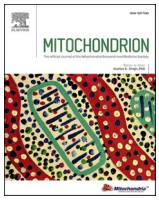
Mitochondrion 80 (2025) 101976

Contents lists available at ScienceDirect

Mitochondrion

journal homepage: www.elsevier.com/locate/mito



Cumulative effects of mutation accumulation on mitochondrial function and fitness

Frédérique Paquin [a], Melania E. Cristescu [b], Pierre U. Blier [a], Hélène Lemieux [c], France Dufresne [a,*]

[a] Département de biologie, Université du Québec à Rimouski, 300 allée des ursulines, Rimouski, Québec G5L 3A1, Canada
[b] Department of Biology, McGill University, 1205 Docteur Penfield, Montréal, Québec H3A 1B1, Canada
[c] Department of Medicine, Women and Children Research Health Institute, University of Alberta, Edmonton, Alberta T6C 4G9, Canada

ARTICLE INFO

Keywords:
Mutation accumulation
Mitochondria
Mitochondria copy number
Fitness
Daphnia

ABSTRACT

The impact of mutations on the mitochondria deserves specific interest due to the crucial role played by these organelles on numerous cellular functions. This study examines the effects of repeated bottlenecks on mitochondrial function and fitness. *Daphnia pulex* mutation accumulation lines (MA) lines were maintained for over 120 generations under copper and no copper conditions. Following the MA propagation, *Daphnia* from MA lines were raised under optimal and high temperatures for two generations before assessing mitochondrial and phenotypic traits. Spontaneous mutation accumulation under copper led to a later age at maturity and lowered fecundity in the MA lines. Mitochondrial respiration was found to be 10% lower in all mutation accumulation (MA) lines as compared to the non-MA control. MtDNA copy number was elevated in MA lines compared to the control under optimal temperature suggesting a compensatory mechanism. Three MA lines propagated under low copper had very low mtDNA copy number and fitness, suggesting mutations might have affected genes involved in mtDNA replication or mitochondrial biogenesis. Overall, our study suggests that mutation accumulation had an impact on life history traits, mtDNA copy number, and mitochondrial respiration. Some phenotypic effects were magnified under high temperatures. MtDNA copy number appears to be an important mitigation factor to allow mitochondria to cope with mutation accumulation up to a certain level beyond which it can no longer compensate.

1. Introduction

Mitochondria have various functions in cell, with the most important one being ATP production by the oxidative phosphorylation (OXPHOS) process. As a large part of cellular energy is produced through OXPHOS, any mitochondrial damage can have important fitness consequences. The effects of specific mutations affecting mitochondrial proteins have long been associated to their deleterious impacts on life history traits such as fertility and survival (Ballard et al., 2007; Howe and Denver, 2008; Estes et al., 2011). It is particularly important to understand the impact of spontaneous mutations on mitochondrial DNA (mtDNA) since it is associated with aging and with mitochondrial diseases (Tuppen et al. 2009). A consequence of the compact genetic configuration of the mtDNA is the increased likelihood that any mutation will impinge upon a coding sequence and may thus alter the functional integrity of a vital protein or RNA (Hahn and Zuryn 2019). The absence of histones makes mtDNA more susceptible to direct oxidative damage by ROS increasing the probability of lesions that lead to mutations. The mechanism by which mtDNA point mutations are generated in somatic tissues is an area of much debate (Lawless et al. 2020). Mutations arise from replication errors by the mitochondrial DNA polymerase (Pol γ) (Itsara et al. 2014) and from the high reactive oxygen species (ROS) environment inside mitochondria. Although, it was initially suggested that oxidative stress was responsible for raising mtDNA mutations, studies have shown a limited role for ROS-induced mtDNA mutagenesis causing point mutations in somatic cells (e.g. Kennedy et al. 2013; Itsara et al. 2014). Oxidation of guanines (8-oxo-G) in mitochondria results in elevated CG → AT transversions through mispairing with adenine (Kino et al. 2020) and the proofreading ability of Pol γ can be reduced up to 20-fold under oxidation (Anderson et al. 2020), suggesting interactions between damage and polymerase fidelity can be difficult to untangle. A high resolution study using duplex sequencing found that the mtDNA

* Corresponding author.
E-mail address: france_dufresne@uqar.ca (F. Dufresne).

https://doi.org/10.1016/j.mito.2024.101976
Received 25 July 2024; Received in revised form 10 October 2024; Accepted 29 October 2024
Available online 31 October 2024

mutation spectrum was dominated by mutations increasing AT content, suggesting oxidative damage could be a driver of mtDNA mutations in *C. elegans* (Waneka et al. 2021).

As mitochondrial complexes are encoded partly by the nuclear and partly by the mitochondrial genes, mutations in either genome can affect mitochondrial functions. The proper functioning of these organelles depends not only on each of these genomes, but on the coordination between both genomes with quasi-independent dynamics (Camus and Dhawanjewar 2023). The asexual mode of transmission should leave the mitochondrial genome vulnerable to mutational meltdown by Muller's Ratchet, a process leading to deleterious mutation accumulation in asexual, nonrecombining lineages (Neiman and Taylor 2009). However, nonsynonymous substitution rates for mitochondrial genes coding for respiratory chain subunits are often lower than substitution rates in nuclear loci (Cooper et al. 2015) implying strong purifying selection against mitochondrial mutations (Stewart et al. 2008). Since mtDNA is present in multiple copies per mitochondrion, newly arisen deleterious mutations may be masked until reaching a threshold. Hence these mutations may be lost from bottlenecks between generations or persist at low frequencies in heteroplasmic states (Camus et al., 2022). The relative influence of selection, bottleneck, and segregational drift modulates heteroplasmy. Reduction in effective mtDNA content during oogenesis has been shown in several animal species (reviewed in Zhang et al. 2018). Strong selective effects in germline transmission have been shown in humans where lower deleterious than neutral mutations were found in 96 multigenerational families (Zaidi et al. 2019). The authors suggested that the increase in mitochondrial respiration late in oogenesis could expose the rare deleterious alleles to selection (Zaidi et al. 2019).

Populations of mitochondrial genomes exist in a nested hierarchy of levels of selection (Rand 2001) and are subject to segregational drift due to random partitioning at cell division, mitochondrial DNA migration within mitochondrial networks and germline bottlenecks in mtDNA copy numbers (Radzvilavicius et al. 2017). The segregational drift at the cell levels increases intercellular variance in mutational load and promotes selection against deleterious mutations (Bergstrom and Pritchard 1998, Radzvilavicius et al. 2017). Mutation accumulation experiments in *C. elegans* lines differing in population size revealed that the frequencies of frameshift and nonsynomymous mutations were correlated with population size, suggesting the importance of selection in removing deleterious mutations (Konrad et al. 2017). Strongly deleterious mtDNA variants passed to offspring would be expected to be rapidly selected against. Yet, evolution can favor transmission of some deleterious mtDNA variants provided they have replication or transmission advantages compared to other mtDNA variants (Estes et al. 2023).

Deleterious effects of nuclear and mitochondrial mutations on genes coding for mitochondria have been shown in humans (Wallace, 2010; Schon et al., 2012; Dowling 2014; Dabravolski et al. 2022; Prabhu et al. 2023) and in animal models such as yeast and *Caenorhabditis elegans* (Liau et al. 2007; Gilea et al. 2021; Estes et al. 2023). A few studies on model organisms have allowed to assess the proximal effects of mutations with large effects on genes linked to ETS (Kujoth et al., 2005; Dingley et al., 2010; Hicks et al., 2012; Leung et al., 2013). These include decrease of membrane potential, change in activities of ETS complexes and ATP concentration, as well as increase in ROS. These mutations with large effects may be removed by selection in natural populations while the impacts of mutations with small effects, the most prevalent ones, are less understood (Denver et al., 2000; Rand, 2001; Melvin and Ballard, 2017). These effects are difficult to directly quantify and only a few studies have succeeded in measuring the consequences of natural variation of mitochondrial genes linked to ETS on mitochondrial functions (Moreno-Loshuertos et al., 2006; Ballard and Melvin, 2011; Pichaud et al., 2012; Correa et al., 2012).

Mitochondria are organelles capable of a plastic response, which play an important role to adjust to normal energetic demands, but also to possible dysfunctions induced by mutations (Ballard and Melvin, 2011; Blier et al., 2014; Lemieux and Blier, 2022). Their compensatory responses can include the increase in the number of mitochondrial proteins, the number of mtDNA copies per mitochondria, the quantity of mitochondria and/or their reorganization, morphology, or in the efficiency of the various OXPHOS pathways (Lee and Wei, 2005; Hood et al., 2006; Verkaart et al., 2007; Bratic et al., 2009; Lemieux and Blier, 2022). Mitochondrial number is strictly regulated to ensure appropriate levels of energy and intracellular signals for the maintenance of normal cellular function and reduction of mtDNA copy number can disrupt mitochondrial energetics as seen in aging and cancer cells (e.g. Schon et al. 2003; Zhang et al. 2017). Mutations can alter the mtDNA copy number directly by interfering with the replication mechanisms of mtDNA (Lee et al., 2004) or indirectly by inducing a raise of oxidative stress (Lee and Wei, 2005). MtDNA copy number increases can reflect compensation for reduced respiration capacity as was found in *Drosophila* lines with a mitonuclear mismatch between a mitochondrial tRNAtyr and its nuclear-encoded mitochondrial tyrosine synthetase (Pichaud et al. 2019). Similarly, *C. elegans* with a mutation (gas-1 fc21 allele) decreasing ETS Complex I efficiency by 75 % showed an increase in mtDNA copy number under extreme drift (Wernick et al. 2016). Different results have been obtained in *C. briggsae* where lower mtDNA/nDNA ratios were found in heteroplasmic mutant lines bearing large deletions in the ND5 gene (Wagner et al. 2020). Mitochondrial quality can also be regulated through mitochondrial fission and fusion. Damaged organelles can fuse with healthy ones and undergo fission to create healthy daughter mitochondria or damaged mitochondria or mitochondria carrying deleterious mutations may be selectively targeted for degradation via mitophagy (Killackey et al. 2020, Suen et al. 2010). Damaged mitochondria can sometimes escape mitophagy due to defects in the mitophagy machinery, altered signaling, or mitochondrial adaptations (Stewart and Chinnery 2021).

Mutation accumulation experiments have been invaluable in providing measures of the rate and spectra of mutations in mitochondrial genomes. Such experiments in *Daphnia pulex* have revealed that their mitochondrial genomes are dominated by insertions and deletions and have a very high mutation rate among eukaryotes (1.73×10^{-7} per nucleotide per generation), with five to ten copies of mitochondrial genomes transmitted per female *Daphnia* per generation (Xu et al. 2012). Yet the phenotypic effects of this high mutation rate are unknown. Our study tests the effects of 100 generations of bottlenecks on mitochondrial functions and fitness. To do so, mitochondrial respiratory capacity, mtDNA copy number, and life history traits were measured in *Daphnia* lines that have been through series of bottlenecks for more than 120 generations and were compared to those that have been kept in a large, competitive population for the same amount of time. The principle of these experiments is to allow the majority of neutral and deleterious mutations to accumulate in the genomes of lines maintained at very low effective population size to minimize selection. Hence mutation accumulation experiment is to some extent akin to the effect of aging on somatic cells since MA lines accumulate mutations in their nuclear genomes with no recombination for hundreds of generations. The *Daphnia* lines used in this study come from two MA treatments, one propagated under mild sublethal copper, and one propagated in the absence of copper. The exposure to mild copper was carried out to elevate mutation rates and help reveal the phenotypic effects of mutations. Significantly higher rates of large-scale deletions and duplication were measured in *Daphnia* MA lines exposed to low concentrations of copper compared to control lines (Chain et al., 2019). We expect that 1) MA lines will show decreased mitochondrial respiration as compared to controls, 2) exposure to low copper will lead to lower fitness, lower mtDNA copy number, and lower mitochondrial respiration, and 3) exposure to high temperature will result in traits with lower phenotypic values.

2. Material and methods

2.1. Animals

The *Daphnia* lines used here came from a mutation accumulation experiment carried out for more than 120 generations. This experiment has been described in detail elsewhere (Flynn et al., 2017). The design of the mutation accumulation experiment consisted in setting 200 lines of a single asexual clone sampled in Windsor, Ontario (Canard pond, Lat. 42°12″, Long. 82°98″). Lines were divided into two treatment groups: 50 were grown under a mild sublethal copper solution (40 g L^{-1}: Winner & Farrell 1976) (MA with copper) and 50 were submitted to mutation accumulation but under optimal conditions (MA without copper). To do so, single daphniids were bottlenecked every generation by keeping a single individual to set up the next generation. This design increased the probability of mutation fixation that do not fully impair reproduction and that are not lethal. A control non-MA group was created by housing 100–250 individuals from the same clone in a 15L tank with the FLAMES medium. The three groups were raised under the same standard condition of 18 °C, a 12/12 h photoperiod, and fed a mixture of three algal species (*Ankistrodesmus* sp., *Scenedesmus* sp. et *Pseudokirchneriella* sp.).

After an average of 120 generations, 10 *Daphnia* lines were randomly chosen from each of the three groups (non-MA, MA non-Cu, MA Cu) and propagated in single progeny under the same standard environmental conditions (18 °C, 12 h/12 h photoperiod, in standard medium without copper) for two generations. Sixteen neonates (less than 24 h babies) from the 2nd clutch of each of the 30 MA lines or non-MA lines were isolated and raised at two temperatures (18 °C; n = 8) and (27 °C; n = 8). *Daphnia* experiments are usually started from 2nd clutch babies as there is less variation than among 1st clutch babies (Teschner et al. 1995). The high temperature (27 °C) was selected in preliminary experiments as it was non-lethal but had significant impacts on life history traits. *Daphnia* were raised in 50 ml Falcon tubes filled with 40 ml FLAME medium and fed every second day with 800 µl of the algal mixture at a concentration of approximately ~ $1x10^7$ cells·ml/1 (Fig. 1). The *Daphnia* were grown for two generations under these conditions prior to the measurements.

*Fig. 1. Experimental design. *Daphnia pulex* mutation accumulation lines (MA) lines were maintained for over 120 generations under both low and no-copper conditions. Following the MA propagation, 10 *Daphnia* from MA lines with and without copper and 10 *Daphnia* maintained in large numbers for the same period (control) were raised at 18 °C with no copper for two generations and placed at 18 °C and 27 °C where life history traits (8 replicates) and mtDNA copy number (6 replicates) were assessed. Mitochondrial respiration was measured at 27 °C on 10 *Daphnia* lines per treatment with 6 replicates for each line).

2.2. Mitochondrial respiration

For the mitochondrial respiration measures, juveniles (less than 48 h old) coming from different mothers raised at 27 °C were transferred in 1 L jars (n = 5) containing 800 mL of FLAMES medium (Celis-Salgado et al., 2008). Mitochondrial respiration was measured on 6 replicate pools of 10 individual *Daphnia* per group using the protocol of Kake-Guena et al. (2015). Briefly, *Daphnia* were rinsed in ice cold BIOPS medium (2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 20 mM imidazole, 20 mM taurine, 6.56 mM $MgCl_2$-$6H_2O$, 5.77 mM ATP, 15 mM phosphocreatine, 0.5 mM dithiothreitol, 50 mM 2-(N-Morpholino) ethanesulfonic acid potassium salt, pH 7.1 at 4 °C). Eggs inside the brood chambers were delicately removed by flushing water inside the female carapace using Pasteur pipettes. *Daphnia* were mechanically permeabilized using mounted needles. In order to ensure complete permeabilization of the cell membrane, the *Daphnia* were then agitated for 30 min on ice in BIOPS supplemented with 50 µg·mL^{-1} saponin. Permeabilized *Daphnia* were immediately transferred into respiration chambers (Oroboros Oxygraph-2 k, Oroboros Instruments Inc., Innsbruck, Austria) containing 2 mL of respiration buffer MiR05 (110 mM sucrose, 60 mM K-lactobionate, 0.5 mM EGTA, 1 g·L^{-1} fatty acid free BSA, 3 mM $MgCl_2$-$6H_2O$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM K-HEPES, pH 7.1, osmolarity 330 mOsm; (Gnaiger et al., 2000)), as well as 5 mM pyruvate and 5 mM malate. Mitochondrial respiration in the LEAK state, without ADP, was first measured. Subsequent addition of substrates and inhibitors in the chamber then allow to measure various states and pathways: ADP (10 mM) for the OXPHOS state (coupled to ADP phosphorylation) of the NADH pathway (N-pathway in the presence of pyruvate and malate entering into complex I), cytochrome *c* (10 µM) addition to test for mitochondrial outer membrane integrity, succinate (10 mM) for the OXPHOS state of the combined NADH and Succinate pathways (NS-pathway; with electron entering into complexes I and II simultaneously), rotenone (0.5 µM) for the OXPHOS state of the Succinate pathway (S-pathway with electron entering into complex II only), antimycin A (2.5 µM) for non-mitochondrial residual oxygen consumption, ROX, after inhibition of complex III, ascorbate (2 mM) and

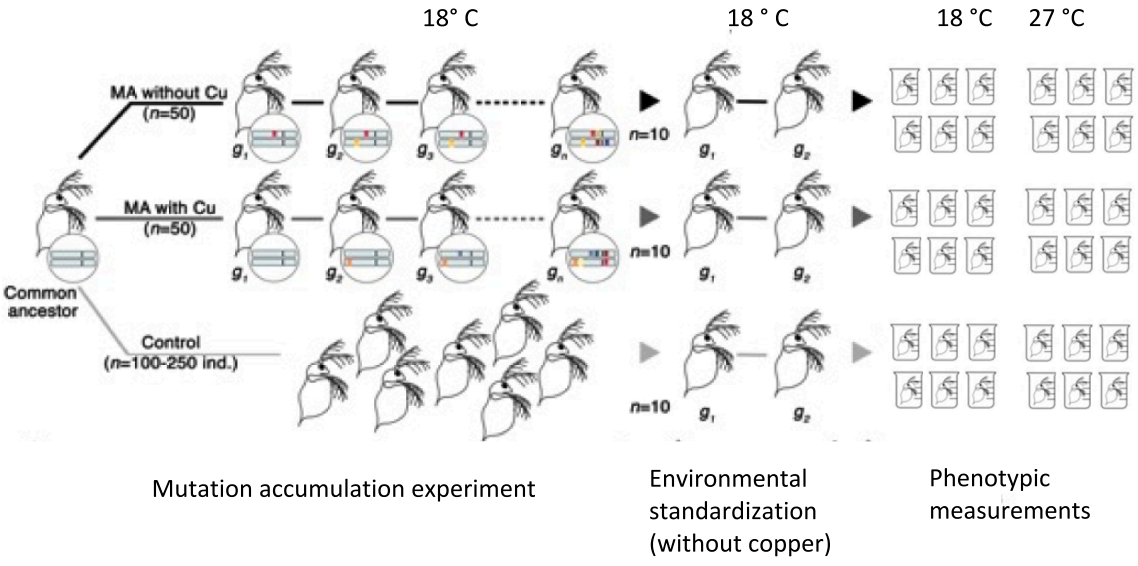


Fig. 1. Experimental design. *Daphnia pulex* mutation accumulation lines (MA) lines were maintained for over 120 generations under both low and no-copper conditions. Following the MA propagation, *Daphnia* from MA lines and *Daphnia* maintained in large numbers for the same period (control) were raised at 18 °C and 27 °C for two generations before assessing phenotypic traits.

N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD; 0.5 mM) for the activity of complex IV, and sodium azide (100 mM) for the chemical background after inhibition of complex IV. Respiration was measured at the rearing temperature of *Daphnia*, 27 °C, and was corrected for oxygen flux due to instrumental background (all states and pathways), for ROX (LEAK and OXPHOS states), and for chemical background (complex IV activity). Datlab software (Oroboros Instruments Inc.) was used for data acquisition and analysis. Respiration flux in *Daphnia* was expressed in pmol O_2 per second per mg of protein or as a flux control ratio (FCR) normalized to maximal OXPHOS capacity in the presence of substrates feeding the NS-pathway. At the end of each experimental run, the content of the chamber was removed and homogenized 8 times on ice with fritted glass potters, and immediately stored at −80 °C for measurement of protein content and citrate synthase (CS) activity.

2.3. Protein content

Protein content was measured using bicinchoninic acid method (Smith et al., 1985) and a plate reader (EnVision® Multilabel Reader, Perkin Elmer Health Sciences Canada, Inc Woolbridge, Ontario). Because the homogenates contained BSA and cytochrome *c*, the protein content of the MiR05 medium supplemented with 10 μM cytochrome *c* was measured and subtracted from the measurement obtained for each homogenate.

2.4. Citrate synthase activity

CS activity was used as a marker of mitochondrial content and measured at 27 °C using the plate reader (EnVision® Multilabel Reader) according to a protocol modified from (Larsen et al., 2012). The absorbance was measured for 8 min. at 412 nm following the reduction of 0.1 mM 5,5′-dithiobis-2-nitrobenzoic acid ($\varepsilon = 13.6\ \text{ml}\cdot\text{cm}^{-1}\cdot\mu\text{mol}^{-1}$) in the presence of 0.113 mM acetyl-CoA, 0.15 mM oxaloacetic acid, potassium phosphate 60 mM buffer (pH 8.0), and 100 μL of homogenate in a well containing a total volume of 250 μL. The activity was measured in triplicates and expressed in international units (IU) per mg of protein, IU being defined as the quantity of CS to form one micromole of product by min.

2.5. MtDNA copy number measurements

The relative mtDNA copy number over nuclear DNA copy number (mtDNA/nucDNA ratio) was measured on *Daphnia* bearing their 5th or 6th clutches and grown under 18 °C and 27 °C. Eggs were delicately expulsed from females by flushing a pipette full of FLAMES medium and blotted dry prior to being frozen at −80 °C until DNA extractions.

DNA was extracted from pools of three mothers using the CTAB (Doyle and Doyle, 1987) and preserved at −20 °C until amplification. Organic extractions such as CTAB are more consistent and accurate to column-based methods for estimating mtDNA/nDNA ratios (Guo et al. 2009). The relative number of mtDNA to nuclear DNA was measured by realtime PCR. Primers for the mitochondrial gene NADH dehydrogenase (*ND5-CanF*-5′- TTAAGAGGTGGTCCGCATTC-3′ and *ND5-CanR*-5′-TTTGTTGGTGTTTCCCTGCT-3′) were designed based on the ND5 sequence from the Canard clone. The nuclear gene Syntaxin (*Stx16*) was used as a reference gene (*Stx16F*-5′-CACATTGGTCGTCCTTAGTCTTG-3′ and *Stx16R*- 5′- TGCTATACGTTACGCTTGTCCTTAC-3′) (Spanier et al., 2010) as it was confirmed to be a single copy number gene and a good candidate for a reference gene (Heckman et al. 2008). The amplification efficacy of each gene was assessed prior to measurements as in Livak and Schmittgen (2001). Briefly, the ΔC_t of the two amplicons (C_T *Stx16* − C_T *Nd5-Can*) was plotted against the log of the DNA dilutions. As the regression slope was close to 0 ($y = 0.014\text{Log}(x) + 6.79$), both genes had similar amplification efficacies and could be reliably used. The PCR mix consisted of 2 μl of DNA (about 50 ng), 7,5 μl of SensiFAST™ SYBR® No-ROX Kit (Bioline), 400 nM of each primer and nanopure water for a final volume of 15 μl reactions ran on a LightCycler 480 II (Roche) with the following PCR conditions: 3 min at 95 °C and 40 cycles of 10 sec at 95 °C, 10 sec at 60 °C and 10 sec at 72 °C. The relative number of mtDNA copy number was calculated with the $2^{\Delta C}_T$ equation (Qiu et al., 2013). Samples were run in duplicates. When the variation coefficient for the C_T (CV_{CT}) was greater than 2 % for one of the genes, the amplification was performed again to keep variation below 2 %.

2.6. Life history assays

Life history traits were measured on the same individuals used for the mtDNA copy number. Tubes were inspected every 24 h for measurements of age (days) and number of babies for the first five clutches. Neonates were removed from the tubes upon hatching and mortality was consistently recorded. The following life history traits were measured: age at 1st clutch release and the average number of juveniles per clutch in clutches 1 to 5. The intrinsic population growth rate (*r*) of each individual was calculated and used as a proxy for fitness (Teschner, 1995). Individual *r* was calculated based on the age at first clutch release and clutch size using the Euler-Lotka equation:

$$\sum e^{-rx} l_x m_x = 1$$

where *x* is the age (in days), l_x is the survival probability on day *x*, and m_x is fertility on day *x*. To include females that did not reproduce prior to dying, the fitness value was presented as the finite growth rate (e^r) rather than *r* because females that do not reproduce do not have an *r* of $-\infty$. Instead, females that did not reproduce were considered to have a fitness value of $e^r = 0$.

2.7. Data analysis

The effect of mutation accumulation on mitochondrial respiration states pathways and steps, CS activity, mDNA/nDNA ratio, and life history traits was tested using the generalized linear mixed model, with the method of maximum likehoods (REML) and the RStudio software (1.0.136; RStudio Inc., version 1.0.136) (Pinheiro et al., 2014). For each variable, the model y = treatment + line (treatment) + ε was analyzed using a two-way ANOVA, with the treatment as a fixed effect and the line as a random factor. The ANOVA was followed by pairwise Tukey comparison.

For some variables, the means between the MA Cu and MA non-Cu groups were not significantly different (p close to 1.000) and these were grouped together to compare with the control group (Ma non-Cu). For these variables, Dunnett test were then used to determine if the mitochondrial traits were different between MA lines from the group non-MA.

For each variable, the variance components were also assessed using a linear generalized mixed model $y = line + \varepsilon$ where the line was a random factor. The intra-lines variance (Ve) represents the environmental variance whereas the inter-line variance (VL) represents the genetic variance. F tests were used to compare the variances between the groups. The genetic variation coefficient (VCL) was generated by normalizing the square root of VL by the treatment average (Houle, 1992).

In order to test if mutation accumulation treatments generated different plastic responses i, genotype x environment (G x E) was tested with the following mixed linear model: $y = Treatment + Temperature + Treatment \times Temperature + Line\ (Treatment) + \varepsilon$ where the terms treatment and temperature, as well as their interaction, were fixed factors, whereas the line was a random factor nested in the treatment. To evaluate the variability of the line plasticity within each treatment, fixed factor two-ways ANOVAs were used: $y = Line + Temperature + Line \times Temperature + \varepsilon$. Significant interactions were interpreted as indicating a difference between the standards of reaction of treatment or lines (Latta et al., 2015; Windig et al., 2004). To confirm the different plastic

responses and evaluate if the differences were due to a change in order of the mean and/or in the level of variance between lines, the standard of reaction were analyzed individually and the variances between lines at each temperature were compared with a F test.

3. Results

3.1. Mutation accumulation affects OXPHOS capacity and the differences are not due to change in mitochondrial content

Various electron transfer pathways were evaluated under OXPHOS state, coupled to phosphorylation of ADP. These included: (1) the NADH-pathway, where electrons are transferred from pyruvate and malate into complex I, (2) the Succinate-pathway, where electrons are transferred from succinate into complex II, and (3) the NS-pathway, where there is simultaneous entry of electrons into complexes I and II. The MA treatment had a significant effect on the OXPHOS rate expressed in flux per mg protein^{-1} (Fig. 2A, B, C). The trend was the same for the NADH (Fig. 2A), the succinate (Fig. 2B), and the NS-pathways (Fig. 2C) with a significantly higher respiration in the non-MA group compared to both MA groups, propagated with or without copper (~9 to 13 % difference). In the NADH pathway, the difference was significant when the control was compared to the MA group with copper (Fig. 2A). For the succinate pathway, significant differences were found between the control and the MA group without copper (Fig. 2B). For the NS-pathway, both MA groups (with and without copper) were significantly lower than the control group (Fig. 2C). Furthermore, for all OXPHOS pathways expressed per mg of proteins, the MA groups, with and without copper,

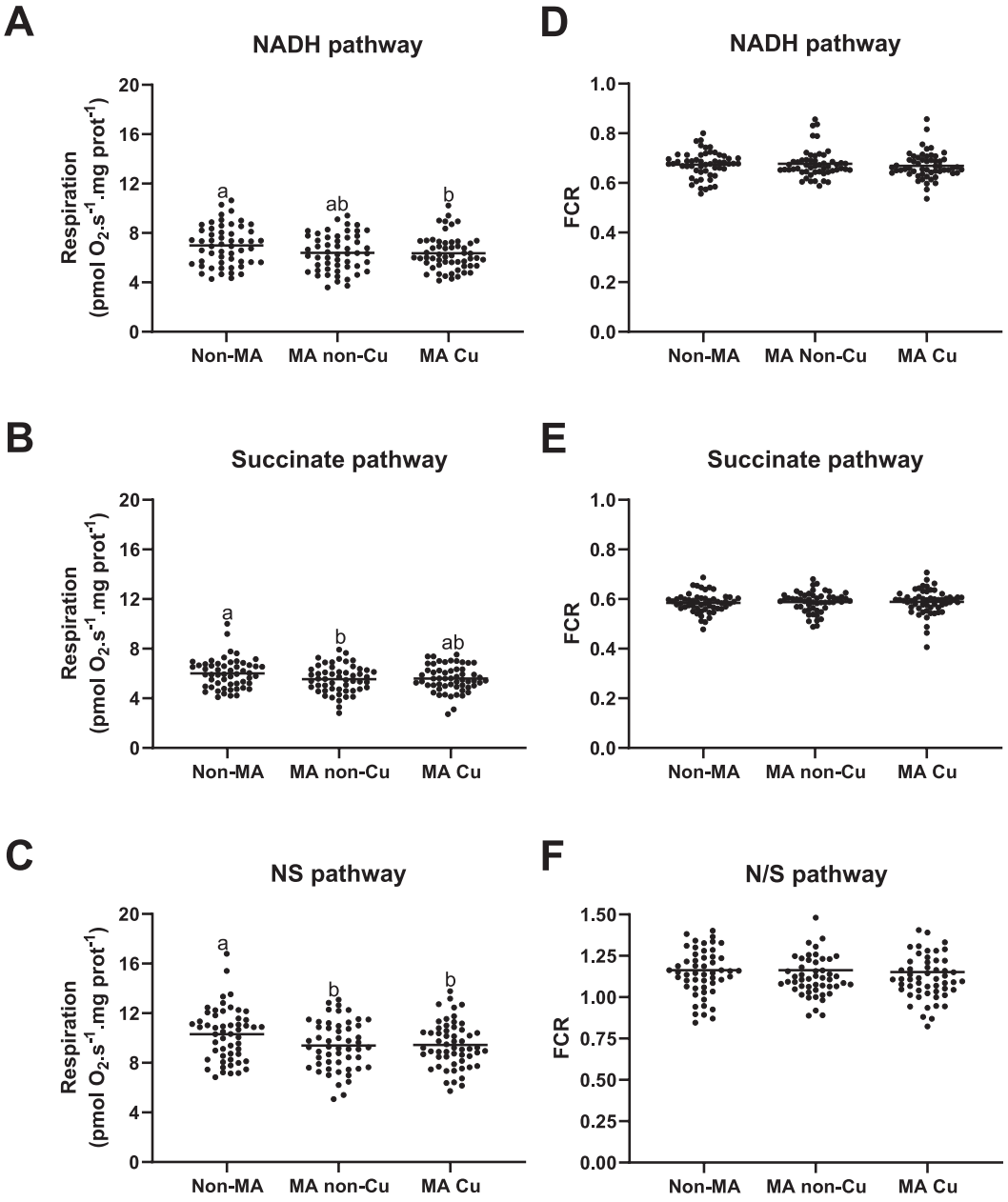


Fig. 2. Effects of mutation accumulation on the capacity of various OXPHOS pathways measured at 27 °C in *Daphnia pulex*. The data are in flux per mg protein for the NADH pathway (A), the Succinate pathway (B) and the combined NADH and Succinates pathways (NS-Pathway, C), in flux control ratio (FCR; normalized to the maximal electron transfer with NADH and succinate pathways activated simultaneously) for the NADH (D) and the succinate (E) pathways, and for the ratio NADH/Succinate pathway (F). Data are presented as dot plots with the horizontal line representing the mean, with N = 54, 53, and 56, for the non-MA, MA non-Cu, MA Cu, respectively. Significant differences between groups within a state or ratio are present when the groups do not share a common letter ($P < 0.05$; Tukey HSD).

did not differ significantly from each other (Fig. 2A, B, C). When the groups MA non-Cu and MA Cu were pooled together, they showed no significant differences with the control group for each of the pathways ($p = 0.818$ for the NADH pathway, $p = 0.896$ for the succinate pathway, $p = 0.954$ for the NS-pathway). When lines were taken individually, none of the MA lines showed significant differences with the mean of the control isolates non-MA (Dunnett tests, $p > 0,05$).

Mitochondrial function was then expressed as a FCR, normalized to maximal OXPHOS flux with substrates feeding electrons into the combined NADH and succinate pathways. The FCR indicates changes of mitochondrial respiratory functions rather than quantitative changes associated with mitochondrial mass (Gnaiger, 2014). For the NADH (Fig. 2D) and for the succinate pathways (Fig. 2E), the FCR were identical between the treatments ($p = 0.818$ and $p = 0.896$ for the NADH and the succinate pathway, respectively). The N/S ratio also reflects the constant relative contribution of the NADH and succinate pathways between the treatments with a $p = 0.954$ (Fig. 2F).

The mitochondrial content was evaluated using CS (Fig. 3A) and complex IV activities (Fig. 3B). Both markers have been shown to be correlated to the mitochondrial content measured with electron microscopy (Larsen et al., 2012). The data showed no effect of mutation accumulation on either marker of mitochondrial content ($p = 0.857$ for CS activity; $p = 0.701$ for complex IV activity). None of the MA line showed differences in CS or complex IV activity compared to the control non-MA lines (Dunnett test, $p > 0.05$). The differences in OXPHOS capacity, estimated by the rate of oxygen consumption per unit of protein mass, could unlikely be dictated by variation in mitochondrial content. MA non-Cu and MA Cu lines did not show higher genetic variance than non-MA lines for the various mitochondrial function variables as expected for mutation accumulation lines (Table 1). The genetic variance (V_L) was not significant in all groups for most of the variables (ANOVA, $p > 0.05$).

3.2. No impact of mutation accumulation on coupling of oxidative phosphorylation

Mitochondrial coupling efficiency was evaluated using the LEAK state measured in the presence of pyruvate and malate, before the addition of ADP, a state representing the respiration compensating for proton leak and slip, cation cycling, and electron leak (Brand et al., 1994; Gnaiger, 2009). The LEAK was expressed as a FCR, normalized to the maximal OXPHOS flux with electrons feeding the NS-pathway (Fig. 3C). A value of 1.00 indicates a fully coupled system, whereas a value of 0.00 indicates a fully uncoupled system (Gnaiger, 2014). Our values in this study were 0.17–0.18 on average (Fig. 3C), demonstrating the good coupling, similar to the average values in previous studies on permeabilized *Daphnia* (Kake-Guena et al., 2015; Kake-Guena et al., 2017) or other small invertebrates such as planarian (Scott et al., 2019). This ratio did not differ between treatments ($p = 0.763$).

3.3. Mutation accumulation does not affect the mitochondrial outer membrane integrity

The integrity of the outer mitochondrial membrane was estimated with the cytochrome *c* control efficiency (FCF*c*, Fig. 3D), which expresses the control of respiration by externally added cytochrome *c*. It is

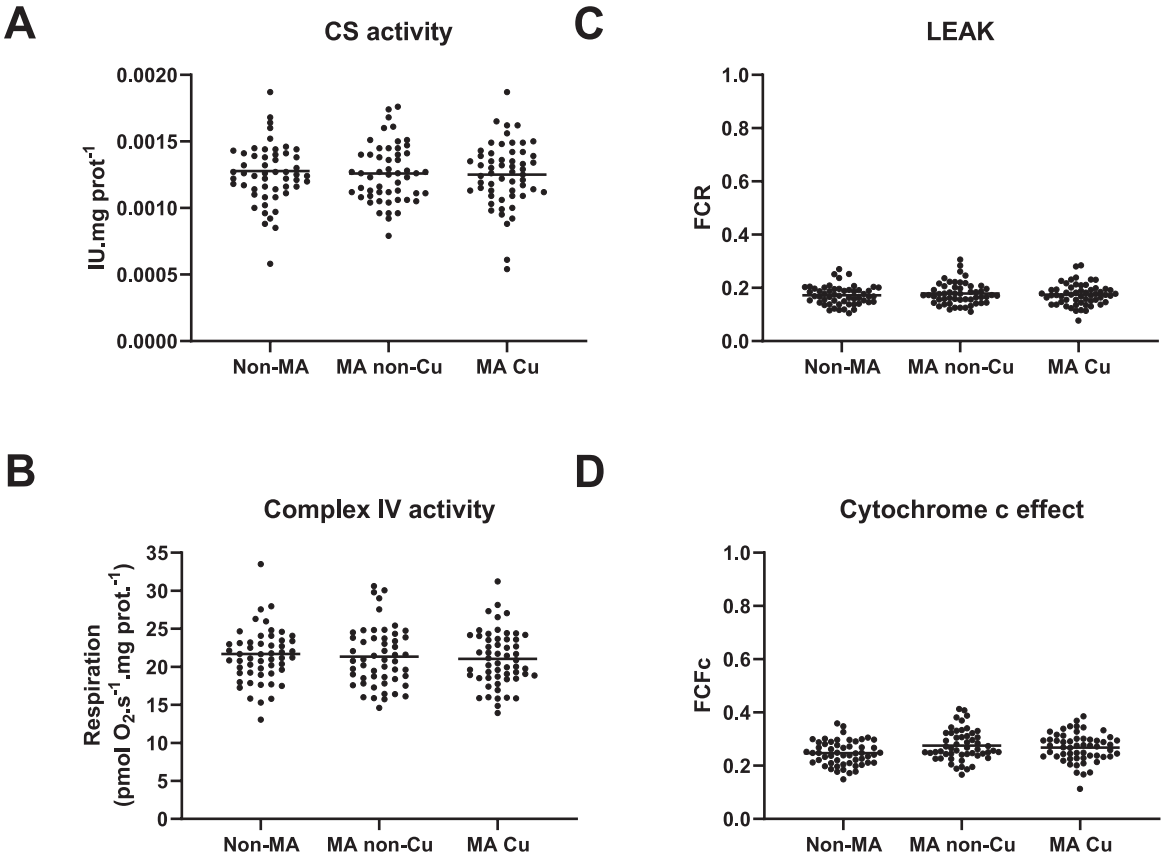


Fig. 3. Effect of mutation accumulation treatment on citrate synthase (CS) activity (A), complex IV activity (B), leak (C), and cytochrome *c* (D) measured at 27 °C in *Daphnia pulex*. CS activity is expressed in international units (IU) per protein mass and complex IV activity is expressed in pmol $O^2.s^{-1}$.mg $prot^{-1}$. The FCR is normalized to the maximal electron transfer with NADH and succinate pathways activated simultaneously. The cytochrome *c* control factor is expressed as (OXPHOS with cytochrome *c* – OXPHOS without cytochrome *c*) / OXPHOS with cytochrome *c*. Data are presented as dot plots with the horizontal line representing the mean, with N = 54, 53, and 56, for the control non-MA, MA non-Cu, and MA Cu, respectively. No significant difference between the treatments were detected.

Table 1
Median (min–max) for genetic variance (V_L), environmental variance (V_e) and genetic variation coefficient (CV_L) for mitochondrial for mutation accumulation treatments (control without mutation accumulation, non-MA; mutation accumulation, MA non-Cu; and mutation accumulation in the presence of copper, MA Cu) performed at 27 °C.

		Non-MA	MA non-Cu	MA Cu
	Mean ± SE	6.95 ± 0.22	6.30 ± 0.20	6.25 ± 0.19
NADH pathway	V_L	<0.001	<0.001	<0.001
pmol $O_2.s^{-1}$.mg prot. $^{-1}$	V_e	2.65	2.09	2.02
	CV_L (%)	<0.001	<0.001	<0.001
	Mean ± SE	5.98 ± 0.16	5.45 ± 0.15	5.48 ± 0.15
Succinate pathway	V_L	<0.001	<0.001	<0.001
pmol $O_2.s^{-1}$.mg prot. $^{-1}$	V_e	1.42	1.21	1.20
	CV_L (%)	<0.001	<0.001	<0.001
	Mean ± SE	10.27 ± 0.29	9.30 ± 0.27	9.34 ± 0.25
NS pathway	V_L	<0.001	<0.001	<0.001
pmol $O_2.s^{-1}$.mg prot. $^{-1}$	V_e	4.56	3.77	3.53
	CV_L (%)	<0.001	<0.001	<0.001
	Mean ± SE	21.68 ± 0.54	21.32 ± 0.54	21.04 ± 0.48
Complex IV activity	V_L	<0.001	2.48[a]	2.00[a]
pmol $O_2.s^{-1}$.mg prot. $^{-1}$	V_e	15.84	12.95	11.27
	CV_L (%)	<0.001	7.38(↑)	6.73(↑)
	Mean ± SE	0.67 ± 0.01	0.68 ± 0.01	0.67 ± 0.01
NADH pathway	V_L	<0.001	<0.001	<0.001
FCR	V_e	<0.01	<0.01	<0.01
	CV_L (%)	2.67	2.49	2.18
	Mean ± SE	0.58 ± 0.01	0.59 ± 0.01	0.59 ± 0.01
Succinate pathway	V_L	<0.001	<0.001[a]	<0.001[*a]
FCR	V_e	<0.01	<0.01	<0.01
	CV_L (%)	2.40	<0.001(↓)	<0.001(↑)
	Mean ± SE	2.14 ± 0.03	2.34 ± 0.06	2.029 ± 0.04
Complex IV activity	V_L	<0.01	0.03[a]	0.02*
FCR	V_e	0.05	0.15[a]	0.08
	CV_L (%)	3.96	7.04 (↑)	5.54
	Mean ± SE	1.27 ± 0.04 (X 10^{-3})	1.26 ± 0.03 (X 10^{-3})	1.25 ± 0.03 (X 10^{-3})
CS activity	V_L	2.59×10^{-16}	4.31×10^{-9} [a]	6.46×10^{-16}
IU.mg prot. $^{-1}$	V_e ($\times 10^{-8}$)	7.86×10^{-8}	4.32×10^{-8} [a]	5.72×10^{-8}
	CV_L (%)	<0.01	5.22 (↑)	<0.01

*Indicates a significant interline variance (ANOVA, p < 0,05).
[a] Indicates a significant differences of the MA treatment compared to the control (F-test, p < 0,05). The arrow indicates the direction of the differences.

calculated from the OXPHOS states in the presence of pyruvate and malate, as following: (OXPHOS with cytochrome *c* – OXPHOS without cytochrome *c*) / OXPHOS with cytochrome *c* (Gnaiger 2014). A ratio of 0.00 suggests full integrity of the mitochondrial outer membrane while a ratio of 1.00 indicate a highly damaged mitochondrial outer membrane. Average values were between 0.25 and 0.28 in our study, indicating some injury to membranes, but comparable to previous studies on permeabilized Daphnia (Kake-Guena et al., 2015; Kake-Guena et al., 2017). Furthermore, the increase in respiration associated with the addition of exogenous cytochrome *c* was similar between the treatments (p = 0.268). In order to avoid any impact of the outer membrane damage on the data, and to ensure that measured rate was not limited by a deficiency in cytochrome *c*, all the OXPHOS states used to compared lines and treatments in our study were the states with cytochrome *c* added.

3.4. MA lines have higher mtDNA copy number than non-MA lines at 18 °C but not at 27 °C

At optimal temperature (18 °C), the MA non-Cu lines had significantly higher (33 %) mean mtDNA/nuclear DNA ratios than non-MA lines (Table 2, p < 0,0001) but the mean ratios of the MA Cu lines did not differ significantly from those of the non-MA lines, even though ratios tended to be larger (16 %; p = 0.068; Table 2, Fig. 4). The important among-line variance in the MA Cu lines might explain the lack of significant differences (Table 2). Notably, this group had two distinct lines (MA Cu4 and MA Cu8) with very low mtDNA/nDNA ratios. The genetic variances (V_L) of the MA lines were significantly larger than those of the non-MA at 18 °C, especially those of the MA Cu lines (F-test, p < 0,001; Table 2). There were no significant differences in mean mtDNA/nDNA ratios among groups at 27 °C (Table 2, p = 0,264). Reaction norms of the mtDNA/nDNA ratios were steep for MA lines and flat from non-MA lines (Supplemental Figure 1). The among-line variance in the MA without copper group was larger at 18 °C than 27 °C (F-test, p = 0,046) and was not significantly different from the control group (p = 0,427). At this high temperature, the genetic variance of the MA Cu group was higher than that of both MA non-Cu and non-MA groups (p = 0.009 and 0.002 Table 2).

3.5. MA Cu lines have lower fecundity than non-Cu and non-MA at 27° C and steeper reaction norms

At 18 °C, the non-MA lines, MA non-Cu lines, and MA Cu lines had mean fecundities of 19.21, 19.07 and 16.96 juveniles per clutch respectively for their first five clutches. The MA lines did not differ significantly in mean fecundity compared to the non-MA group (mixed linear model, $F_{2,\ 27} = 0.94$, p = 0.40; Fig. 5). At 27 °C, the mean fecundities of all groups decreased, reaching 12.88, 9.65 and 8.31 juveniles per clutch for the non-MA, MA non-Cu, and MA Cu line (linear mixed models, non-MA, $F_{1,140} = 99.48$, p < 0.001, MA non-Cu, $F_{1,140} = 402.47$, p < 0.001, MA Cu, $F_{1,140} = 268.84$, p < 0.001). Mutational treatment had a significant effect on the mean fecundity at high temperatures (linear mixed model, F2, 27 = 4.54, p = 0.020). When compared to the non-MA group, the mean absolute clutch size was reduced by 25 % in MA non-Cu lines (but the difference was not significant) and was reduced by 36 % in MA Cu lines ($t_{27} = 2.93$, p = 0.018).

The interaction between temperature and treatment was significant for mean fecundity (mixed linear model, $F_{2,\ 447} = 8.52$, p < 0.001). Visually, the steeper reaction norms for MA lines than for the non-MA group suggest that *Daphnia* with accumulated mutations were more susceptible to thermal stress (Supplemental Figure 2). This was supported by the.

significantly lower mean relative fecundity under high temperatures than under optimal conditions for both non-MA Cu and MA Cu treatments (Fig. 2, paired t-tests, MA control, t9 = 6.61, p < 0.001 and MA Cu, t9 = 6.312, p < 0.001).

At both temperatures, the among-line variance for fecundity was not significantly different for MA non-Cu lines than for the non-MA group (F-tests, 18 °C, F = 3.02, p = 0.057; 27 °C, F = 2.04, p = 0.152), but was greater for MA Cu lines than for non-MA or MA non-Cu (Table 2). The MA Cu treatment translated into an increased among-line variance

Table 2
Among-line variance (V_L), coefficient of genetic variation (CV_L) and intra-line variance (V_e) for mean phenotypic traits measured for MA lines and non-MA control lines.

	Temp.	Treatment	Mean ± SE	V_L	CV_L (%)	V_e
Age at first clutch (days)	18 °C	Non-MA	11.615 ± 0.170	1.121	9.115	1.269
		MA non-Cu	12.075 ± 0.183	0.293[a]	4.484 (↓)	2.411[a]
		MA Cu	12.975 ± 0.234[a] (↓)	1.842[b]	10.460	2.684[a]
	27 °C	Non-MA	6.899 ± 0.134	0.261	7.402	1.185
		MA non-Cu	6.725 ± 0.200	2.457[a]	23.309 (↑)	0.975
		MA Cu	7.692 ± 0.199	1.072[a]	13.458 (↑)	2.097[a]
Mean clutch size (clutches 1 to 5)	18 °C	Non-MA	19.205 ± 0.553	2.884	8.843	21.835
		MA non-Cu	19.073 ± 0.515	8.748	15.507	13.242[a]
		MA Cu	16.955 ± 0.752	32.841[ab]	33.800 (↑)	15.360[a]
	27 °C	Non-MA	12.878 ± 0.418	3.973	15.477	10.360
		MA non-Cu	9.653 ± 0.384	8.090	29.467	4.400[a]
		MA Cu	8.305 ± 0.578[a] (↓)	21.728[a]	56.126 (↑)	6.910
Fitness (e^r)	18 °C	Non-MA	1.269 ± 0.023	0.002	3.107	0.041
		MA non-Cu	1.290 ± 0.004	0.000[a]	1.366 (↓)	0.001[a]
		MA Cu	1.227 ± 0.023	0.005[b]	5.591	0.037
	27 °C	Non-MA	1.499 ± 0.021	0.002	2.893	0.033
		MA non-Cu	1.495 ± 0.015	0.015[a]	8.084 (↑)	0.006[a]
		MA Cu	1.336 ± 0.039	0.057[ab]	17.938 (↑)	0.068[a]
ND5/Stx16 ($2^{\Delta C}_T$)	18 °C	Non-MA	92,31 ± 2,40	18,44	4,65	328,21
		MA non-Cu	123,23 ± 3,15[a]	137,66*[a]	9,52 (↑)	469,82
		MA Cu	107,20 ± 3,17	305,28*[a]	16,30 (↑)	317,39
	27 °C	Non-MA	96,76 ± 2,06	60,32*	8,03	194,77
		MA non-Cu	95,96 ± 2,07	68,38*	8,62	187,51
		MA Cu	87,22 ± 2,93	336,17*[ab]	21,02 (↑)	195,20

[a] Indicates that the MA treatment mean or variance was significantly different from that of the non-MA control (F-test, $p < 0,05$). [b] Indicates that the mean or variance of MA Cu treatment is significantly different from that of MA non-Cu (F-test, $p < 0,05$), *Indicates a significant interline variance (ANOVA, $p < 0,05$).

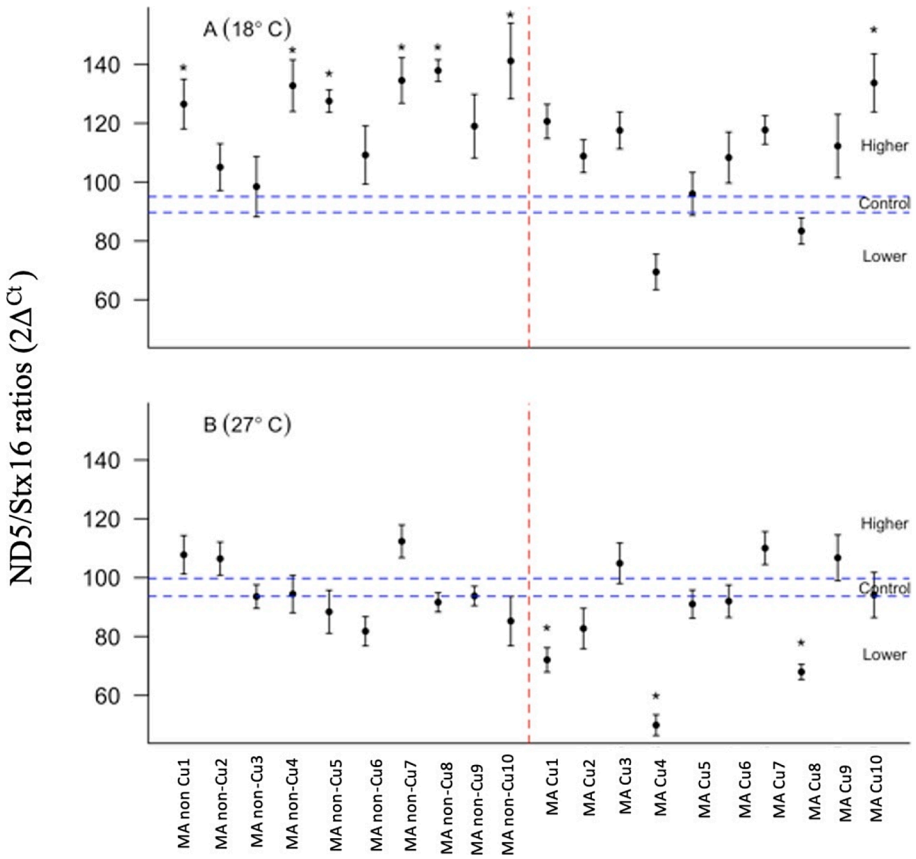


Fig. 4. ND5/Stx16 ratios for each MA line raised at 18 °C (A) and 27 °C (B). Lines that differ from the mean are indicated by a * (Dunnett test, $p < 0,05$).

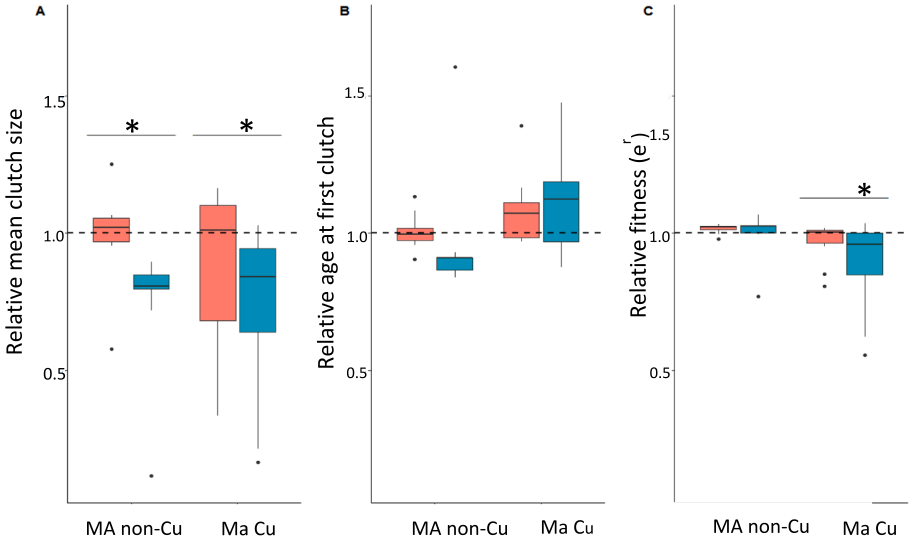


Fig. 5. Box plot of life history traits and fitness in mutation accumulation lines raised under low copper (MA Cu) and no copper (MA non-Cu) relative to the non-MA control mean (hatched line) when measured under optimal (red) and high (turquoise) temperature. Horizontal lines indicate significant differences. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Table 2) as three (18 °C) and four (27 °C) lines out of ten showed significantly different fecundity compared to the non-MA mean (Fig. 6).

3.6. MA Cu lines are older at maturity than MA non-Cu and non-Ma at 18 °C and show higher line variance at 27 °C

When raised at lower temperatures, the mean ages of the first clutch of the non-MA, MA non-Cu, and MA Cu groups were 11.63, 12.08 and 13.01 days. Mutational treatment had a significant effect on this trait at 18 °C (mixed linear model, $F_{2,\ 27} = 3.56$, $p = 0.042$). Pairwise comparisons showed that MA lines raised under copper were, on average, significantly older (~11 %) upon release of their first clutch than the non-MA control group (Tukey, $p = 0.024$). The reproductive delay was maintained over the four subsequent clutches. The MA non-Cu lines had an intermediate mean age at the first clutch and were not significantly different from either the MA Cu or the non-MA groups (Tukey, $p > 0.05$). When raised at 27 °C, the mean age of the MA Cu lines was 11 % lower than that of the non-MA control group at first clutches; however, this difference was not statistically significant (mixed linear model, $F_{27,\ 207} = 2.01$, $p = 0.15$). At this higher temperature, we observed accelerated reproduction for all treatments (mixed linear model, non-MA, $F_{1,} =$, $p <$ 0.001, MA non-Cu, $F_{1,} =$, $p < 0.001$, MA Cu, $F_{1,} =$, $p < 0.001$), but no significant interaction between treatment and temperature (mixed linear model, $F_{2,\ 441} = 2.25$, $p = 0.106$). Accordingly, when comparing the mean relative age at the first clutch, there were no significant differences between benign and hightemperatures for either MA treatment

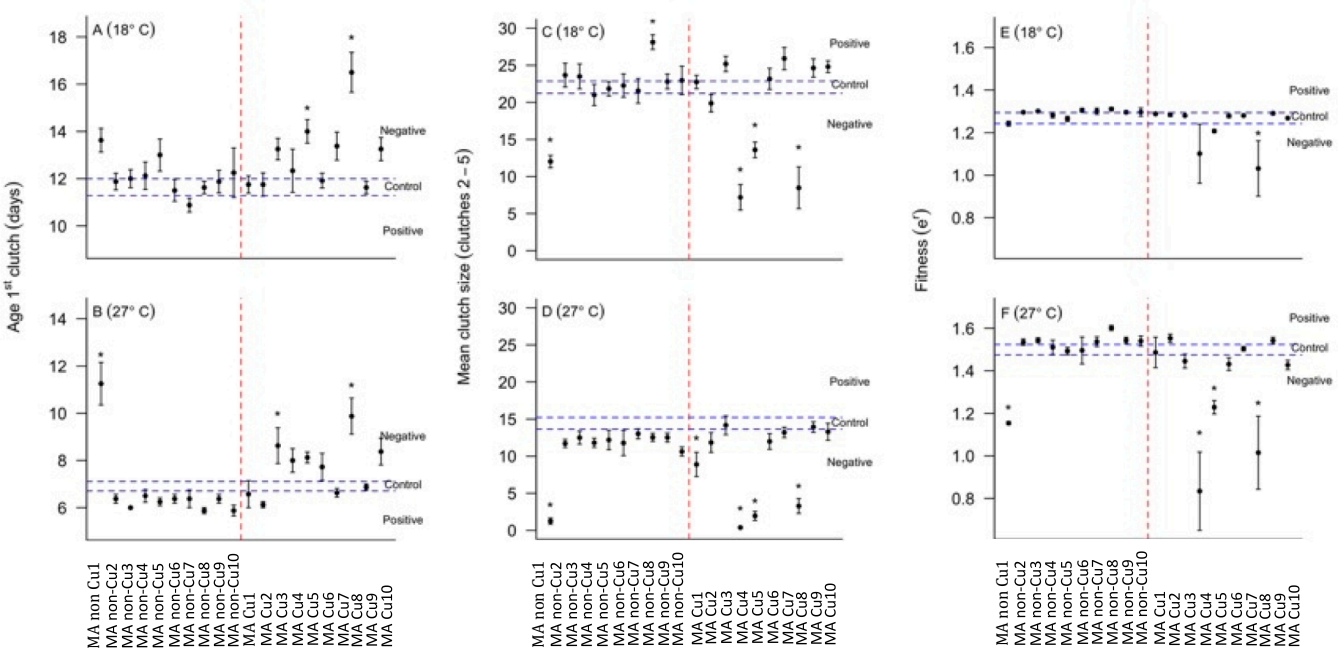


Fig. 6. Life history traits (mean ± standard errors) of mutation accumulation lines raised at 18 °C (A, C, E) and 27 °C (B, D, F). Hatched blue lines represent the mean and standard error of non-Ma lines. Lines that differ from the non-MA lines mean are * (Dunnett test, $p < 0{,}05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 5, paired t-tests, MA non-Cu, $t_9 = 0.75$, $p = 0.473$ and MA Cu, $t_9 = 0.74$, $p = 0.48$).

The among-line variance for age at the first clutch was higher for MA lines than for the non-MA group at 27 °C only (Table 2). Dunnett's test revealed that significant differences with the non-MA mean were limited to one line at 27 °C in the MA non-Cu treatment, and two lines at 18 °C and 27 °C for the MA Cu treatment).

3.7. No fitness differences among MA Cu, MA non-Cu and non-MA at both temperatures but significant temperature × treatment interaction at 27 °C

At 18 °C, *Daphnia* from the non-MA, MA non-Cu and MA Cu treatments had mean fitness values of 1.27, 1.29, and 1.23, respectively. The mean fitness of *Daphnia* from all treatments increased significantly under high temperature, reaching 1.50 for non-MA and MA non-Cu and 1.33 for MA Cu lines (linear mixed models, non-MA, $F_{1,140} = 57$, $p < 0.001$, MA non-Cu, $F_{1,140} = 503$, $p < 0.001$, MA Cu, $F_{1,140} = 9$, $p < 0.001$). Mutational treatment had no significant effect on fitness at 18 °C or 27 °C (linear mixed models: 18° °C, $F_{2,\ 27} = 1.85$, p = 0,18; 27 °C, $F_{2,\ 27} = 2{,}97$, p = 0,07). However, there was a significant temperature × treatment interaction for this trait (linear mixed model, $F_{2,\ 447} = 8.52$, $p = 0.034$), suggesting that the general fitness increase at 27 °C was not equivalent across the treatments. A paired *t*-test showed that the relative mean fitness was significantly different between temperatures for MA Cu, but not for MA non-Cu lines (MA control, $t_9 = 0.87$, $p = 0.409$; MA Cu, $t_9 = 2.41$, $p = 0.04$). The mean relative fitness of MA non-Cu lines was virtually the same at 18 °C and 27 °C (1.02 vs 1.00) but decreased from 0.97 to 0.89 for MA Cu lines (Fig. 2). Visual observation of reaction norms revealed that plastic responses across MA Cu lines were highly variable, and some lines even showed a decreased fitness at 27 °C compared to 18 °C (Supplemental Figure 2), as was also the case for one MA non-Cu line.

At 18 °C, the among-line variance of the MA treatments was not higher than the non-MA control group variance (Table 2). At this temperature, the variance of the MA Cu group was higher than that of the MA non-Cu group. High temperatures had a greater impact on the among-line variances in fitness for the MA lines than for the non-MA group. At 27 °C, the MA non-Cu V_L was significantly higher than the selective-control V_L, and the MA Cu V_L was significantly higher than both the non-MA and MA non-Cu V_L (Table 2).

The increased among-line variance of the MA non-Cu treatment was imputable to one line that showed an important fitness decrease at 27 °C, which had a significantly lower fitness compared to the selective-control mean at this temperature. In the MA Cu treatment, one line had lower fitness than the selective control means at 18 °C (Dunnett's test) and three at 27 °C (Dunnett's test).

3.8. MA Cu lines with low fecundity also have low mtDNA copy number

Three of the 10 MA Cu lines (MA Cu1, MA Cu4, MA Cu8) had lower mean clutch size than non-MA lines as well as lower fitness values ($p <$ 0,001). In addition, these lines stood out as having very low mtDNA/nucDNA ratios. Interestingly, there was a significant correlation between mean clutch size and mtDNA/nucDNA ratios in the copper MA lines at 18 °C ($R^2 = 0.85$, $p < 0.001$) (Fig. 7) but not in the non-copper MA lines. When the temperature was increased to a high target of 27 °C, the relationship between mean clutch size and mtDNA copy number remained significant ($R^2 = 0.55$, $p = 0.008$; Fig. 7).

4. 4.Discussion

4.1. Mitochondrial respiration

Our study assessed if spontaneous mutation accumulations (nuclear and mitochondrial) affect mitochondrial performance and fitness traits. To do so, the mitochondrial respiratory capacity, mtDNA copy number, and life history traits were measured in *Daphnia* lines that have accumulated mutations for 120 generations in the presence and absence of copper. Our results showed that mitochondrial respiration, measured *in situ* by providing substrates to feed the ETS through the NADH and the succinate pathways, was 10 % lower in both MA lines as compared to controls. This difference was present when the ETS was fed via either NADH or succinate pathway, or both pathways simultaneously. Because the FCR for the NADH or the Succinate pathway and the ratio NADH/Succinate pathway were the same between lines, one possible explanation of the change in respiration in flux per mg of protein could be a change in mitochondrial content between the lines. However, since the respiration rates of complex IV and CS activity per mg protein (two markers of mitochondrial contents) did not differ between groups, this corroborates the hypothesis that mutation accumulation does affect the function of respiratory system upstream of Complex IV. Also, a drop by approximately 10 % in the catalytic capacity of both NADH and succinate pathways suggest that the small deficiency in MA lines affects a step common to these two pathways. Knowing that CI OXPHOS as well as CII OXPHOS decreased while CS and CIV did not change (both markers of mitochondrial content), the decrease in OXPHOS could be related to a loss of function at a trait common to CI and CII OXPHOS, which could be Complex III.

Two main processes can explain the lower OXPHOS capacity in the MA lines compared non-MA lines: 1) the cumulated effects of deleterious mutations that have occurred in the MA lines and have been fixed during the experimental period or 2) the improvement of the mitochondrial OXPHOS capacity of non-MA isolates following positive selection. While our experimental design does not allow us to eliminate completely the

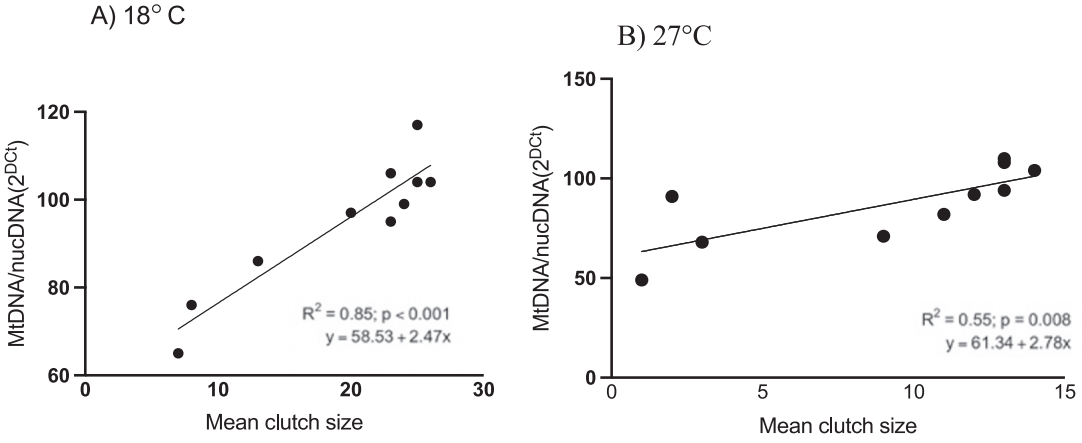


Fig. 7. Relationship between mean clutch size and mtDNA/nucDNA ratios in mutation accumulation lines raised at 18 °C (A) and 27 °C (B), both with copper.

hypothesis that controls without MA have been subjected to positive selection for mitochondrial function, we consider the first hypothesis as most parsimonious. The sequencing analyses performed previously on our MA lines have shown a higher level of nuclear mutations in these lines when raised under low copper compared to the control non-MA isolates (Chain et al., 2019; Flynn et al., 2017) that translated into higher age at maturity, decreased fecundity, and lower mtDNA copy number in our study. MA experiments generally report a decrease in the mean value of complex phenotypic traits (Halligan & Keightley, 2009; Huey et al., 2003; Schaack et al., 2013). The mtDNA has not been sequenced in our lines but mtDNA generally shows a higher mutation rate than nuclear DNA (e.g. Haag-Lieutard et al., 2008). Mutation accumulation in mtDNA can cause adverse effects to mitochondria with damages to the components of the respiratory chain resulting in mitochondrial dysfunction and increased ROS damages (Wallace 2010). The ratio of wild type to mutant mtDNA within a cell (heteroplasmy) leads to a "threshold effect" in mitochondrial function. A certain percentage of mutated mtDNA must accumulate before the cell exhibits dysfunction (Klucnika and Ma 2019). In humans, mtDNA deletions have been shown to increase across post-mitotic tissues during aging and can lead to a loss of respiratory capacity of cells (Kraytsberd et al. 2006) though it is not a universal response. During mutation accumulation experiments, mutations in the nuclear DNA are fixed in single lines reproducing ameiotically for more than hundreds of generations. As such, their effects could be similar to what is seen in aging cells where *de novo* somatic mutations accumulate with age. Since there are over 1500 nuclear −encoded mitochondrial genes compared to only 37 mitochondrial genes, there are far more opportunities for mutations perturbing mitochondrial respiration to be in the nuclear genome. As we found no significant interline differences in mitochondrial respiration, it is likely that the mutational effects on this trait are generated by the cumulated effects of numerous mutations of weak effects rather than by a few mutations of greater effects. This contrasts with our life history results where significant variation in traits among lines was due to a few lines that suffered large scale mutations. Our results suggest that mitochondria are good at eliminating big mutations by purifying intra-individual selection. MA lines raised under low copper and no copper did not differ in OXPHOS capacity, stressing again the importance of purifying selection on the mitochondrial genome. Interestingly, the expression of Hsp60, a mitochondrial stress protein has been shown to increase in MA lines of *Daphnia magna* exposed to thermal stress suggesting a role for HSPs in the buffering of mutations (Scheffer et al. 2022). Leuthner et al. (2022) found that, in *C. elegans*, mtDNA was resistant to damage-induced single nucleotide mutations after hundreds of generations of exposure to cadmium, even in absence of key mitophagy genes and despite an increase in nuclear DNA mutation rate.

4.2. MtDNA copy number

The higher copy number of mtDNA at 18 °C in most MA lines compared to control non-MA lines suggests the existence of a compensatory response following mutation accumulation. Increases in mtDNA copy number is one of the most frequently observed compensatory mechanism in mitochondria and has been linked with pathologies, aging, as well as various sources of exogenous stress such as temperature and contaminants (Bratic et al., 2009; Crane et al., 2013; Fetterman et al., 2017; Rumsey et al., 2017). Modest mitochondrial dysfunction could induce a retrograde response favoring the replication of mtDNA via an increase in the level of ROS (Lee and Wei, 2005; Moreno-Loshuertos et al., 2006). A high level of mtDNA would allow to maintain a functional excess aimed at ensuring the mtDNA integrity and a good level of gene expression, thus protecting the organism against a decline in energy metabolism (Lee and Wei, 2005; Atamna et al., 2018). In *Drosophila*, the negative effects of the mutations on mitochondrial respiration are mitigated by increasing their mtDNA content during development (Correa et al., 2012). At 27 °C, the mtDNA copy number did not differ between the MA lines and the control lines, suggesting that under this high temperature, the mitochondria could no longer compensate leading to the repressed mitochondrial respiration observed in all the MA lines. An alternative hypothesis for the increase in mtDNA copy number at the lower temperature is that selection has favored the proliferation of mtDNA haplotypes that have a replication advantage in MA lines. Bottlenecking across generations reduces selection on individuals but does not eliminate intracellular selection among mtDNA copies. As a result, it is possible that MA lines tilt the balance towards selection for intracellular replication advantage. The positive relationship of mtDNA copy number with fitness would be congruent with this explanation. Several studies have reported the existence of selfish mitochondrial mutants that can outcompete beneficial mitochondrial genomes and rise to high frequencies because the strength of selection on the mitochondrial genomes for cellular function is weaker than the strength of selection to replicate (Havird et al., 2019). Taylor et al. (2002) have shown that defective mitochondrial genomes of yeast can accumulate because of a within cell replication advantage when among cell selection for efficient respiration is relaxed. They found a strong and inverse relationship between yeast population size and the success of mutant mitochondrial genomes (petites) with a replication advantage. Mitochondrial mutations were favored by natural selection within cells but were consistently kept at very low frequencies in large populations. Similarly, ND5 deletion mutants *C. briggsae* were found to have a transmission advantage in MA experiments (Clark et al. 2012). MA experiments conducted in *C. elegans* revealed the propagation of a selfish mitochondrial mutant. This mutant had important deletions in 2 protein coding mitochondrial genes and showed a 35 % increase in mtDNA copy number that may help in its proliferation despite fitness disadvantage to the host (Sequeira et al. 2024).

The very strong relationship between mtDNA copy number and fecundity in the MA lines with copper suggests that there is an optimal level of mtDNA to maintain below which fitness is compromised. Three of the copper lines (MACu1, MACu4, MACu8) did not have the same compensatory response as the other MA lines at 18 °C and their mtDNA/nDNA ratio was even more reduced at 27 °C. The more important deleterious effects of accumulated mutations could represent a sufficient stress to compromise the compensatory response of mtDNA in these lines, even under optimal thermal conditions. Whole genome sequencing of these outlier lines (respectively lines 268, 281, 291 in Chain et al. 2018) revealed that copper led to an increased number of large-scale deletion events (Chain et al. 2018). The accumulated deleterious mutations in these lines may hinder mtDNA replication. This effect could be mediated by an excessive increase in oxidative stress caused by these mutations (Lee and Wei, 2005). An alternate assumption, but not mutually exclusive, would be that the control region in the D-loop – identified as hot-spot mutations in this species prevent the efficient replication of mitochondrial genome (Xu et al., 2012). Such mutations could affect the compatibility between replication promotor sites in this region and the mitochondrial polymerase (Burton et al., 2006). Decrease in the number of copies of mtDNA have previously been associated to mutations in the D-loop region of cancer cells (Lee et al., 2004). In a study involving more than 274,832 whole genome sequences derived from human tissues, Gupta et al. (2023) found that nuclear haplotypes could influence mtDNA copy number and heteroplasmy. Heteroplasmic indels maternally inherited as mixtures with relative levels were associated with 42 nuclear loci involved in mtDNA replication, maintenance and novel pathways. The authors suggested that these nuclear loci could act by conferring a replicative advantage to specific mtDNA molecules instead of by DNA mutagenesis.

4.3. Life history traits

Life history traits were measured under optimal and high temperatures to assess if the expression of spontaneous mutations were impacted by environmental variation. High temperatures accelerated sexual

maturation, increased fitness, and reduced clutch size in all three groups. Our results are consistent with those of Davenport et al. (2021) for *Daphnia* MA lines raised at 20 °C and 24 °C, except that we report decreased fecundity at our higher temperature. Significant interactions between mutation accumulation (treatment) and temperature for fecundity and fitness indicate that some mutational effects depended on the thermal environment. As predicted, the major effects of high temperature on clutch size and fitness were to exacerbate the cumulative deleterious effects of spontaneous mutations. In both MA treatments, exposure to thermal stress led to a similar decline in relative fitness (18 °C vs. 27 °C). The average decline in fitness of the MA lines propagated with copper compared to the non-MA group varied from 3 % at 18 °C to 10 % at 27 °C. This decline was accompanied by an increase in genetic variance at 27 °C, as expected, if high conditions exacerbated the mutational effects.

Our study is one of the first to use a mutation accumulation approach to examine the effects of mutations on mitochondrial respiration. We report lower rates of mitochondrial respiration in all MA lines when compared with the non-MA controls, likely associated with the accumulation of inherited mutations. We also show that mtDNA copy number can compensate, to some extent, the deleterious effects of mutations on mitochondria under optimal temperature. Under high temperatures, the compensatory response of increased mtDNA copy number was impaired, resulting in a decrease in respiratory capacity. Further work investigating processes underlying mitochondrial quality needs to be carried out using mutation accumulation approaches.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The mutation accumulation experiments was made possible by a NSERC CREATE training program on Aquatic Ecosystem Health to MEC and a NSERC scholarship to FP. PB, FD, and HG helped finance the project with their NSERC discovery grants. We thank Jullien Flynn for her help with growing MA lines and Jigar Trivedi for commenting on an earlier version of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mito.2024.101976.

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