 1*b*). The greatest difference we found was in AOX capacity, which functions primarily as a stress mediator, preventing mt damage from excessive reactive oxygen species (ROS) production [20,25,26]. Mitochondria from fast species are possibly primed to mitigate oxidative or other stressors that could impede chimeric ETS function. Electron flow through AOX is typically activated when metabolic flux through the chimeric cytochrome pathway complexes is inhibited [4]. Impeded electron flow through the cytochrome pathway causes an overly reduced ETS and has the potential to produce excessive ROS [27]. A historic, relatively rapid increase in mt mutation rate during *Silene* evolution [15,19] may have caused a dramatic shift in oxidation state due to inhibition of electron flow through the cytochrome pathway [28] resulting in an increased reliance on AOX respiration that is maintained in some contemporary lineages.

Rescue of mitochondrial function from deleterious mt mutations could arise from compensatory changes in N-DNA (e.g. 'mitonuclear coevolution' [29,30]) or plasticity in alternative metabolic pathways. Here, we show that nuclear responses to accelerated mt mutation rates in *Silene* may extend beyond molecular evolution, to mt physiology as well. Although there is currently no evidence of positive selection on AOX or accessory NADHs in fast species, the responses observed here may be due to molecular evolution in the nuclear genes encoding these proteins and/or ancestral plastic responses in mt physiology. We speculate that increased AOX respiration allows for adequate ATP production and maintenance of redox homeostasis to prevent the over-production of ROS, in conjunction with complementary N evolution that may act to mitigate harmful effects on chimeric ETS complex function.

In addition to the physiological differences in some fast versus slow species we found here, responses to increased mt mutation rates may also include complementary N mutations. Nuclear coevolution in response to elevated mt evolution rates has been well documented in *Silene* [18,19,31]. Fast species show elevated rates of evolution and signatures of positive selection in N encoded, mt-interacting genes [19]. However, coevolutionary responses in AOX, DHex and other accessory nuclear mt proteins should be investigated further to complement the changes in mitochondrial physiology observed here.

Differences in FCF values attributed to fast mutation rates were subtle and not universal and may be driven by select species (figure 1*b*). Most notably, *S. subconica* relied heavily on AOX and DHex, supporting previous comparisons of *S. subconica* and *S. conica* [21]. It is unclear whether mt mutation rates remain elevated in fast species, which may explain interspecific variation if individual species or lineages have slowed to different degrees. Furthermore, low effective population size in some fast species like *S. subconica* may cause inefficient selection on mt genes and further reliance on nuclear-encoded complexes. Future work could focus on examining species-level differences in mitochondrial dynamics, including quantifying ETS protein content and gene expression, within fast species.

We found that higher AOX values were associated with lower OXPHOS efficiency across mutation speeds (figure 2*a*). Electron flow through AOX is inherently inefficient for

OXPHOS because AOX does not translocate protons across the inner mitochondrial membrane required for phosphorylation of ADP [8]. Additionally, flux through AOX bypasses CIII–CIV, which do translocate protons, leaving only CI to contribute to the requisite proton gradient (figure 1*a*). We also found that the correlation between AOX and DHex flux was stronger in fast species than slow species (figure 2*b*). DHex and AOX can form a supercomplex which cycles electrons rapidly to shift the redox balance to an oxidized state, which disfavours ROS production [26]. Therefore, it is possible that increased reliance on AOX and DHex in fast species is a plastic nuclear response to oxidative stress caused by mt mutation accumulation. This agrees with previous studies implicating these alternative ETS complexes in environmental stress responses [9,20,32,33] but extends the response to 'domestic' stressors.

Predictions about the impact of mt mutations on mt function are varied: efficient mt selection results in fixation of only neutral or advantageous mutations and has recently been suggested to be common despite classic mutation accumulation theory [34–36]. Alternatively, compensatory evolution in the nuclear genome may offset deleterious mt mutations, making them effectively neutral (or even advantageous) [37,38]. Here, we find evidence that despite drastically elevated mt mutation rates, overall mt function is minimally impacted in fast *Silene* species. This is likely due to both mito–nuclear coevolution and nuclear-mediated responses in mt physiology, most notably increased reliance on AOX. Our results highlight the importance of considering physiological outcomes when making predictions about the importance of mito–nuclear interactions.

Data accessibility. The data and code used to produce the results presented here are available at https://doi.org/10.6084/m9.figshare.12771260.v2.

Authors' contributions. J.H. conceived and designed the study. All authors collected the data. J.H. and R.W. carried out the statistical analyses, interpreted the results and drafted the manuscript. All authors critically revised the manuscript, gave final approval for publication and agree to be held accountable for the work performed therein.

Competing interests. We declare we have no competing interests.

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