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Age-related tolerance to paraquat-induced parkinsonism in *Drosophila melanogaster*

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ABSTRACT

Paraquat (PQ) is a widely used herbicide that can cross the dopaminergic neuronal membrane, accumulate in mitochondria and damage complex I of the electron transport chain, leading to neuronal death. In *Drosophila melanogaster,* PQ exposure leads to the development of parkinsonism and is a classical model for studying Parkinson's Disease (PD). Muscle mitochondrial dysfunction, affecting survival and locomotion, is described in familial PD in *D. melanogaster* mutants. However, no study has shown the effects of PQ-induced parkinsonism in *D. melanogaster* regarding muscle ultrastructure and locomotor behavior at different ages. Thus, we evaluated survival, locomotion, and morphological parameters of mitochondria and myofibrils using transmission electron microscopy in 2 and 15-day-old *D. melanogaster,* treated with different PQ doses: control, 10, 50, 100, 150, and 200 mM. PQ100mM presented 100% lethality in 15-day-old *D. melanogaster*, while in 2-day-old animals PQ150mM produced 20% lethality. Bradykinesia was only observed in 15-day-old *D. melanogaster* treated with PQ10 mM and PQ50 mM. However, these results are unlikely to be associated with changes to morphology. Taken together, our data indicate pathophysiological differences between PQ-induced parkinsonism and familial parkinsonism in *D. melanogaster* (resultant from gene mutations), demonstrating for the first time a differential susceptibility to PQ in two developmental stages.

1. Introduction

Paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride) (PQ) is a broad-spectrum herbicide applied against annual or perennial weeds affecting cotton, cocoa, soybean, rice, and other crops, which is widely used in more than 100 countries (Rashidipour et al., 2019). Although safe for soil microorganisms and plant roots, when used for extensive periods it is highly toxic for humans and the environment (Frimpong et al., 2018), thus, PQ has been banned in several countries and regions, including South Korea and the European Union (Cha et al., 2016; Bang et al., 2017).

Motor deficits and severe muscle integrity disruption, mainly in myofibrils and mitochondria of indirect flight muscles (IFMs) (Fig. 1), were found in familial Parkinson's disease (PD) in *Drosophila* (Pesah, 2004; Yang et al., 2006; Greene et al., 2003; Klein et al., 2014; Si et al., 2019). This reduction in mitochondrial function promoted by PD is also observed in rodents, since, in transgenic mice, *α-synuclein* overexpression impairs mitochondrial function (Song et al., 2004; Martin, 2006).

In addition to the numerous cases of PQ poisoning that have been

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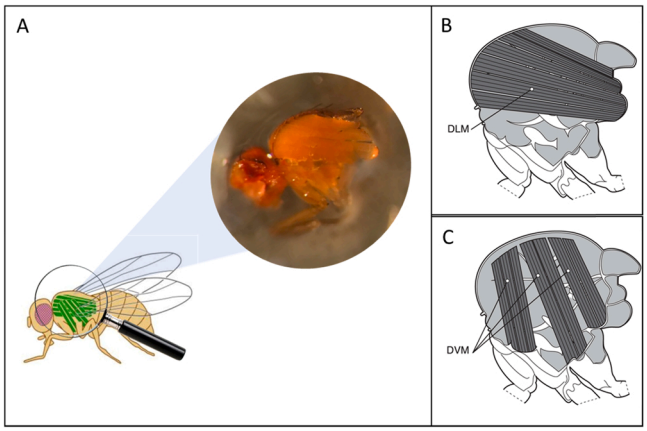

Fig. 1. *D. melanogaster* chest and its indirect flight muscles (IFMs). A) sagittal section through the chest of a specimen showing IFMs, which are divided into two antagonistic sets of muscles that run from front to back. B) shows the dorso-longitudinal muscles (DLMs); C) shows the dorso-ventral muscles (DVMs). Figure adapted from Hedenstro (2014) (Hedenstro, 2014) and Swank (2011) (Swank, 2012) containing an image from the authors.

reported globally, PQ is considered a key risk factor for PD in humans (Huang et al., 2019; Tsai, 2013). PD is a progressive neurodegenerative disorder (Greene et al., 2003; Vila et al., 2008; Faivre et al., 2019) characterized by loss of dopaminergic neurons in the substantia nigra pars compacta (Teismann et al., 2003; Dickson, 2018; Verma and Ravindranath, 2020), resulting in a significant striatal dopaminergic deficit which generates motor dysfunctions such as tremors, akinesia, bradykinesia, rigidity and postural instability (Verma and Ravindranath, 2020; Lin et al., 2002; Deng et al., 2008; Ziviani et al., 2010; Guo, 2012). In the laboratory, exposure to PQ is an established method of inducing parkinsonism in animal models, since it can cross dopaminergic neuronal membrane, accumulate in mitochondria, damage the complex I of the electron transport chain and consequently lead to neuronal death (Vila et al., 2008). *Drosophila melanogaster* is a widely used animal model to study PD in its different forms, such as familial PD related to mitochondrial alterations, mutations in homologous genes shared with humans (such as *α-synuclein, DJ-1*, *PINK1*, *parkin*, *LRRK2* and *VPS35* (Greene et al., 2003; Klein et al., 2014; Deng et al., 2008; Ziviani et al., 2010; Guo, 2012; Krüger, 2004; Petit et al., 2005; Park et al., 2006; Clark et al., 2006; Park et al., 2009; Hao et al., 2010; Malik et al., 2015; Bardai et al., 2018)), and parkinsonism induced by herbicides, such as PQ (Hosamani and Muralidhara, 2013).

The cellular basis of familial PD pathogenesis includes mutations in genes directly or indirectly associated with mitochondrial homeostasis or mitophagy mechanisms (Ryan et al., 2015). The *PINK1*, *parkin*, and *DJ-1* genes, which are related to autosomal recessive forms of PD, seem to work cooperatively in the same pathway related to mitophagy processes, with *parkin* acting downstream from *PINK1*, while *DJ-1* functions as an important redox sensor (Park et al., 2006; Clark et al., 2006; Ryan et al., 2015; Trempe and Fon, 2013). Furthermore, mutations in these genes lead to similar phenotypes, such as mitochondrial morphology defects and IFM degeneration. Genes related to autosomal dominant PD have also been shown to modulate mitochondrial functions and mitophagy processes. An example is *LRRK2* which, despite not being directly involved in muscle degeneration or mitochondria alterations, interacts with *parkin* and *PINK1;* suggesting that dominant genes in PD can act cooperatively in the same pathway as recessive genes (Venderova et al., 2009; Xiong and Yu, 2018; Lee et al., 2007).

Another example of such interaction is *VPS35*, a gene involved in a late onset, autosomal dominant form of PD. *VPS35* mediates the degradation of mitochondrial proteins; being related to the quality control of mithocondria (Malik et al., 2015). Mutants for this gene show defective larval development at the neuromuscular junctions, leading to reduced larval locomotion. Recently, *VPS35* has been associated with processes in which *parkin* and *PINK1* are the central promoters (Malik et al., 2015). Animals doubly heterozygous for *VPS35* and *parkin* had significantly worse climbing ability, and this synergy was age-dependent, being apparent only old animals (Malik et al., 2015). This indicates that these two genes may act partially in a common pathway that is related to the aging process (Malik et al., 2015). Interestingly, these animals also showed PQ sensitivity. However, while there appears to be a genetic interaction between *VPS35* and *parkin*, no such interaction has been reported with *PINK1*, indicating that despite working in the same pathway, *PINK1* and *parkin* have common but independent functions (Malik et al., 2015). When *VPS35* was overexpressed in homozygous animals for *parkin*, it was able to rescue several phenotypical alterations such as the impaired climbing activity and PQ sensitivity (Malik et al., 2015). Mutations in the SNCA, the gene encoding *α-synuclein*, and *LRRK2,* both related to autosomal dominant PD, have also been shown to modulate mitochondrial function, but in the brain. Abnormal *α-synuclein* expression is related to oxidative stress, mitochondrial dysfunction and dopaminergic neurodegeneration (Sarkar et al., 2021).

PQ-induced parkinsonism in *Drosophila,* especially in mature animals (over 10 days old) (Hosamani and Muralidhara, 2013; Bagatini et al., 2011), displays both motor and central alterations similar to those reported in PD patients, including neurodegeneration of dopaminergic neurons, akinesia and bradykinesia (Hosamani and Muralidhara, 2013; Bagatini et al., 2011; Chaudhuri et al., 2007; Inamdar et al., 2012). However, most studies involving this Parkinson-inductor agent in *D. melanogaster* have focused on biochemical evaluations of mitochondria in general rather than the effects on myofibrils and their mitochondria (Hosamani and Muralidhara, 2013).

Thus, our goal was to analyze the effects of different PQ doses on very young (2-day-old) and mature (15-day-old) *D. melanogaster*, considering survival and locomotion; while performing an ultrastructural analysis of myofibrils and mitochondrial morphology using transmission electron microscopy (TEM) associated with planar morphometry and stereology.

2. Material and methods

2.1. Drosophila strain and culture maintenance

For this study, we used 240 female Canton-S wild-type *Drosophila melanogaster* acquired from the Bloomington *Drosophila* Stock Center, Indiana University, USA. The flies were maintained in an incubator at 25 ± 2 °C under a 12-hour light/dark cycle, with 60–70% humidity, in housing pots containing standard medium prepared with 10 g agar, 10 g methylparaben (antifungal), 100 g rye flour, 50 g brown sugar, 1 L water, and dried yeast. This medium, without PQ, was provided to the control group. The experimental groups received the same medium with the addition of 10, 50, 100, 150 or 200 mM doses of PQ (1,1′-dimethyl-4,4′-bipyridinium ion, Syngenta).

2.2. Experimental groups

Flies were divided into 12 groups according to age, 2 or 15-day-old, and PQ treatment doses, 0 (control), 10, 50, 100, 150, and 200 mM. Pupae hatching day was considered the flies' first day of life. The number of flies used in each test is shown in Table 1. First, the survival curve was evaluated, with the surviving animals being used for both the locomotor tests and transmission electron microscopy (TEM) analysis. Survival rate and locomotor behavior were evaluated 2 days after PQ treatment; consequently, these parameters were assessed in flies aged 4 and 17 days. The control and PQ 50 mM groups were chosen for TEM analysis, since a significant difference was found between survival rates when comparing 2 and 15-day-old *Drosophila melanogaster* with this dose (Table 1).

Table 1
Experimental groups and number of flies used in each analysis for each age group.

Experimental Groups			2-day-old *D. melanogaster*	
			Evaluated Parameters	
	Survival (n = 120)	Open field (n = 96)	Climbing assay (n = 60)	TEM (n = 10)
Control Group	(n = 20)	(n = 20)	(n = 20)	(n = 5)
PQ10mM	(n = 20)	(n = 20)	(n = 20)	–
PQ50mM	(n = 20)	(n = 20)	(n = 20)	(n = 5)
PQ100mM	(n = 20)	(n = 20)	–	–
PQ150mM	(n = 20)	(n = 16)	–	–
PQ200mM	(n = 20)	†	†	–
Experimental Groups			**15-day-old *D. melanogaster***	
			Evaluated Parameters	
	Survival (n = 120)	Open field (n = 44)	Climbing assay (n = 59)	TEM (n = 10)
Control Group	(n = 20)	(n = 20)	(n = 20)	(n = 5)
PQ10mM	(n = 20)	(n = 16)	(n = 20)	–
PQ50mM	(n = 20)	(n = 8)	(n = 19)	(n = 5)
PQ100mM	(n = 20)	†	†	–
PQ150mM	(n = 20)	†	†	–
PQ200mM	(n = 20)	†	†	–

Legends: † = 100% of lethality.

2.3. Open field test

An open field test (OFT) was performed between 09.00 and 12.00 h, in a dark room with temperature set to 25 ± 2 °C and humidity at 60–70%. A negatoscope provided a homogenous light source to avoid negative or positive phototaxis.

A single fly was placed into an empty 60 mm Petri dish, and the movement was recorded with a tripod-mounted digital video camera (Sony DCR-PJ5). The locomotor activity was evaluated for four minutes, the two first minutes were considered exploratory locomotion and the next two minutes were considered basal locomotion. To quantify locomotor behavior, the Anymaze® software was used (www.anymaze.com); the evaluated parameters being distance traveled, average speed, time mobile, and average speed during time mobile.

2.4. Climbing assay

The climbing test was performed to further assess locomotor behavior in the flies exposed to PQ. The test was carried out under the same temperature, humidity and luminosity conditions as the open field test. For the test, we built an apparatus consisting of a 3D printed base and lid supporting 3 glass vials (14 cm × 1.5 cm) with rulers (14 cm) attached next to the vials, mimicking a graduated cylinder (Fig. 2). The test was conducted according to Bajracharya and Ballard (2018), Bourg and Lints (1992) with modifications. Briefly, one fly was placed one each glass vial, and then the apparatus was shaken three times in a distinct, non-rhythmic pattern, to displace the flies to the bottom of the vial. A tripod-mounted digital video camera (Sony DCR-PJ5) was used to record the flies' subsequent movements. The locomotor activity was evaluated for two minutes counting from the last shake. The parameters evaluated were: time to move (in seconds), maximum height reached in 10 s, maximum height reached in 20 sec, maximum height reached within 120 sec (2 min), number of inactive flies, number of flies surpassing the minimum height of 2 cm, number of flies surpassing the maximum height of 8 cm, falls (number of flies that fell from the glass wall), and the number of flies that went to the bottom of the glass tube (Bajracharya and Ballard, 2018; Bourg and Lints, 1992).

2.5. Transmission electron microscopy (TEM)

The flies evaluated in the locomotor assays were euthanized by hypothermia (20 min at −10 °C) and fixed in a 2% paraformaldehyde and 2.5% glutaraldehyde mixture buffered with 0.12 M phosphate (pH 7.2–7.4). It was decided to use females in the is study because they are larger than males, facilitating fiber dissection. The indirect flight muscles (IFMs) were dissected as follows. The wings, abdomen, and legs were removed using very fine spring scissors (Vannas, 2 mm, Fine Science Tools). A pin (Insect pin, 0.03 mm, Fine Science Tools) was placed between the head and the thorax to stabilize the fly. To expose the IFMs, the thorax was cut along the sagittal plane using a scalpel blade (#23, 30 mm, Fine Science Tools) (Fig. 1A).

The dissected thorax was once again fixed in the same, previously described, fixative solution for at least one hour, and post-fixed in osmium tetroxide (Electron Microscopy Science) 2% diluted in 0.2 M phosphate buffer (pH 7.2–7.4) for 45 min before dehydration. Samples

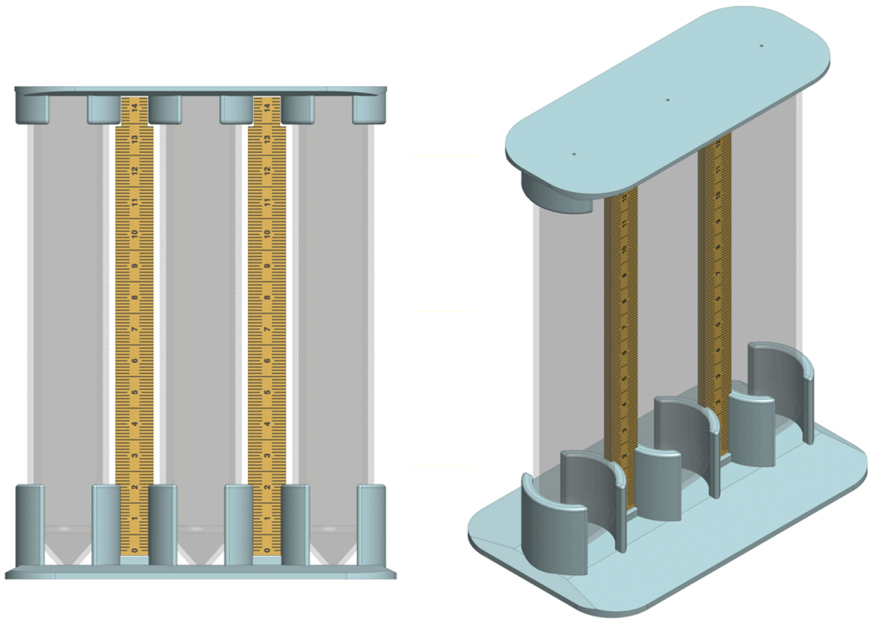

Fig. 2. Purpose-built climbing test apparatus.

were dehydrated in a graded acetone series (30 – 100%) and embedded in araldite (Durcupan ACM, Fluka) for 72 h at 60 °C. Thin sections (100 nm) were stained with 2% uranyl acetate followed by lead citrate (Meira Martins et al., 2014). The TEM analysis was performed in the IFMs as a whole, without subdividing the dorso-longitudinal muscles (DLMs) and dorso-ventral muscles (DVMs).

Ultrastructural analysis was performed using Tecnai G2 T20, FEI transmission electron microscopy. The morphological evaluation of the TEM images, by planar morphometry and stereology, was performed using Image Pro Plus software (IPP 6.1, Media Cybernetics, Silver Spring, USA) as described below.

2.6. Mitochondrial and myofibrillar densities

To estimate the mitochondrial and myofibril densities, we undertook quantitative analysis by planar morphometry, similar to the Neubauer chamber. Two randomized squares measuring 22.95 μm^2, (the areas of interest - AOIs), were overlaid on each TEM image (3600X), and mitochondria and myofibril located inside each square or intersected by the upper and/or left edges of the squares were counted.

2.7. Percentage of area covered by mitochondria and myofibril

The percentage of area covered by mitochondria and myofibril was estimated using the stereological point-counting method (Ilha et al., 2008; Lazzarotto Rucatti et al., 2015; Fernandes et al., 2016). Whereby, a grid mask 220 × 220 (a grid of crosses with equidistant intervals) with an area/point value of 1.96 μm^2 was placed over the TEM images. When the mitochondria or myofibril images were located in the upper right quadrant of the cross they were counted.

2.8. Mitochondrial and myofibrillar area

To estimate the mitochondrial and myofibril areas, we used the same TEM images and AOIs previously described in Section 2.5 above; a 60 × 60 grid mask with an area/point value of 0.14 μm^2 was placed over these images. All the crosses within the border of any mitochondrion or myofibril were counted together with any cross whose right upper quadrant touched the mitochondrion or myofibril border. The area of each mitochondrion or myofibril was obtained by multiplying the number of crosses counted by the area/point of each cross. The percentage area occupied by mitochondria and myofibril was obtained using the following equation: Percentage of area occupied by the mitochondria or myofibril = (number of crosses counted in each mitochondrion or myofibril/total number of crosses counted in the image) × 100.

2.9. Mitochondrial and myofibrillar Shape Z

The same TEM Images and AOIs previously analyzed were then used to calculate mitochondrial and myofibrillar coefficient shape (Shape Z). The shape Z, i.e. the shape coefficient (Kiêu et al., 1999; García-Fiñana et al., 2003) was used to obtain the degree of mitochondrial and myofibrillar elongation. This stereological parameter was obtained using the following equation:

$$Shape \quad Z = \frac{P}{\sqrt{\hat{A}}},$$

Where P is the mitochondrial or myofibrilar perimeter and Â is the mitochondrial or myofibrilar area. The mitochondrial and myofibrilar perimeters and areas were obtained by delineation, using IPP 6.1 software. Low Shape Z values indicate a more rounded shape, while high values indicate a more elongated shape. In these analyses performed, ten images were evaluated per group.

2.10. Gray scale analysis of mitochondria and myofibril

The mitochondrial and myofibrillar electron density was measured using the grayscale tool (Xavier et al., 2005). The same images used in the previous analyses were used to determine the regional grayscale (255 shades of gray). Two equal-sized AOIs measuring 0.26 μm^2 were placed over each mitochondrion and myofibril to determine the grayscale. The image analysis software (IPP 6.1) determines the grayscale of the AOIs based on the number of pixels in the digitized images. According to this analysis, 0 = absolute black and 255 = absolute white, and therefore the higher the electron density, the lower will be the values obtained in the grayscale analysis. Conversely, an increase in the grayscale indicates lower electron density, which correlates with a possible loss of mitochondrial crests. The same rationale was used to analyze myofibrils.

2.11. Statistical analysis

Two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison *post-hoc* test was used for between-group comparisons in all the analyzed parameters ($p < 0.05$). Statistical analyses were performed using IBM SPSS Statistics 25 (IBM, Armonk, NY, USA).

3. Results

3.1. 15-day-old D. melanogaster are more susceptible to acute PQ exposure

We found 2-day-old *D. melanogaster* were more resistant to PQ than 15-day-old *D. melanogaster*, in which higher PQ doses implied higher mortality. In 2-day-old flies, the survival rate at the PQ10 mM, PQ50 mM, and PQ100 mM doses was 100%, while PQ150mM induced 20% mortality, and at PQ200 mM there were no survivors. By contrast, in the 15-day-old *D. melanogaster*, the mortality rate was much higher throughout the dosage range, with 20% mortality at PQ 10 mM, 60% at PQ50 mM and 100% at PQ100, 150 and 200 mM (Fig. 3).

3.2. Acute PQ treatment only affects locomotor behavior in 15-day-old D. melanogaster

Our assessment of the locomotor behavior in the OFT considered two different subtypes of locomotion, namely: exploratory locomotion, which refers to process by which the flies habituate themselves to the novel environment by gathering of information; and basal locomotion, which refers to their movements when they are already familiar with the

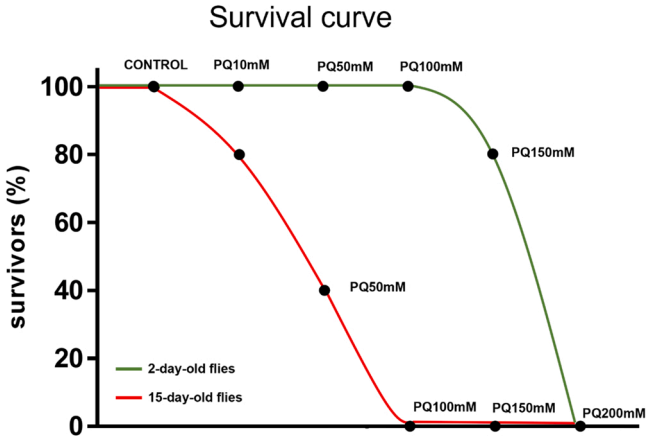

Fig. 3. Survival Curve of 2 and 15-day-old *D. melanogaster* exposed to different paraquat (PQ) doses: 0 (control) 10, 50, 100, 150 and 200 mM, for 48 h (n = 20 flies for each PQ concentration).

testing environment (Kaur et al., 2015). Regarding all the analyzed parameters, no changes were observed in the 2-day-old experimental flies compared to controls, during either the exploratory or basal activity periods (Figs. 4 and 5). By contrast, analyzing 15-day-old animals, PQ10 mM, and PQ50 mM doses caused a significant reduction in both exploratory and basal activity, when compared to controls ($p < 0.05$) (Figs. 4A, B, D and 5A, B, C, D).

Regarding exploratory locomotion, mature animals treated with PQ10 mM and PQ50 mM showed reduced distance traveled, average speed, and average speed during the time mobile when compared to the control group ($p < 0.05$) (Fig. 4A, B, D). No changes were observed in the time mobile in 2 and 15-day-old animals in any of the doses analyzed (Fig. 4C).

Comparison between very young and mature flies showed that 15-day-old flies have significantly reduced time mobile when compared to 2-day-old flies exposed to a 10 mM PQ concentration ($p < 0.01$) (Fig. 4C). Regarding the average speed during the time mobile, both PQ10mM and PQ50mM led reduced speed 15-day-old flies compared to 2-day-old flies ($p < 0.01$) (Fig. 4D).

Regarding basal locomotion, only 15-day-old flies showed a significant reduction when compared to controls, in all the evaluated parameters: distance traveled, average speed, mobile time, and average speed during the mobile time ($p < 0.05$) (Fig. 5). Moreover, a between-group comparison showed that 15-day-old flies treated with PQ50mM presented a significant reduction in distance traveled compared to 2-day-old flies ($p < 0.05$). Also, 15-day-old flies treated with 10 mM showed reduced mobile time compared to 2-day-old flies ($p < 0.05$).

In the climbing test, we only used flies exposed to the PQ10 mM and PQ50 mM doses, because at these concentrations the flies survived and displayed alterations to both basal and exploratory locomotion. No difference was observed in the time flies took to move in the climbing test (time to move – Fig. 6A). Very young flies treated with PQ10 mM showed higher maximum height reached at both 10 and 20 s when compared to mature flies ($p < 0.05$ and $p < 0.001$, respectively; Fig. 6B and C respectively). Furthermore, very young flies treated with PQ50 mM showed higher maximum height reached at 20 s ($p < 0.01$ – Fig. 6C). Very young flies treated with both PQ10 mM and PQ50 mM also reached greater height at 120 ss (maximum height at 120 s) than mature flies ($p < 0.05$, Fig. 6D). The other evaluated parameters (number of inactive flies, number of flies surpassing the minimum height of 2 cm, number of flies surpassing the maximum height of 8 cm, the number of falls and the number of flies that went to the bottom of the tube) did not differ between groups (Fig. 6E, F, G, H and I).

3.3. Acute PQ treatment did not affect mitochondrial or myofibrillar morphology

Concerning the planar and stereological quantification of ultrastructural analysis, in 2 and 15-day-old flies treated with PQ, no differences were found in the mitochondrial and myofibrillar morphological parameters evaluated in the TEM images: density, percentage of area covered, area, shape Z, and gray scale (Fig. 7). Similarly, in the qualitative analysis, no signs of myofibrillar or mitochondrial degeneration were found, with both groups showing variable mitochondrial area and no mitochondrial ridge loss. The IFM myofibrils from the experimental groups presented normal morphology, identical to that of the control group, with no changes to fibril patterns or spacing (Fig. 8).

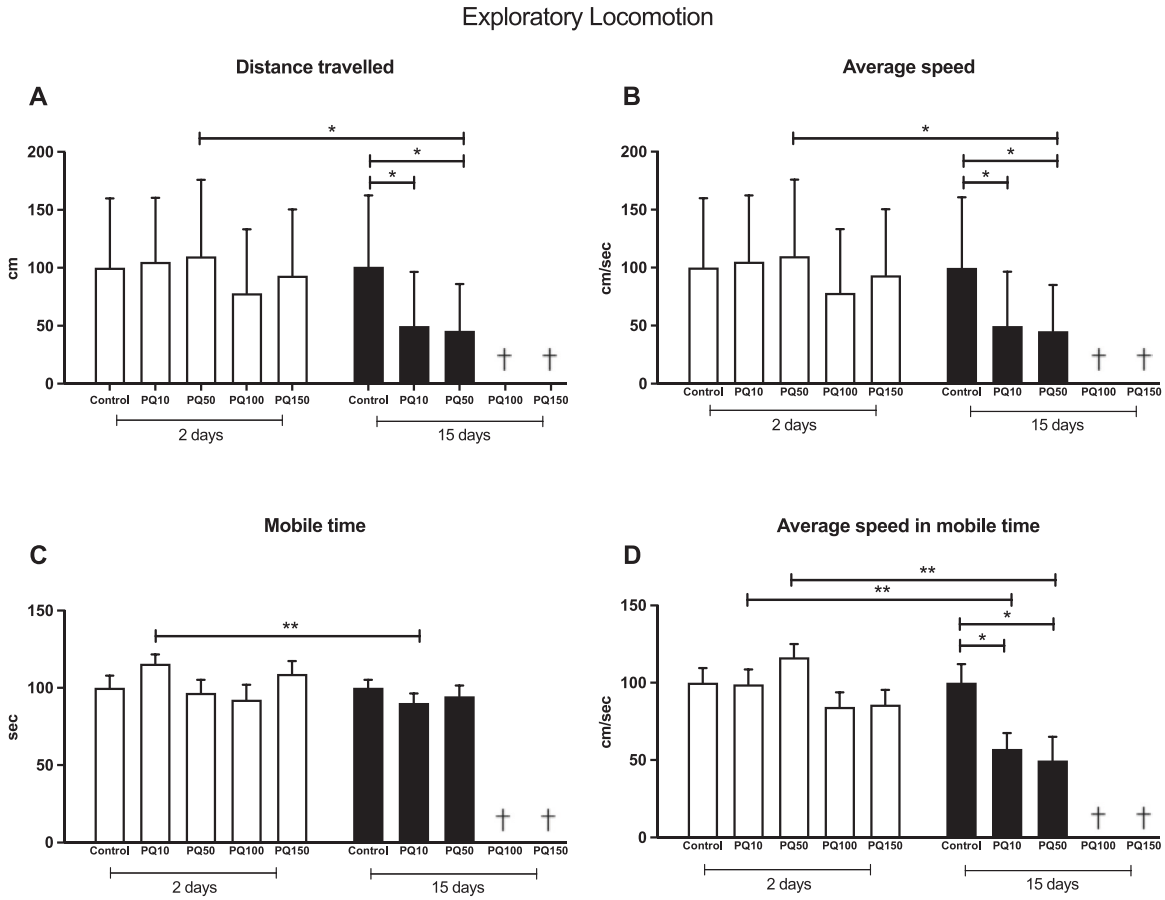

Fig. 4. Exploratory locomotion evaluation in 2 and 15-day-old *D. melanogaster*. The graph depicts data from control and surviving flies from both 2 and 15-day-old flies exposed to different paraquat (PQ) doses: 10, 50, 100, and 150 mM, for 48 h. Two-way ANOVA-Tukey test. Data are presented as mean ± s.e.m. * $p < 0.05$; * * $p < 0.01$.

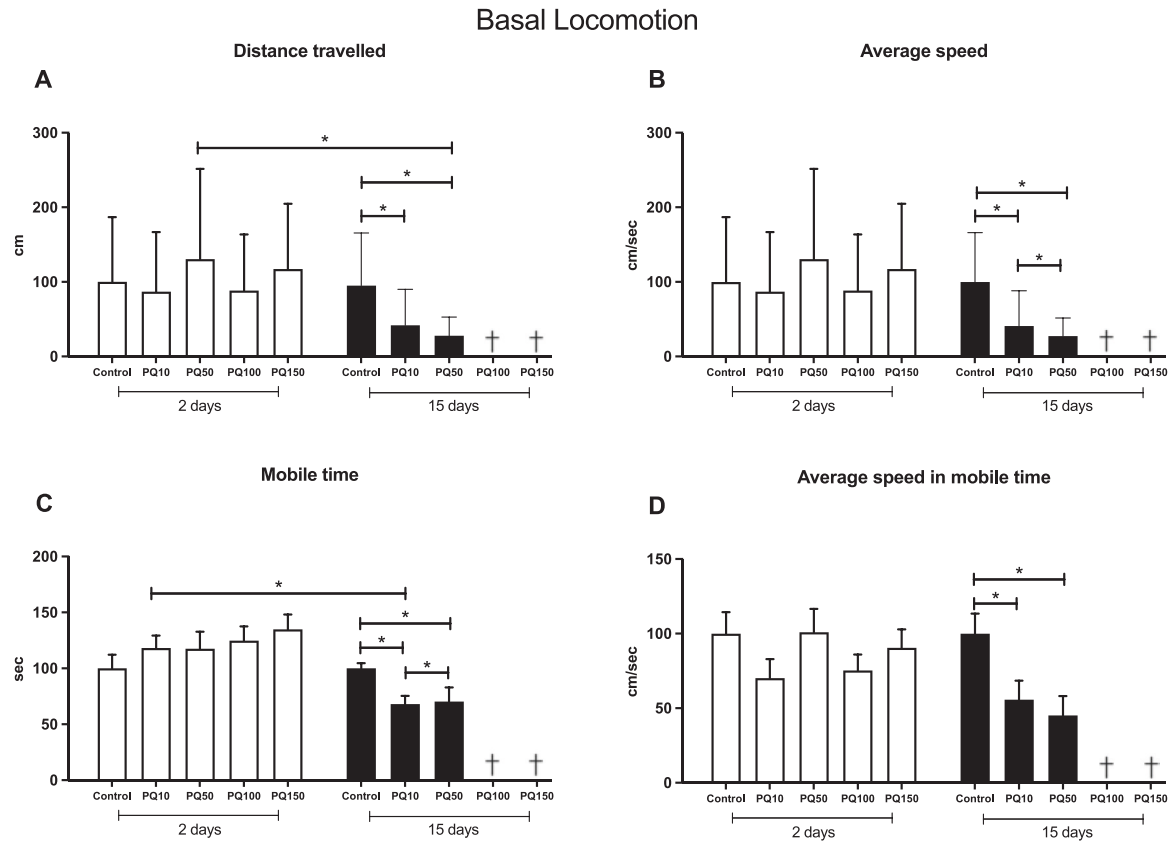

Fig. 5. Basal locomotor evaluation in 2 and 15-day-old *D. melanogaster*. The graph depicts data from control and surviving flies exposed to different concentrations of paraquat (PQ): 10, 50, 100, and 150 mM, for 48 h. Two-way ANOVA-Tukey test. Data are presented as mean ± s.e.m. * $p < 0.05$.

4. Discussion

As far as we know, this is the first study to evaluate ultrastructural alterations in muscles following PQ-induced parkinsonism in *D. melanogaster*. While no significant alterations were found in the assessed parameters, we found a reduced susceptibility to effects of PQ in 2-day-old flies as observed by the absence of alterations to locomotion parameters, climbing ability and survival. Our results corroborate previous findings demonstrating that PQ tolerance in wild-type flies decreased significantly during the first two weeks of adult life, since, when exposed to PQ for 24 h, the survival rate is 95% among 1-day-old flies and only 8% among 15-day-old flies. Locomotor behavior is closely associated with the functioning of the dopaminergic system. When there is any alteration to the number of dopaminergic neurons (i.e. death of dopaminergic neurons) and consequently the dopaminergic system is disrupted, locomotor behavior is impaired. Accordingly, the literature shows that exposure to PQ affects the dopaminergic system in *D. melanogaster*, impairing the locomotor behavior (Chaudhuri et al., 2007; Weber et al., 2012; Shukla et al., 2014; Jimenez-Del-Rio et al., 2010; Li et al., 2005; Somayajulu-Niţu et al., 2009).

One hypothetical explanation for these findings is related to levels of DAMB, a D1-like dopamine receptor mostly expressed in mushroom cells of D. *melanogaster*, whose signaling has been shown to be an important regulator of behavior in drosophila (Han et al., 1996). DAMB expression progressively increases over development, doubling its levels in 15-day--old flies (Cassar et al., 2015). Interestingly, DAMB-mutant flies were surprisingly resistant to PQ at all ages, indicating the involvement of this receptor in the mechanisms related to toxin sensitivity and oxidative stress induced by PQ. Dopamine levels have been shown to increase in flies exposed to PQ, even in acute manner, leading to overactivation of DAMB, which in turn triggers oxidative stress in neurons (Cassar et al., 2015). This evidence is supported by studies demonstrating PQ tolerance in flies treated with DAMB antagonists (Cassar et al., 2015). In normally developing flies, this effect is avoided due to the fine regulation of DAMB expression by dopamine levels (Cassar et al., 2015). The excessive oxidative stress generated by PQ exposure is seen as increased sensitivity to the herbicide (Cassar et al., 2015). Thus, the 15-day-old flies, which have twice the amount of DAMB, will be more susceptible to a sudden increase in dopamine levels due to PQ exposure. This susceptibility is seen not only as increased lethality, but also as the impaired locomotor behavior reported by us in 15-days old flies. However, this hypothesis is a merely speculative and further studies are necessary to test the "DAMB theory" in relation to our results.

Another hypothesis is related to the expression of *split ends (spen)* - RNA binding protein, orthologous to *SPEN/SHARP* in humans (Girard et al., 2020). *SPEN/SHARP* is expressed by astrocytes and is significantly overexpressed in the central nervous system of PD patients compared to healthy individuals (Girard et al., 2020). In *Drosophila, spen* expression in glial cells mediates their tolerance to PQ treatment, since its expression is upregulated following PQ treatment (Girard et al., 2020). The down-regulation of *spen* in glial cells has been shown to increase the sensitivity of male flies to PQ, while its upregulation protected against PQ toxicity (Girard et al., 2020). Moreover, flies heterozygous for *spen* loss-of-function mutation exhibited more significant PQ-induced mortality when compared to control flies. One function associated to *spen* is as a regulator of fat storage, including the accumulation of lipid droplets in glial cells, which might have a role in triggering oxidative stress (Girard et al., 2020). In mice, the literature shows *spen* expression is reduced with age. However, whether this is the case with drosophila is unknown (Yabe et al., 2007).

It is possible to speculate that the *spen* and DAMB mechanisms might act in parallel to produce the tolerance to PQ observed in young flies. In such a scenario, the age-related lower DAMB expression seen in young *D. melanogaster* (i.e. 2-day-old flies) would protect them against the PQ

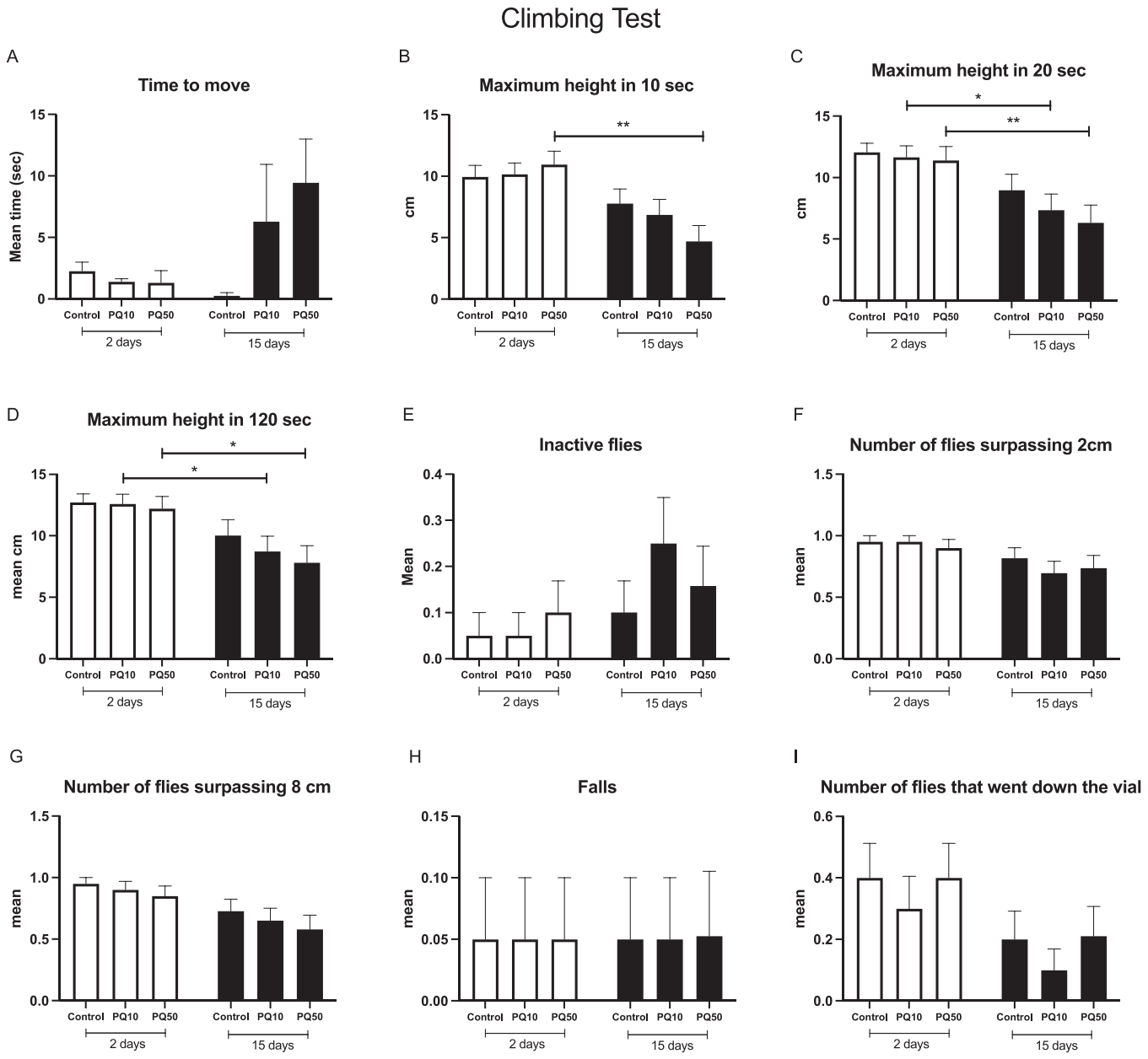

Fig. 6. Climbing test in 2 and 15-day-old *D. melanogaster*. The graph depicts data from control and surviving flies from both 2 and 15-day-old flies exposed to two different paraquat (PQ) doses: 10 and 50 mM, for 48 h. Two-way ANOVA-Tukey test. Data are presented as mean ± s.e.m. * $p < 0.05$; * * $p < 0.01$.

effects in combination with the protective effect of the increase in *spen* expression in response to PQ exposure. However, as flies age, DAMB expression increases, rendering the flies more sensitive to the effects of PQ. In this situation, the increased DAMB expression would outweigh the protective effect of *spen* expression, thus diminishing the protection against the effects of PQ, resulting in increased sensitivity to PQ. Nevertheless, experiments using double mutants or flies heterozygous for these molecules need to be conducted to test the involvement of both mechanisms in the greater tolerance to PQ observed by us in 2-day-old flies.

In our study, no changes were observed in *D. melanogaster* mitochondria and myofibrils after acute PQ treatment. This is in contrast with data from familial PD, where muscular and mitochondrial dysfunctions are described (Yang et al., 2006; Greene et al., 2003). Ultrastructural analysis of IFMs from 1 and 2-day-old *D. melanogaster parkin* mutants revealed severely disrupted muscle integrity, showing pronounced muscular disintegration leading to impaired flight and climbing abilities (Greene et al., 2003). In *D. melanogaster PINK1* mutants, IFM ultrastructural analysis also demonstrated irregular arrangement of the densely swollen myofibrils and mitochondria with outer membrane loss, reducing muscle content and presenting mitochondrial impairment caused by apoptosis in these muscles. However, when *PINK1* is expressed again, these mutant phenotypes are rescued (Park et al., 2006). Thus, the locomotor deficits in 15-day-old flies treated with PQ are probably associated to dopaminergic neuronal loss, corroborating the DAMB and *spen* hypothesis. Moreover, the data presented here might reflect the natural differences between distinct PD forms – familial (resultant from mutations in *PINK1* and *parkin* genes) and PQ induced – which might involve different biochemical mechanisms and alterations.

It should be noted that we exposed the flies to an acute PQ treatment, which might be another factor explaining our results. Interestingly, TEM analysis showed degenerative changes in the brain of *D. melanogaster* exposed to chronic PQ treatment rather than acute treatment, in which

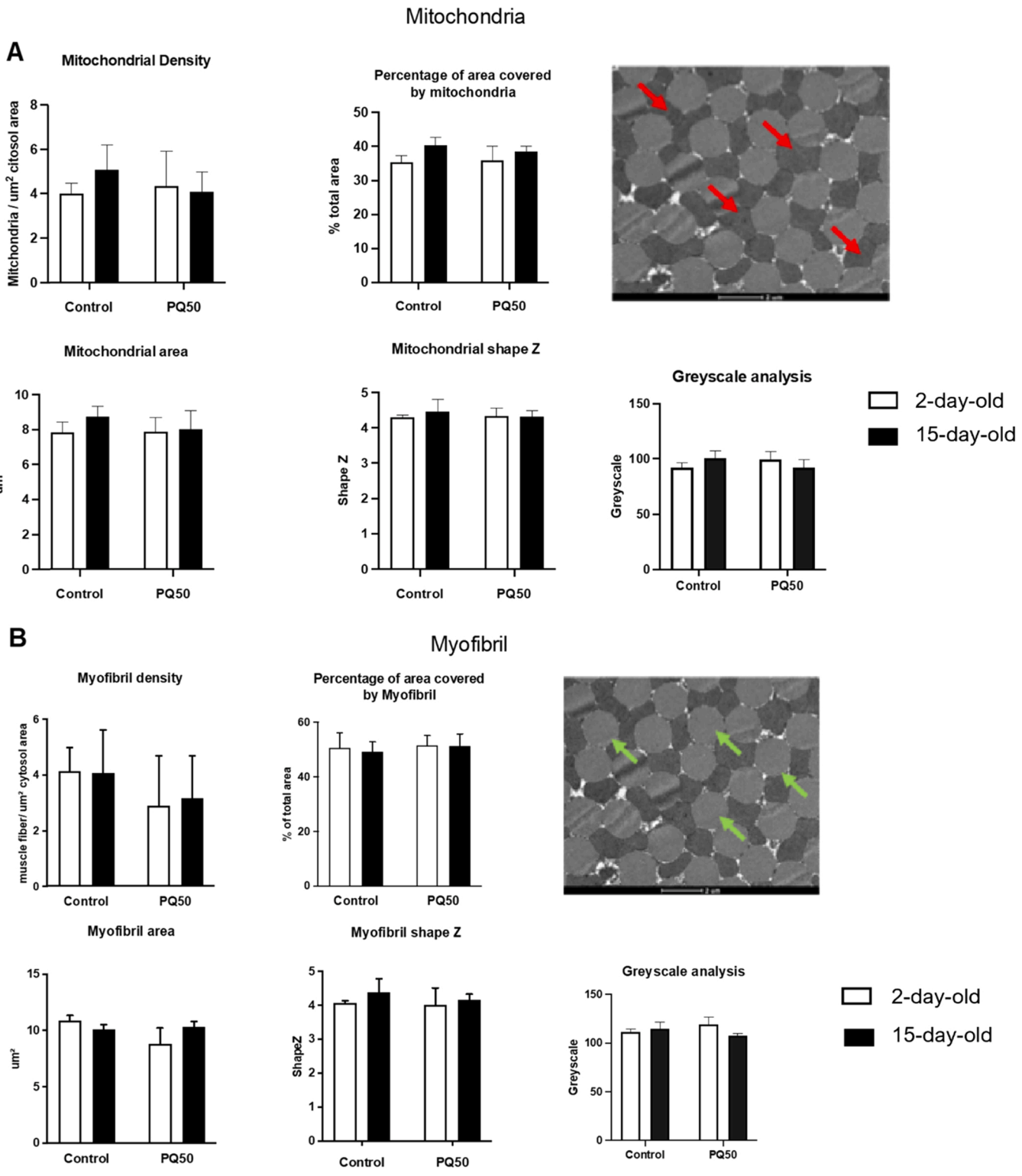

Fig. 7. Quantitative analysis of TEM images obtained from IFMs of both control *D. melanogaster* and those treated with paraquat (PQ) 50 mM dose at 2 and 15 days old. Panel A shows the quantitative analysis of mitochondria. Red arrows indicate mitochondria in a TEM image. Panel B shows analysis of myofibrils. Green arrows indicate myofibrils in a TEM image. No qualitative or quantitative differences were found in either the mitochondria or myofibrils regarding any of the analyzed parameters: density, percentage of area covered, area, shape Z or gray scale. Two-way ANOVA - Tukey test. Data are presented as mean ± s.e.m. *Scale bar* = 2 μm.

mitochondria presented many vacuolated areas and fragmented crests compared to the group treated with acute doses (Niveditha et al., 2017). Even though the brain is much more vulnerable to mitochondrial dysfunction than muscle and myofibrils, the cerebral mitochondria of flies exposed to acute PQ treatment showed no change. Thus, it is expected that the mitochondria from myofibrils of *D. melanogaster* acutely treated with PQ would not affected. Therefore, in future studies, it would be interesting to assess the chronic effects of PQ treatment in *D. melanogaster* myofibrils and mitochondria.

There are some caveats related to the present study. Food

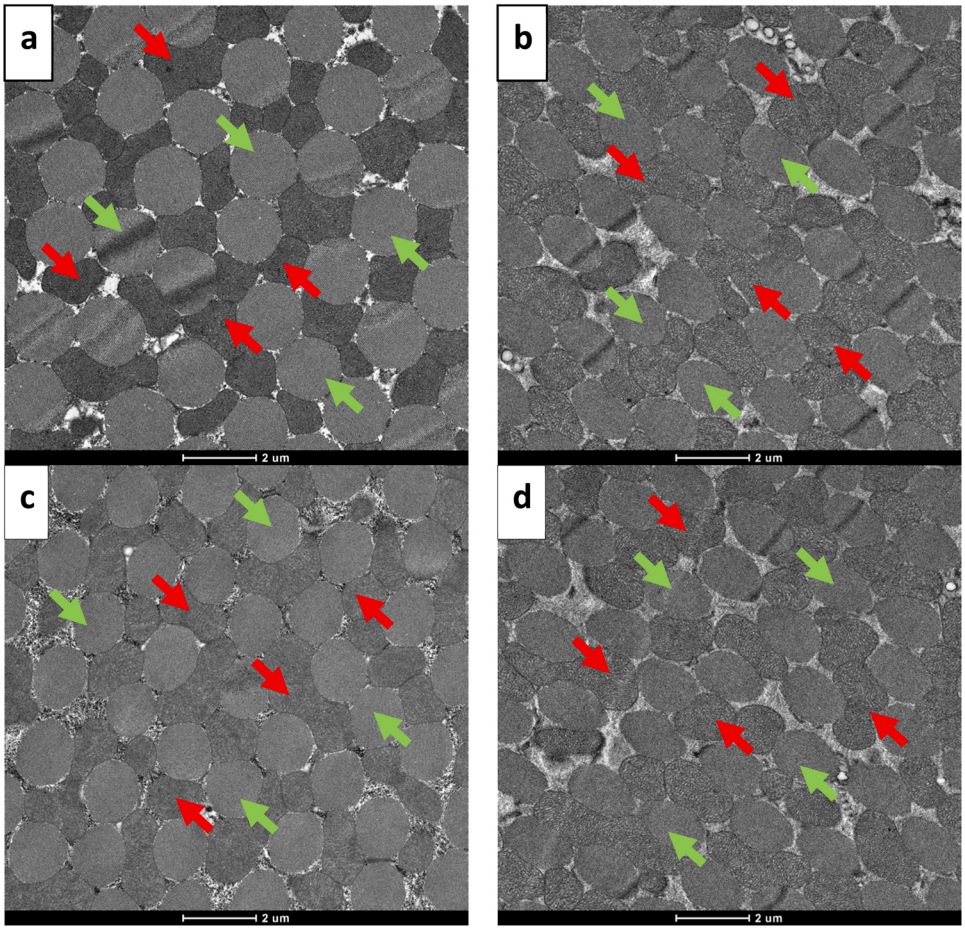

Fig. 8. Morphological, morphometric and stereological evaluation of transmission electron microscopy (TEM) images obtained from Indirect Flight Muscles (IFMs) of both control *D. melanogaster* and those treated with a paraquat (PQ) 50 mM dose at different ages, 2 and 15 days old. No qualitative or quantitative differences were found in either the mitochondria or myofibrils regarding any of the analyzed parameters: density, percentage of area covered, area, shape Z, and grayscale. (a) control and (b) PQ 50 mM dose in 2-day-old flies; (c) control and (d) PQ 50 mM dose in 15-day-old flies. Green arrows indicate myofibrils, while red arrows indicate mitochondria. *Scale bar* = 2 μm.

consumption has been suggested as an essential factor leading to increased resistance to PQ in mature flies. It has been postulated that mature *D. melanogaster* might consume more food, thus increasing their intake of PQ in relation to young flies, consequently leading to more pronounced PQ effects in those flies. On the other hand, a previous study showed that dietary PQ strongly inhibits food intake in flies, as over 12 h, flies fed with PQ ate 75% less than controls (Ja et al., 2007). Moreover, the anorexigenic effect of PQ varies according to age, being more pronounced in 2-day-old *D. melanogaster* compared to mature flies, leading to increased PQ effects in mature flies. The data in the literature is controversial, and since we were unable to estimate food intake, it remains a possible explanation for our findings.

5. Conclusions

The data presented here allow us to conclude that very young *D. melanogaster* (2-day-old) are tolerant to PQ-induced parkinsonism, as observed by the absence of changes to locomotion and survival. On the other hand, mature *D. melanogaster* (15-day-old) were very sensitive to PQ effects, as showed by the reduced survival rate and behavioral alterations. Moreover, our data indicates that PQ-induced parkinsonism does not produce the myofibrillar or mitochondrial alterations seen in familial PD, which result from *PINK1* and *parkin* gene mutations. New experiments should be conducted to precisely understand the pathways involved in PQ-induced parkinsonism.

CRediT authorship contribution statement

PFRN, AW and LLX – study design; data interpretation and manuscript elaboration. PFRN, BBM, LVP, LTN, VACMM, LTN, LCV, RBS, JHS, GPK, ECRR, ELSS, MQV and PBB conducted experimental procedures. PFRN, LTN, RGM, AW and LLX – data collection and interpretation.

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.

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