THE MITOCHONDRIA-TARGETED HYDROGEN SULFIDE DONOR AP39 IMPROVES HEALTH AND MITOCHONDRIAL FUNCTION IN A *C. ELEGANS* PRIMARY MITOCHONDRIAL DISEASE MODEL

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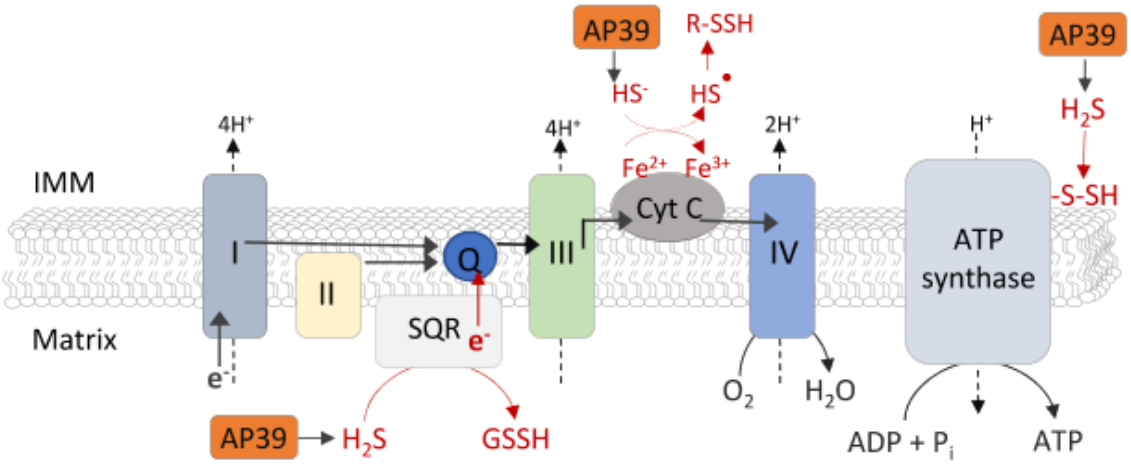
GRAPHICAL ABSTRACT



SUMMARY

Primary mitochondrial diseases (PMD) are inherited diseases that cause dysfunctional mitochondrial oxidative phosphorylation, leading to diverse multisystem diseases and substantially impaired quality of life. PMD treatment currently comprises symptom management, with an unmet need for therapies targeting the causative mitochondrial defects. Molecules which selective target mitochondria have been proposed as potential treatment options in PMD but have met with limited success. We have previously shown in animal models that mitochondrial dysfunction caused by the disease process could be prevented and / or reversed by selective targeting of the 'gasotransmitter' hydrogen sulfide (H_2S) to mitochondria using a novel compound, AP39. Therefore, in this study we investigated whether AP39 could also restore mitochondrial function in PMD models where mitochondrial dysfunction was the cause of the disease pathology using *C. elegans*. We characterised several PMD mutant *C. elegans* strains for reduced survival, movement and impaired cellular bioenergetics and treated each with AP39. In animals with widespread electron transport chain deficiency (*gfm-1(ok3372)*), AP39 (100 nM) restored ATP levels, but had no effect on survival or movement. However, in a complex I mutant (*nuo-4(ok2533)*), a Leigh syndrome orthologue, AP39 significantly reversed the decline in ATP levels, preserved mitochondrial membrane potential and increased movement and survival. For the first time, this study provides proof-of-principle evidence suggesting that selective targeting of mitochondria with H_2S could represent a novel drug discovery approach to delay, prevent and possibly reverse mitochondrial decline in PMD and related disorders.

CONCISE TAKE-HOME MESSAGE: Mitochondria-targeted sulfide delivery may represent a novel approach to PMD drug discovery and/or treatment.

INTRODUCTION

Primary mitochondrial diseases (PMD) are inherited, genetic-based disorders that cause impaired mitochondrial oxidative phosphorylation [1]. They present as childhood- and adult-onset, at an estimated frequency of 1:4300 live births [2], and cause significantly impaired quality of life, encompassing both high morbidity and mortality [3,4]. With over 350 mitochondrial and nuclear genes identified as causing PMD to date, PMD are etiologically and phenotypically diverse [3].

Presently, there is an unmet clinical need for PMD-specific medicines, with treatments centred primarily on symptom management, rather than causative mitochondrial defects. Clinical trials on resveratrol, to improve mitogenesis [5,6], acipimox, to restore NAD^+ levels [7], and gene therapies to correct for heteroplasmic mitochondrial DNA disorders [8,9] have proven inconclusive. Additionally, the only drug currently licensed to treat PMD is idebenone, a coenzyme Q_{10} analogue used to treat visual impairment in Leber's hereditary optic neuropathy [5], which is not licensed universally due to limited evidence of effectiveness [10].

A role for hydrogen sulfide (H_2S) in mitochondrial function can be traced back around 1.5 billion years to mitochondrial evolution where, in the sulfur-rich environment, H_2S oxidation provided an electron source to fuel ATP production [11,12]. Although mitochondria have subsequently evolved to function in an oxygen-rich environment, conservation of H_2S as an electron source remains, with H_2S being the only known inorganic substrate of the electron transport chain [13,14,15]. Impaired endogenous H_2S biosynthesis contributes to the pathophysiology of several conditions including hypertension, atherosclerosis, and neurodegenerative disease [16-19], and application of exogenous H_2S has been shown to alleviate disease phenotypes in numerous disease models [20,21].

Mitochondria are a primary target of H_2S signalling, where H_2S stimulates many aspects of mitochondrial function, including mitochondrial bioenergetics [12,22,23], mitochondrial biogenesis and morphology [24, 25], protection against oxidative stress [26] and stabilisation of apoptosis [27,28]. Moreover, several mitochondrially-mediated mechanisms have been established for H_2S including: electron donation to the ETC *via* the sulfide:quinone oxidoreductase (SQR) enzyme system [14,15, 29]; persulfidation of cysteine residues on proteins (addition of a thiol group to an accessible cysteine residue: R-SH $\rightarrow$ R-SSH), such as ATP synthase F1 subunit α, leading to enhanced activity [30,31,23] and; binding and/or reducing metal centres of iron heme proteins, e.g. ferric cytochrome c leading to increased persulfidation of mitochondrial proteins and cytoprotection [28].

Given the cytoprotective role of H_2S in mitochondria, we developed a novel mitochondria-targeted slow-release H_2S delivery molecule AP39, which contains the H_2S-releasing moiety anethole dithiolethione (ADT-OH) and exploits a triphenylphosphonium (TPP^+) motif to accumulate in the mitochondria [32-34]. AP39 protects mitochondrial function and improves disease phenotypes in several *in vitro* and *in vivo* models of disease, including protection against oxidative stress

following hyperglycemic injury [33], neuroprotection in Alzheimer's disease and following cardiac arrest [35,36] and protection against ischemia reperfusion injury in renal and heart transplant models [37,38]. These effects of AP39 occur at doses/concentrations several orders of magnitude lower than non-targeted H_2S generating molecules [32-34]. Since drug-induced mitochondrial toxicity is a key risk factor in PMD drug safety assessment [4], the low effective dose renders AP39 a promising H_2S-based therapy. These studies endorse a therapeutic role for mitochondria-targeted H_2S where mitochondrial dysfunction is a secondary feature of disease. As such, mitochondria-targeted H_2S donors might also alleviate symptoms where mitochondrial dysfunction is the primary cause of disease. We, therefore, sought to determine if AP39 alleviates mitochondrial dysfunction in PMD, using mitochondrial mutant *C. elegans* as an established model for understanding human disease [39] and, specifically, as an *in vivo* model of PMD [40].

MATERIAL AND METHODS

The mitochondria-targeted H_2S generating molecule AP39 was synthesised as previously described by us [32]. Unless otherwise stated, reagents were purchased from Sigma-Aldrich Company Ltd. (Dorset, UK). Culture plates and 96 well plates were purchased from Greiner Bio-One Ltd. (Gloucestershire, UK).

2.1. *C. elegans Strains and Culture*

The following strains were used in this study: N2 wild-type, VC1947 (*nuo-4*(*ok2533*) III/hT2 [*bli-4(e937)* let-?(*q782*) qIs48] (I;III).); VC2701 (F29C12.4(*ok3372*)/mT1 II; +/mT1 [*dpy-10*(*e128*)] III); VC294 (*sdhb-1*(*gk165*)/mIn1 [mIs14 *dpy-10*(*e128*)] II); LB21 (*nuo-1*(*ua1*)/mIn1 [*dpy-10*(*e128*) mIs14] II). Strains were obtained from the *Caenorhabditis* Genetics Center (CGC, University of Minnesota). For routine culture and experiments *C. elegans* were incubated at 20°C on NGM agar plates containing OP50 *E. coli* as food source [41]. To note, *nuo-4* and *gfm-1* animals displayed developmental delays of approximately 8 hours to young adult stage. Both strains were therefore synchronised 8 hours before N2's, where morphological characteristics were used to determine young adult stage rather than chronological age to ensure age-matched populations. Whilst *nuo-4* mutants showed no alterations in gross morphology, twice as many L1's are needed to obtain equal quantities of young adult worms to wild-type. In contrast, *gfm-1* animals displayed mixed phenotypes of normal and dumpy morphology (see supplementary fig.1). It should be noted that these defective animals were included in analysis throughout and experienced the same developmental delays as animals or normal phenotype. 2ml drug plates were prepared with either 100nM AP39 or respective DMSO vehicle control (0.001%) and stored at 4°C for ≤ 16 hours before use. For all experiments, synchronised *C. elegans* populations at larval stage L1 were isolated by gravity synchronisation and transferred onto drug plates at 20 worms per plate. Day 0 young adults were defined as L4 + 1d.

Strains were grown to young adult stage (day 0) and subsequently transferred to fresh drug plates at 20 worms per plate every other day for the duration of the experiment. Worms were transferred by collecting worms from plates in M9 [41] and washing a minimum of three times by gravity sink to remove offspring. Following transfer any remaining offspring were removed using a needle.

2.2. *C. elegans Survival Assay*

For each strain 20 L1 worms were plated onto 2ml plates. Live and dead worms were scored from Day 0 onwards, with worms scored as dead when no response was observed following tapping with a needle**.** Following transfer of worms, live worms were counted as the reference for subsequent scoring and overall survival was calculated as % live worms. n ≥ 200 animals were per assay across two separate experimental replicates.

2.3. *C. elegans Movement Assay*

Movement was measured using the WMicrotracker One (Phylumtech, S.A. Santa Fe, Argentina). Worms were collected from plates in M9, washed three times and added to a 96 well plate in 100μl M9. Worms were incubated at 20°C for 10 minutes prior to assaying and movement over the following 30 minutes measured and normalised to counts per worm. At least three wells with 15 worms per well were assayed per condition, and a minimum of two separate assays performed. n ≥ 90 animals per condition, per time point.

2.4. *Measuring basal oxygen consumption rate (OCR) in C. elegans*

Basal OCR was measured using the Agilent Seahorse XFe24 Analyzer with the Seahorse XFe24 FluxPak (Agilent Technologies LDA UK Limited, Cheshire, UK). Assays were performed according to the manufacturer's instructions. Worms were collected from plates in M9, washed three times and added to the 24 well culture plate. Basal OCR was measured using eight cycles of the following: mix plate for 3 minutes, pause for 3 minutes, measure for 3 minutes. To minimize the effect of outliers due to unstable OCR measurements [42,43], outliers were identified using the ROUT method (Q 1%), (GraphPad Prism 8) and omitted from analysis. OCR was normalised to OCR/min/worm. Five wells with 20 worms/well were assayed per condition and a minimum of two separate assays performed. n ≥ 200 animals per condition, per time point.

2.5. *C. elegans ATP Assay*

ATP levels were measured using the CellTiter-Glo® 2.0 assay (Promega, Hampshire, UK). Worms were collected from plates in M9, washed three times and left in a final volume of 30 µl M9. Samples were freeze-thawed in liquid N_2 three times, before storing at -80°C. Prior to the assay samples were spun in a microcentrifuge at 15,000 rpm for 10 minutes at 4°C. Supernatant was collected and ATP levels measured in triplicate according to the manufacturer's instructions on a PHERAstar plate reader (BMG LABTECH Ltd., Bucks., UK). Sample protein levels were measured by Bradford assay (Bio-Rad Laboratories Ltd., Hertfordshire, UK), and ATP levels normalised to mg protein. 70 worms were assayed in triplicate per condition, n ≥ 140 animals per condition, per time point.

2.6 Mitochondrial membrane potential *in situ*

Mitochondrial membrane potential was assessed using the lipophilic potentiometric fluorophore JC-10 (Enzo Life Sciences, UK) and worms imaged by fluorescence microscopy as described previously (43). Animals were synchronised to L1 by gravity flotation and grown to young adult (day 0) containing vehicle or AP39 100 nM) and transferred to fresh plates every 2 days. On days of imaging, 50 animals (per condition) were picked into 40 µl of JC-10/S-medium (83 µM) and incubated in the dark at 20^{o} C for 4 hours (43). Animals were then washed and centrifuged 2-3 times in M9 before pipetting out 20 µl on a slide and immobilising *via* a cover slip. Each animal was visually assessed and ~20 - 30 representative images were taken before photo-bleaching could occur. Images were taken with a 40 x objective and were all exposed to light emission for 1 second with a gain of 3.4d.B. Images were then analysed in ImageJ for pixel intensity in muscle cells.

2.7. *Statistics*

Statistics were performed in GraphPad Prism 8, with significance determined by paired t-test or two-way ANOVA with post hoc Sidak's multiple comparisons test. For image analysis (e.g. mitochondrial membrane potential) data were analysed as a two-way ANOVA with Tukey's multiple comparison.

RESULTS

C. elegans mitochondrial mutant strains display variable changes in lifespan and movement rate

For this study, *C. elegans* strains containing mutations that span complex I (*nuo-1* and *nuo-4*), complex II (*sdhb-1*), as well as multiple ETC deficiency (*gfm-1*), were used to reflect the multiple

mitochondrial variations in, and varying human forms of, PMD (Table 1). Characterising the mitochondrial mutants revealed reduced survival in the *nuo-4(ok2553)* and *gfm-1(ok3372)* strains compared to N2 wild-type animals ($P < 0.0001$; $P< 0.0001$) (median lifespan 4.6, 6.5 and 8.4 days, respectively), with no effect on survival observed in the *sdhb-1(gk165)* or *nuo-1(ua1)* strains (Fig. 1A). Given that muscle is rich in mitochondria and assessment of muscle activity by movement assays is an excellent indicator of whole animal health [50], movement assays were subsequently performed in the wild-type and mutant strains. Reduced movement was observed in the *nuo-4(ok2533)*, *gfm-1(ok3372)* and *nuo-1(ua1)* mutants compared to N2 on day 2 ($P < 0.0001$; $P < 0.0001$; $P < 0.001$), with the *nuo-1(ua1)* mutant also displaying reduced movement on day 6 ($P < 0.01$) (Fig. 1B). No change in movement was observed in the *sdhb-1(gk165)* strain (Fig. 1B). Taking into consideration the consistently lowered movement rates up to day 6 of adulthood, plus the relevance of childhood – early adulthood as critical clinical time frames for human PMD [51], assessment of movement later into the lifespan was deemed unnecessary.

AP39 restored survival and movement only in nuo-4 complex I mutants.

As the *nuo-4(ok2533)* and *gfm-1(ok3372)* strains showed both reduced survival and movement rates compared to N2 (Fig. 1), they were selected for the further study of the effects of AP39 on animal health, survival and mitochondrial function. Survival and movement were measured from day 0 to day 3, as from the initial characterisation altered phenotypes were observed over this time range. In the absence of AP39, reduced survival was again observed in the *nuo-4* and *gfm-1* strains compared to N2 on day 2 ($P < 0.01$; $P < 0.05$) and day 3 ($P < 0.001$; $P <0.001$) (Fig. 2A), and reduced movement observed in *nuo-4* and *gfm-1* animals on day 2 ($P < 0.01$; $P < 0.001$) (Fig. 2B). The addition of AP39 led to increased survival in *nuo-4* animals on day 3 ($P < 0.001$), with no effect on survival in N2 and *gfm-1(ok3372)* mutants (Fig. 2A). The effect of AP39 on movement showed a similar trend, with AP39 increasing movement in the *nuo-4(ok2533)* strain on days 2 and 3 ($P < 0.01$; $P < 0.05$), and no effect of AP39 on movement in N2 and the *gfm-1(ok3372)* strain (Fig. 2B).

AP39 restored ATP levels and mitochondrial membrane potential ($\Delta\Psi_m$) in nuo-4 and gfm-1 mutants

To examine the effect of AP39 on cellular bioenergetics, basal OCR and ATP levels were measured in N2 and the *nuo-4(ok2533)* and *gfm-1(ok3372)* mitochondrial mutant strains (Fig. 3A). In the absence of AP39, lower basal OCR were observed in the *nuo-4* strain compared to N2 on day 0 ($P < 0.01$), with increased OCR compared to N2 on days 2 and 3 ($P < 0.05$). The *gfm-1(ok3372)* strain exhibited reduced OCR on day 0 compared to N2 ($P < 0.0001$), with no effect on subsequent days (Fig. 3A). AP39 addition had no effect on OCR in N2 or the mutant strains throughout the assay. In the absence of AP39, decreased ATP levels were observed in the *nuo-4* strain compared to N2 on

day 0 ($P < 0.01$), but not on subsequent days (Fig. 3B), and no changes in ATP levels were observed in the *gfm-1* strain compared to N2 throughout the assay (Fig. 3B). Addition of AP39 had no effect on ATP levels in the N2 wild-type strain, however, on day 0 AP39 addition led to a 2.2-fold (+/- 0.12) and 1.9-fold (+/- 0.02) increase in ATP levels in the *nuo-4(ok2533)* and *gfm-1(ok3372)* strains, respectively ($P < 0.0001$) (Fig. 3B). Muscle mitochondrial membrane potential was also reduced only in *nuo-4* mutant *versus* controls at all timepoints and, similarly to total ATP levels, *gfm-1(ok3372)* animals showed no change (Figs. 4A and B). AP39 treatment rescued this *nuo-4(ok2533)* membrane potential defect at days 0, and 2 days post-adulthood ($P < 0.0001$) and the age-associated mitochondrial decline in wild-type animals was restored at day 3 of adulthood (Fig. 4B).

DISCUSSION

PMD are inherited diseases that lead to dysfunctional mitochondrial oxidative phosphorylation, causing a range of multisystem diseases that lead to substantially impaired quality of life. With treatment for PMD being limited to symptom management, there is an unmet clinical need for PMD-specific therapies that address the underlying mitochondrial defects. Here we have provided evidence that the mitochondria-targeted H_2S delivery molecule AP39 has therapeutic efficacy for attenuating health and mitochondrial defects in a C. *elegans* model of complex I-based PMD, which was not observed in mutants characterised by multi-ETC complex failure.

Examining four mitochondrial mutants revealed reduced survival and movement rates in *nuo-4(ok2533)* and *gfm-1(ok3372)* animals, reduced movement in *nuo-1(ua1)* mutants, and no change *sdhb-1* mutants. Such varying survival rates are unsurprising, as defects in ETC proteins in *C. elegans* have been shown to lead to both reduced and extended lifespan [52]. Moreover, our observation of impaired movement in mitochondrially defective animals is in line with the very high mitochondrial content and metabolic requirements of the neuromuscular system [50]. Thus, whilst characterising all available *C. elegans* PMD orthologues [40] will markedly assist understanding the complexity of orthologous human PMDs, these findings help establish *nuo-4(ok2533)* and *gfm-1(ok3372)* mutants as promising models for exploring novel PMD interventions.

AP39 restored ATP levels and reversed the decline in survival and movement observed in *nuo-4* mutants, whereas in *gfm-1* animals, AP39 restored ATP levels, but had no effect on survival or movement. The *nuo-4(ok2533)* mutation is in the *C. elegans* orthologue of human *NDUFA10,* encoding NADH:ubiquinone oxidoreductase subunit A10. This subunit, while having no catalytic activity, is thought to be required for the correct assembly of complex I [53]. The *gfm-1* mutation is in the *C. elegans* orthologue of human *GFM1*, encoding a mitochondrial GTPase required for translation elongation. Mutations in *GFM1* cause widespread ETC deficiency and impaired maintenance of mitochondrial DNA [54]. The varied effects of AP39 observed may, therefore, reflect a capacity for H_2S to act through mechanisms downstream of complex I, such as the SQR enzyme

system or persulfidation of ATP synthase, that are sufficient to protect against complex I deficiency in *nuo-4(ok2533)* animals, but not to protect against the widespread mitochondrial dysfunction in *gfm-1(ok3372)* animals. This raises the possibility of personalised H_2S-based medicines for specific forms of PMD, the full extent of which across all *C. elegans* PMD orthologues [40] would prove an interesting focus for future research.

Although inconclusive, the finding that AP39 failed to alter basal OCR rates in both forms of PMD, but restored ATP levels in *nuo-4(ok2533)* and *gfm-1(ok3372)* animals, potentially reflects H_2S-mediated improvements in mitochondrial efficiency. Indeed, a similar phenomenon was reported following AP39 treatment of endothelial cells under hyperglycaemic conditions [33], where an AP39-induced reduction in proton leak caused the increased ATP levels independently of an increase in OCR. Additionally, mitochondrial H_2S has been shown to persulfidate two key cysteine residues (C244; C294) on ATP synthase F1 subunit a, leading to increased ATP synthase activity [23], which would be anticipated to increase ATP levels without necessitating a parallel increase in OCR. Additionally, selective mitochondrial protein *S*-persulfidation by AP39 [55] is catalysed by specific reduction of cytochrome *c* [56] where persulfidation protects mitochondrial proteins such as MnSOD from inactivation by detrimental oxidants such as peroxide and peroxynitrite [57]. Thus, mitochondrial-H_2S might serve to improve cellular bioenergetics independently of mitochondrial respiration *per se* and could be sufficient to account for the maintenance of movement observed in the *nuo-4* animals. Although our current data strongly suggest mitochondrial protection by AP39 in these *C. elegans* models, additional assays which assess mitochondrial mass and respiratory function (e.g. beyond basal respiration alone) would be useful therapeutic readouts, and other studies such as electron microscopy would be useful to confirm our findings further in future studies

In conclusion, we report for the first-time evidence that as a concept, mitochondria-targeted H_2S may represent a novel potential avenue to explore in drug development as a therapeutic strategy against specific forms of PMD. Additional studies are required to establish optimal doses and timings of compound administration, as well as the efficacy of mitochondrial H_2S across the full breadth of PMD mutations, and we are currently developing additional approaches to both target mitochondria and generate sulfide and related species to address this. Nonetheless, in the context of drug-related mitochondrial toxicity as a vital hurdle in PMD treatments [4], the very low (nM) doses required for positive health effects of mitochondria-targeted H_2S advocates AP39 and related compounds as a promising new forward-translational drug class against PMD and conditions of mitochondrial dysfunction.

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TABLES AND FIGURES

C. elegans Strain	*C. elegans* gene	Human gene	Human protein	Human Phenotype
LB21	*nuo-1*	*NDUFV1*	NADH:ubiquinone oxidoreductase core subunit VI	Mitochondrial complex I deficiency Leigh syndrome [44,45]
VC294	*sdhb-1*	*SDHB*	Succinate dehydrogenase complex iron sulfur subunit B	Mitochondrial complex II deficiency Leukodystrophy, isolated complex II deficiency [46]
VC1947	*nuo-*	*NDUFA10*	NADH:ubiquinone oxidoreductase subunit A10	Mitochondrial complex I deficiency Leigh syndrome [45,47]
VC2701	*gfm-1*	*GFM1*	Mitochondrial elongation factor G	Combined oxidative phosphorylation deficiency Leigh-like syndrome [45,48,49]

Table 1. Mitochondrial mutant *C. elegans* strains employed in this study and corresponding human primary mitochondrial disease.

FIGURE 1

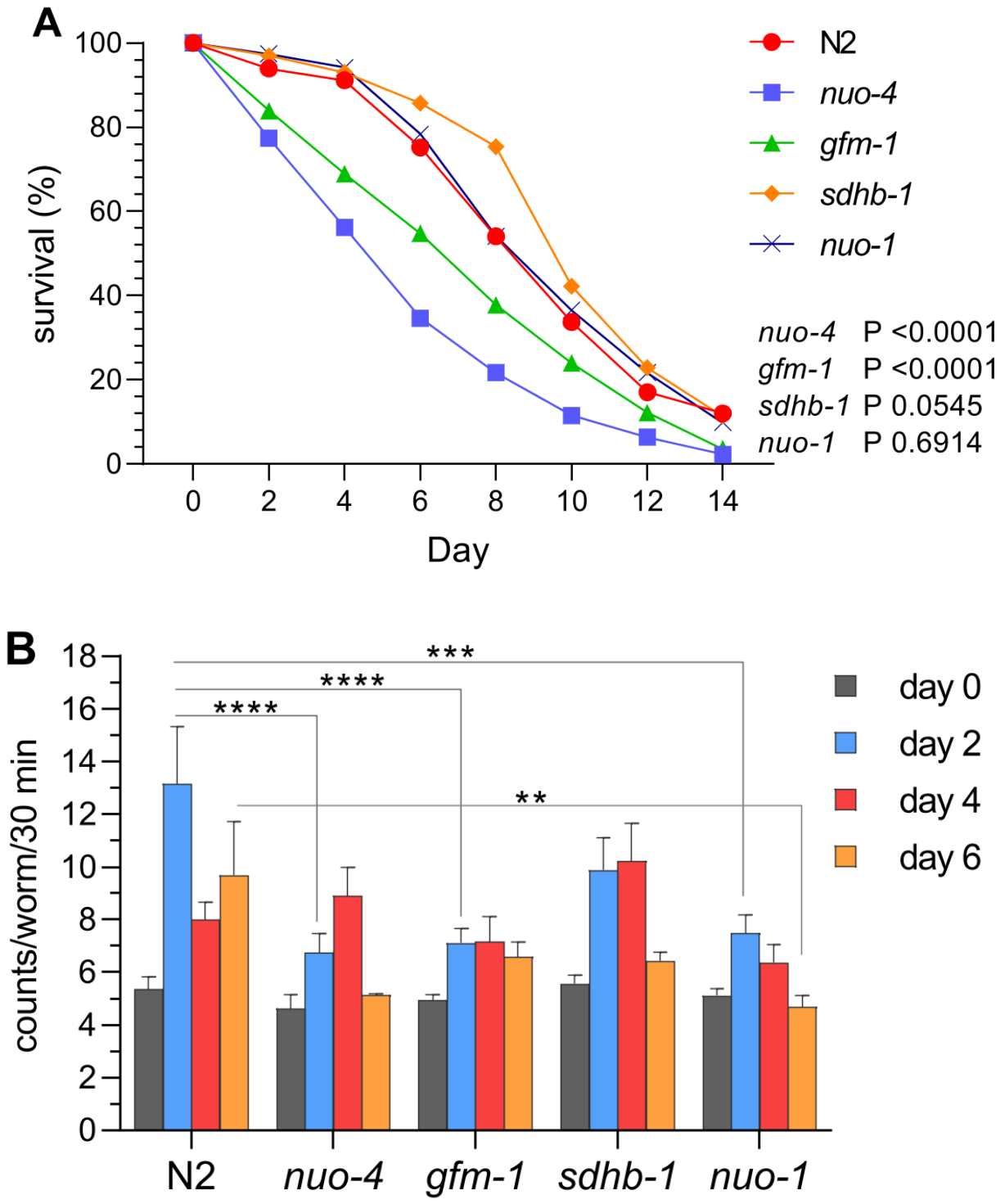


Figure 1: Mitochondrial mutant C. elegans display altered lifespan and movement rates. (A) Survival assay of wild-type (N2) and *nuo-4(ok2533), gfm-1(ok3372), sdhb-1 and nuo-1(ua1)* mitochondrial mutant strains. n ≥ 200. P values for comparison with N2 (two-way ANOVA). (B) Movement assay of wild-type (N2) and *nuo-4*, *gfm-1*, *sdhb-1* and *nuo-1* mitochondrial mutant strains. Data are mean ± SEM, n ≥ 90. ** P < 0.01 *** P < 0.001; **** P < 0.0001, (two-way ANOVA with post hoc Sidak's multiple comparisons test).

FIGURE 2

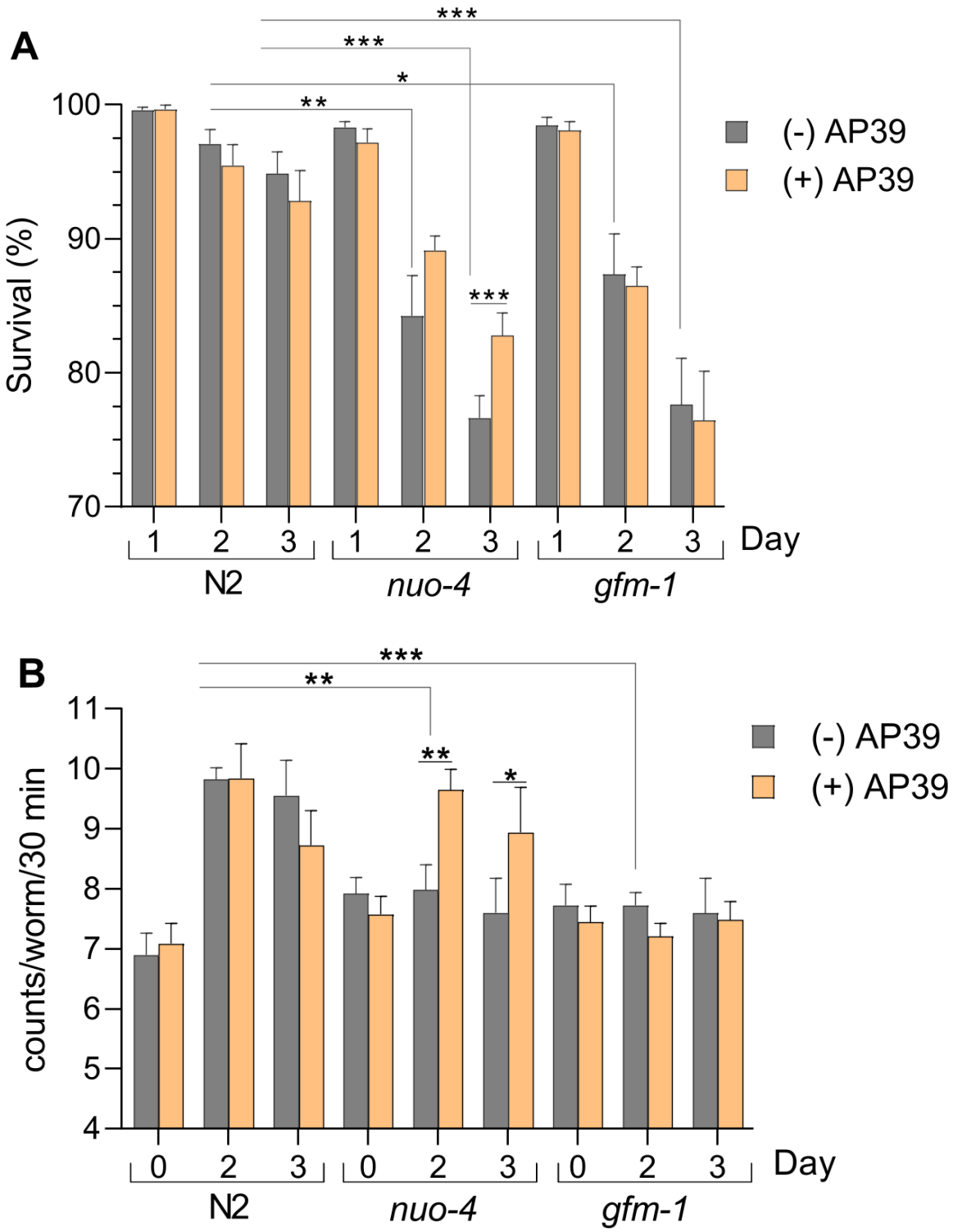


Figure 2: AP39 increased survival and movement only in *nuo-4* mitochondrial mutants. (A) Survival assay of wild-type (N2) and *nuo-4(ok2533)* and *gfm-1(ok3372*) mitochondrial mutant strains in the absence or presence of AP39 (100 nM), n ≥ 200. (B) Movement assay of wild-type (N2) and *nuo-4* and *gfm-1* mitochondrial mutant strains in the absence or presence of AP39 (100 nM), n ≥ 90. Data are mean +/- SEM, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Paired t-test (A); two-way ANOVA with post hoc Sidak's multiple comparisons test (B)).

FIGURE 3

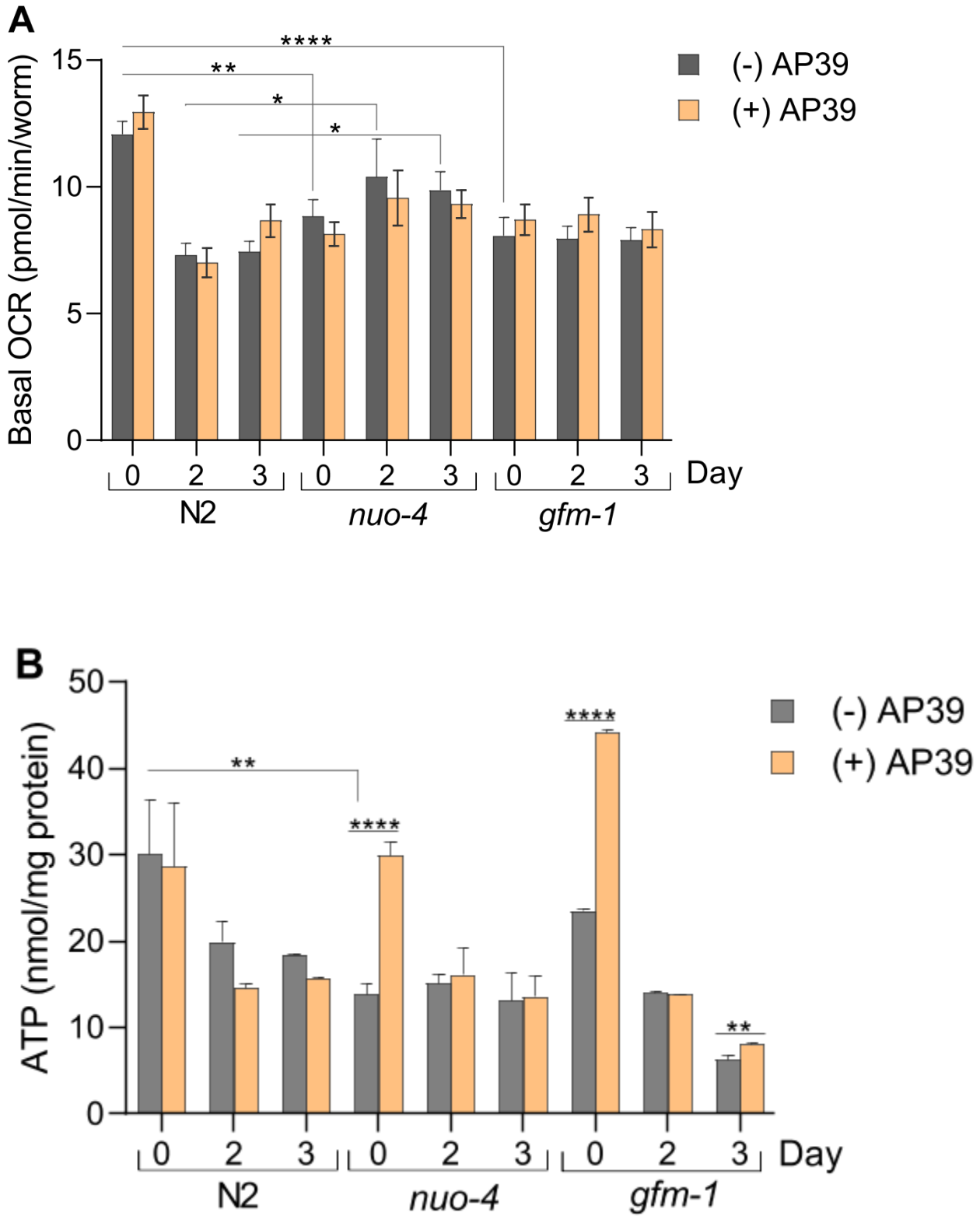


Figure 3: AP39 increased ATP levels but not basal OCR in *nuo-4 and gfm-1* mutants. (A) Basal OCR of wild-type (N2) and *nuo-4(ok2533)* and *gfm-1(ok3372*) mitochondrial mutant strains in the absence or presence of AP39 (100 nM), n ≥ 200. (B) ATP assay of wild-type (N2) and *nuo-4* and *gfm-1* mitochondrial mutant strains in the absence or presence of AP39 (100 nM), n ≥ 140. Data are mean ± SEM, * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001 (two-way ANOVA with post hoc Sidak's multiple comparisons test).

FIGURE 4

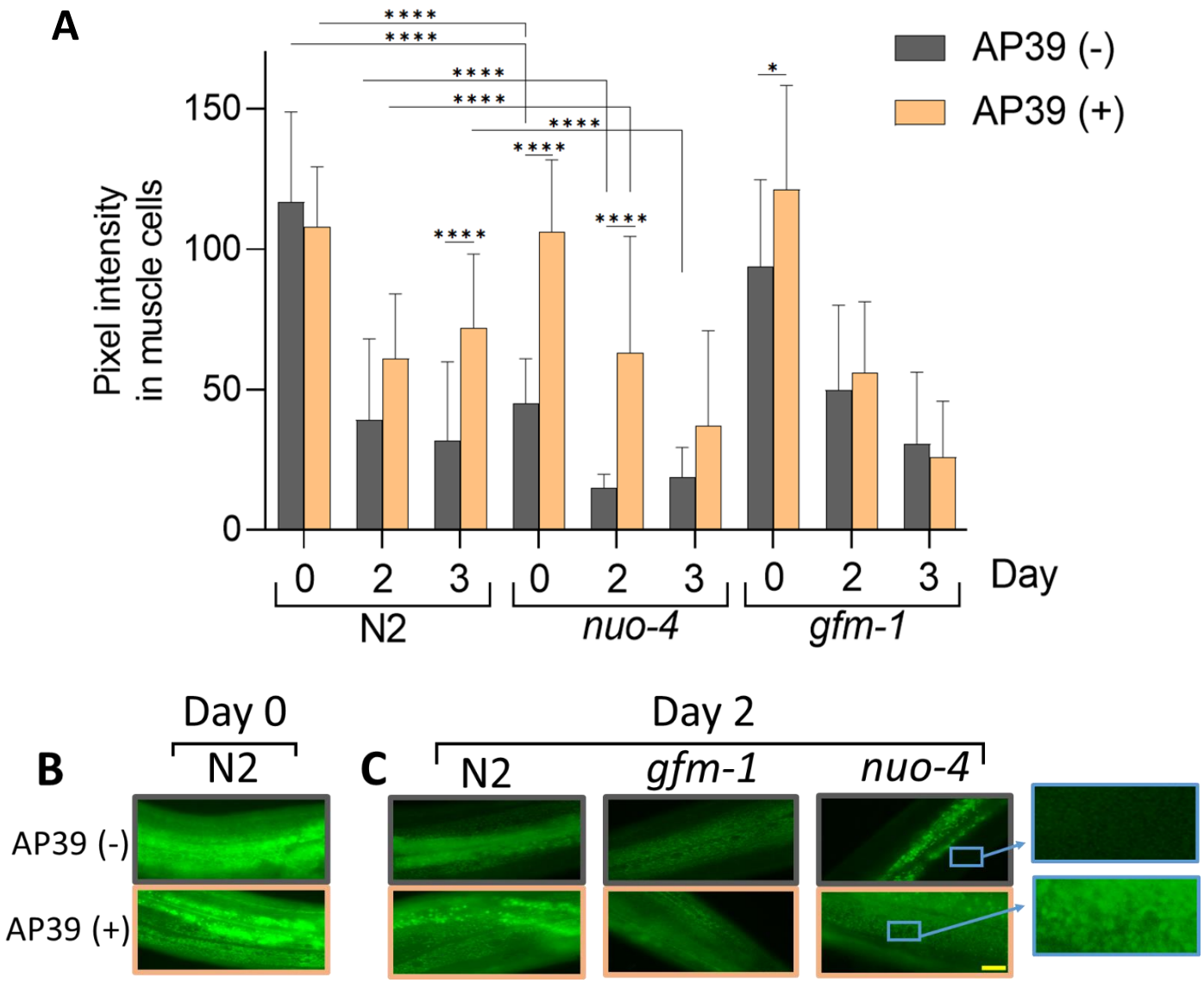
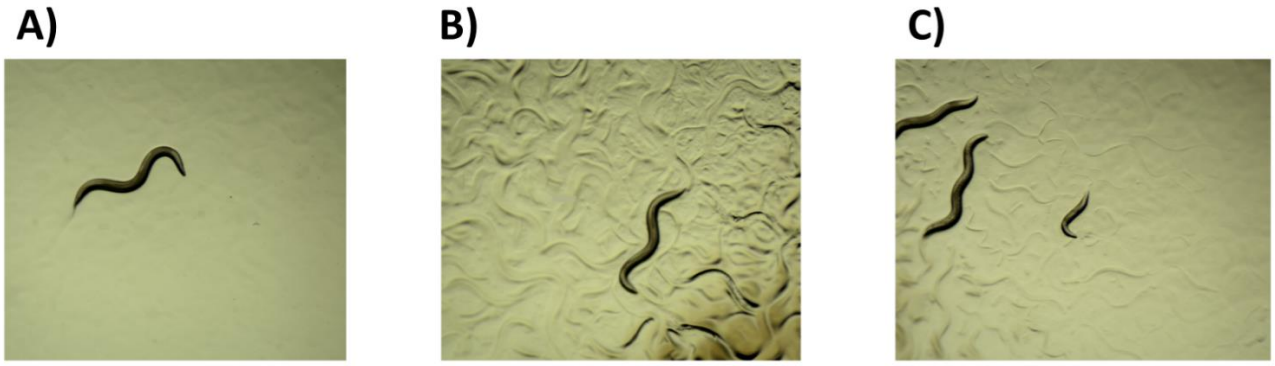


Figure 4: AP39 improved mitochondrial membrane potential in *nuo-4(ok2533)* and *gfm-1(ok3372*) mitochondrial mutant strains, and delayed age-related mitochondrial decline in wild-type animals. (A) Pixel quantification of JC-10 uptake within animal muscle cells. **(B)** Representative images of wild-type animals in the absence (top row) and presence (bottom row) of 100nM AP39 at day 0 showing strong mitochondrial uptake in muscle cells in both conditions. **(C)** Day 2 images of all conditions where *nuo-4(ok2533)* animals showed uptake predominantly within the intestine, where exposure to 100 nM AP39 improved muscle mitochondria uptake. Cut away images are magnified from muscle cells to demonstrate enriched JC-10 uptake with AP39 treatment (note, a brightness illumination of 30% was applied for clarity in cut away images alone). Data are mean ± SD, *n* ~ 30 animals per condition/time point, asterisks denote * P<0.01, **** P<0.0001. Two-way ANOVA with Tukey's multiple comparisons, GraphPad. Yellow scale bar in panel C represents 20μm.

Supplementary Figures



Supplementary Figure 1: Representative images showing N2 (A), *nuo-4* (B) and *gfm-1* (C) animals at young adult stage (day 0). Both *nuo-4* and *gfm-1* animals were synchronysed as L1's approximately 8 hours earlier than N2's due to developmental delays. Additionally, 50% more animals were seeded per plate in the *nuo-4* condition with a clear impairment of some animals to reach young adult. In *gfm-1* mutants, the majority population are of normal phenotype, with some animals displaying morphological impairments as seen in figure C above. It should be noted these animals were included in the experiment and experienced equal developmental delays. Age synchronisation across strains to animal development rather than chronological age ensures any differences in health-related measurements result from strain specific mutations rather than age-related perturbations.