



Research article

Mancozeb impairs mitochondrial and bioenergetic activity in *Drosophila melanogaster*



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ABSTRACT

Mancozeb (MZ) is a broad-spectrum fungicide used worldwide in several crops. Neurological disorders in humans and animals have been associated with exposure to this compound by mechanisms still not fully understood. *Drosophila melanogaster* represents a reliable model in toxicological studies, presenting genetic and biochemical similarities with mammals. In this study, *D. melanogaster* flies were exposed for 15 days to MZ through the food (5 and 10 mg/mL). After that period, the efficiency of mitochondrial respiration complexes and metabolic markers were analyzed and evaluated. Flies presented weight loss, lower glucose, trehalose, and glycogen levels, and augmented levels of triglycerides concerning control (non-treated group). Acetyl-CoA Synthetase (ACeCS-1) and Acyl-Coenzyme Synthetase (ACSL1) contents were unchanged by MZ treatment. Mitochondrial respiration of flies was targeted by MZ treatment, evidenced by a decrease in oxygen consumption and bioenergetics rate and inhibition in mitochondrial complexes I/II. These results suppose that an impairment in mitochondrial respiration jointly with reduced levels of energetic substrates might be a mechanism involved in MZ deleterious effects, possibly by the limitation of ATP's availability, necessary for essential cellular processes.

1. Introduction

Mancozeb (MZ) is a broad-spectrum fungicide used to prevent crop damage in the field and during storage and transport of seeds. It belongs to the ethylene-bis-dithiocarbamate group of molecules containing Manganese and Zinc in its structure (Environmental protection Agency, 2005). MZ presents low acute toxicity and short environmental persistence, according to EPA, 2005. Despite this, many studies reported environmental and health damage associated with exposure to this compound. Among these studies, it was demonstrated genotoxicity, steatosis, and oxidative stress by MZ exposure in different human cells (Balaji et al., 2014; Pirozzi et al., 2016; Srivastava et al., 2016). When present in water, MZ increased Manganese (Mn) accumulation and induced behavioral and biochemical alterations in carps (Costa-Silva et al., 2018). Metabolites like Etilenotiurea (ETU) were in part associated

with MZ toxicity. Augmented ETU concentrations in urine were correlated with lesser verbal learning in children living near banana crops and potentially exposed to MZ (van Wendel de Joode et al., 2016).

This study demonstrates that *Drosophila melanogaster* is susceptible to MZ when exposed for a prolonged period (15 days). This condition induced mortality and oxidative stress with modulation of the activity and mRNA steady-state levels of antioxidant enzymes (Saraiva et al., 2018). The biochemical mechanisms involved in MZ toxicity are not fully understood. Studies demonstrated that mitochondria are one of the main targets of this fungicide. It was reported induction of ROS, loss of mitochondrial transmembrane potential, and apoptotic cell death in human cell lineage exposed to MZ (Kumar et al., 2019). Additionally, MZ causes impairment in isolated mitochondria' respiration rate (Zhang et al., 2003).

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The present study aims to complement MZ toxicity's current understanding using *Drosophila melanogaster* as a model to investigate mitochondrial viability and respiration, and energetic substrates.

2. Materials and methods

2.1. Materials

All the chemicals used in the Respirometry analysis were acquired from Sigma Aldrich® (São Paulo, SP, Brazil). Flours used for fruit flies medium were obtained from commercial suppliers. Mancozeb (80 % purity, Enzeb 800 WP) was purchased from Sabero Organics America S.A (Belo Horizonte, MG, Brazil). Colorimetric kits for measurement of glucose, trehalose, glycogen, triglycerides were obtained from Labtest, MG, Brazil. Anti-Acetyl-Coenzyme A Synthetase (ACeCS1) and anti-Acyl-Coenzyme Synthetase (ACSL1) and anti- β actin antibodies were purchased from Cell Signaling Biotechnology (Danvers, MA, EUA). ECL Western Blotting Substrate Kit was obtained from Promega (Madison, WI, USA). All other chemicals were from the highest analytical grade available.

2.2. *Drosophila* strains culture and procedures

The flies used in the experiments was *Drosophila melanogaster* wild-type (strain Harwich) from our breeding. *Drosophilas* were maintained in a room with a controlled temperature of 25 ± 1 °C, 12 h dark-light photoperiod, and 50–60% relative humidity. The standard diet was composed of cereal flour, cornflour, milk, salt, water, antifungal agent (Nipagin®) and supplemented with dried yeast as previously described by Paula et al. (2016).

2.3. Exposure of flies to mancozeb

Male flies 48 h post-hatching were exposed for 15 days to 0, 5, and 10 mg/mL of Mancozeb, mixed in the cornmeal. The groups were composed of 30 individuals. The concentrations used in this study were described by Saraiva et al. (2018), when MZ decreased in 50 % and 80 % the fly survival and led to a modulation of mRNA steady-state antioxidant enzyme levels with cell stress markers.

2.4. High-resolution respirometry

Oxygraph-2K (O2K, OROBOROS Instruments, Innsbruck, Austria) was used to quantify mitochondrial energetics functions. All experiments were performed at 24°C using DatLab 4.0 software (Oroboros Inc., Austria), with continuous stirring at 750 rpm. This technique was carried out according to Miwa et al. (2003) and Carvalho et al. (2017).

2.4.1. Mitochondrial isolation

Thirty male fruit flies were immobilized by chilling on ice and homogenized into a cold pestle glass with 2 mL ice-cold medium containing 250 mM sucrose, 0.1 % free fatty acid bovine serum albumin, 2 mM EGTA, and 5 mM Tris-HCl pH 7.4. The homogenate was filtered with nylon membrane (10 μ m pore size) and then centrifuged at 200 x g for 3 min. The supernatant was collected and centrifuged at 9.000 x g for 10 min. The mitochondrial pellet was carefully suspended in 2 mL of ice-cold albumin free isolation medium, followed by centrifugation at 9,000 x g for 10 min. The final pellet was resuspended in 100 μ l of albumin free isolation medium; this resuspension contained the isolated mitochondria and was used in the respiration assay described below. All the above procedures were performed at 4 °C, according to previously described for Carvalho et al. (2017).

2.4.2. Mitochondrial respiration assays

It was evaluated by titration of a series of substrates and inhibitors the mitochondrial function reflected as a change in the respiration states. The

analysis of mitochondrial bioenergetics in *D. melanogaster* mitochondria was carried out according to Gnaiger et al. (2012). Oxidizable substrates were used in all experiments, like L-Proline+Pyruvate+Malate and Succinate. After signal stabilization, the basal respiration was supported by endogenous substrates, the Complex I (CI)-mediated Leak (LEAK) respiration was determined using 5 mM pyruvate, 5 mM L-proline, and 1 mM malate. CI-mediated OXPHOS (CIOXPHOS) was determined using ADP (2.5 mM). The functional integrity of the outer mitochondrial membrane (CIcOXPHOS) was determined by the addition of exogenous cytochrome c (cyt c) from the equine heart (10 μ M). Outer mitochondrial membrane disruption is associated with cyt c release, thus the respiration stimulated when cyt c is added is proportional to membrane damage (Pichaud et al., 2013). Respiratory control ratios (RCR = CIOXPHOS/CIcLEAK) and the increase of oxygen flux after injection of Cyt c (CIcOXPHOS/CIOXPHOS) were used as quality control of isolated mitochondria. The convergent electron flow during the maximal OXPHOS respiration (CIc+CIcOXPHOS) was determined with substrates of CIc and CII (10 mM Succinate). The electron transport system (ETS) respiration represents the non-coupled respiration using FCCP (optimum concentration reached between 0.5 and 1.5 μ M), CIc+CII-mediated ETS respiration (CIc+CIcIETS) was determined using FCCP (optimum concentration reached between 0.5 and 1.5 μ M). CII-mediated ETS respiration (CIcIETS) was determined with 0.5 μ M rotenone. The addition of 2.5 μ M antimycin A inhibited complex III, resulting in nonmitochondrial respiration, the residual oxygen consumption (Rox) with small contributions from electron leak in the uncoupled state.

2.5. Metabolic markers

The evaluation of metabolic markers of *D. melanogaster* was performed with the use of colorimetric kits. Twenty flies were homogenized in 100 μ l of 20mM HEPES at pH 7.0 in PowerLyzer homogenizer with one metallic bead per tube at 1.000 x g for 30 s and posteriorly centrifuged at 14.000 x g for 30 min. The samples were kept at 4°C during the preparations until the end of the tests. After centrifugation, the clear supernatant was used to analyze glucose, trehalose, glycogen, triglycerides according to the manufacturer's protocol (Labtest®, MG, Brazil). The results were adjusted according to protein concentration in the samples and expressed in percentage of control.

2.6. Protein quantification

Protein levels in the sample were evaluated by the method of Bradford (1976).

2.7. Western Blotting

Groups of 30 whole flies were mechanically homogenized at 4°C in 200 μ l of buffer (pH 7.0) containing 50 mM Tris, 1mM EDTA, 20mM Na₃VO₄, 100 mM sodium fluoride, and protease inhibitor cocktail. Then, the homogenate was centrifuged for 10 min at 1000 x g at 4 °C, and the supernatant was collected. After protein determination according to Bradford (1976), 4% SDS solution, 25 % glycerol, and 8% β -mercaptoethanol were added to samples to a final concentration of 405 μ l. Samples were frozen for further analysis. The proteins were separated by SDS-PAGE using 10% gels and then electrotransferred to nitrocellulose membranes as previously described (Paula et al., 2012). Membranes were washed in Tris-buffered saline with Tween (100 mmol/L Tris-HCl, 0.9% NaCl and 0.1% Tween-20, pH 7.5) and incubated overnight (4°C) with specific primary antibodies anti-Acetyl-Coenzyme A Synthetase (ACeCS1; 1:1000) and anti-Acyl-Coenzyme Synthetase (ACSL1; 1:1000) and anti- β actin (1:1000). Subsequently, the membranes were washed in Tris-buffered saline with Tween and incubated for 1 h at 25°C with anti-rabbit Ig-secondary antibodies. Antibody binding was visualized using the ECL Western Blotting Substrate Kit. Band staining density was quantified using the Scion Image software (Scion Image for Windows).

Results are expressed as the optical density of ACSL1 or ACeCS1/optical density of respective β -actin.

3. Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by Bonferroni *post hoc* test. Differences between groups were considered statistically significant when $p < 0.05$.

4. Results

4.1. MZ reduces body weight, glucose, trehaloses, and glycogen and increases triglycerides in *D. melanogaster*

Studies carried out with rodents demonstrated that exposure to MZ could alter triglycerides and glucose levels in the serum (Yahia et al.,

2019). This fact could lead to metabolic disturbances. Herein it was wonder if MZ could cause similar effects in flies. It is showed that exposure of *D. melanogaster* to MZ led to a reduction of 9.5 folds in the bodyweight compared to the control. Similar effects were observed in both concentrations (Figure 1A). At 5 mg/mL, the levels of glucose, trehalose, and glycogen decreased by 35%, 43%, and 33%, respectively (figure 1B, C, and D). At 10 mg/mL a decrease of 27%, 46% and 49% respectively was observed. On the other hand, MZ led to an increase in triglyceride levels (37% at 5 mg/ml of MZ and 20% at 10 mg/mL) in Figure 1E.

4.2. Acyl-Coenzyme Synthetase (ACSL1) and Acetyl-Coenzyme A synthetase (ACeCS1) were evaluated in flies exposed to MZ

MZ induced critical alterations in triglyceride content, with a base on this data, it was investigated the content of two enzymes related to lipid

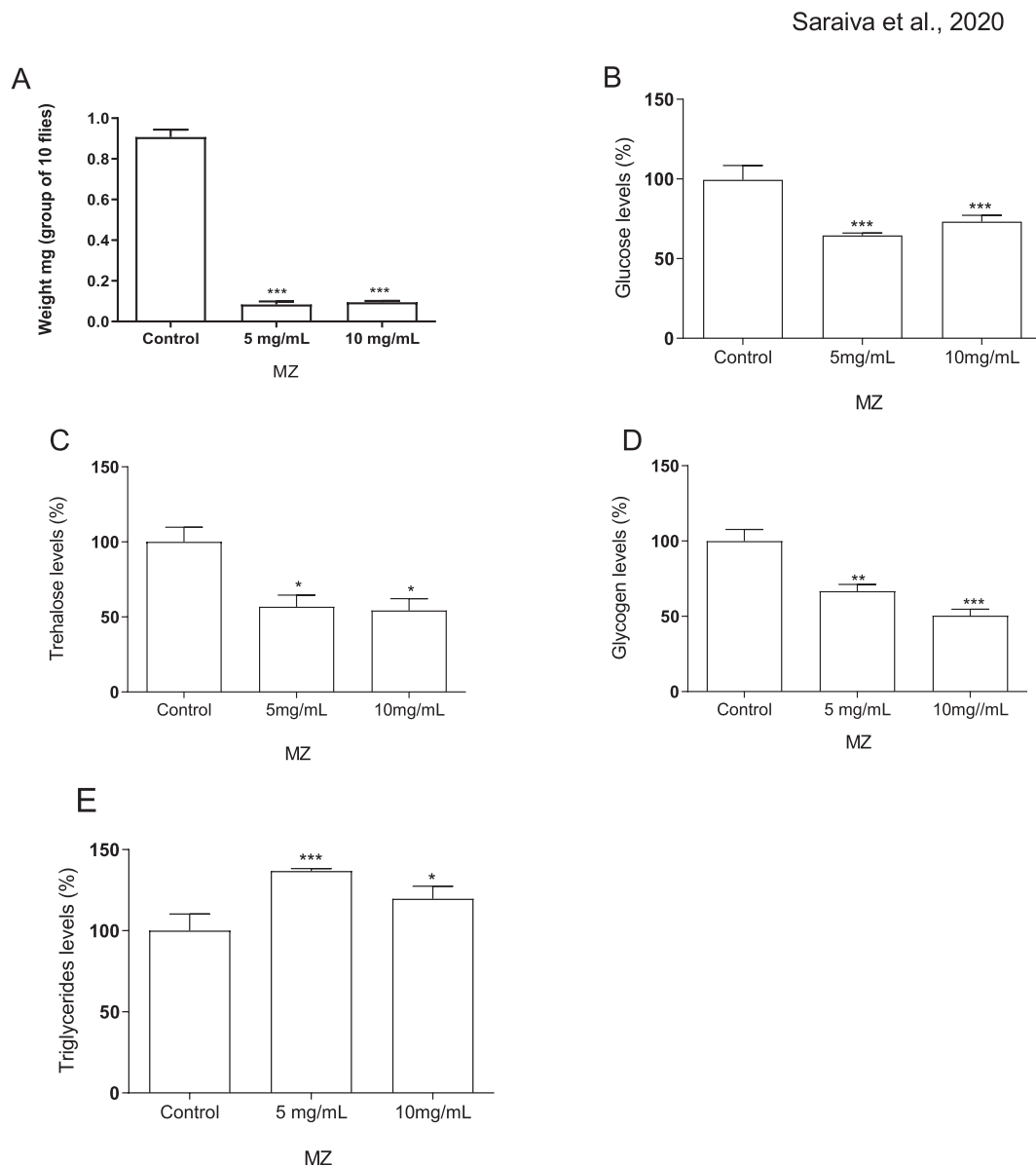


Figure 1. Evaluation of glucose, trehalose, glycogen, triglycerides, and weight of flies in response to MZ exposure. After treatment, a group of 10 flies was weighted and expressed in mg (A); Glucose (B), Trehalose (C), Glycogen (D), and Triglycerides (E) were measured and expressed as an average of 3–5 experiments \pm S.D. in percent of control. Experiments were repeated 3–5 times. Statistical difference between groups was evaluated by one-way ANOVA, followed by Bonferroni's posthoc test. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$ em relation to control group.

biosynthesis. The levels of Acetyl-CoenzymeA Synthetase (ACeCS 1) and Acyl-Coenzyme Synthetase (ACSL) were corrected by β -actin content, as visualised by Figure 2 A and B, no significant alterations concerning control were observed.

4.3. Mancozeb exposition reduced the O_2 flux to mitochondria by high-resolution respirometry (HRR)

Aiming to investigate MZ's effects at the mitochondrial level, the mitochondrial respiration was analyzed using high-resolution respirometry (HRR) (Figure 3), evaluating the oxygen consumption rate in isolated mitochondria. The basal and CILEAK respiration values indicated no significant differences between the control and experimental groups. When the OXPHOS was induced with saturating ADP concentration, all MZ concentrations tested were able to decrease CIOXPPOS and CIIc + CIIOXPHOS. When mitochondrial respiration was uncoupled using FCCP a significant reduction in ETS at CIIc + CIIETS was verified. MZ did not change CIIETS when comparing to the control group. Antimycin A (AmA) decreased the O_2 flux to the basal levels.

4.4. Mitochondrial functionality and integrity evaluation by RCR and CYT_c values

The mitochondrial efficiency represents the capacity to convert animal's resources into ATP and can be calculated as the respiratory control ratio, RCR. MZ at 5 mg/mL was able to reduce the RCR as demonstrated in Table 1. The integrity of the mitochondrial outer membrane was evaluated with the addition of cytochrome c; thus, the respiration rates with and without cyt c were compared. A satisfactory ratio indicating membrane integrity should be less than 1.1 (Carvalho et al., 2017). The respiration rates were similar in all preparations, before and after cyt c addition, attesting integrity of mitochondria (Table 1).

4.5. MZ decreases bioenergetics capacity of mitochondria

Mitochondrial bioenergetics capacity was analyzed by subtracting the ADP-induced Cl_{OXPHOS} values from Cl_{LEAK} values (Figure 4). Treatment with 5 mg/mL of MZ reduced by 35% the bioenergetics mitochondrial capacity whereas 10 mg/mL decreased by 32% the bioenergetics capacity.

5. Discussion

Mancozeb (MZ) is a broad-spectrum fungicide acting in a multi-site mode, indicated for fungi control in several crops (EPA, 2005). *Drosophila melanogaster* is considered a reliable model in toxicological studies due to its physiological, genetic, and biochemical similarities with vertebrates reproducing many effects in response to chemicals (Igboin et al., 2012; Inamdar et al., 2012; Wunderlich et al., 2014). The effect of long-term consumption of MZ in fruit flies was previously reported by the authors, leading to mortality, behavioral alterations, and altered oxidative stress markers as antioxidant enzymes and gene expression (Saraiva et al., 2018). Herein, male fruit flies were exposed to MZ for fifteen days, and then the mitochondrial bioenergetic capacity was evaluated besides metabolic markers.

The evaluation of biochemical markers showed significant changes in the lipid and glucose profiles. MZ exposure for 15 days increased triglycerides and reduced glucose, glycogen, and trehalose. These results corroborate with Yahia et al. (Yahia et al., 2014, 2019), who found that MZ increased triglycerides and decreased serum glucose in rats exposed to this fungicide. In the present study, MZ induced weight loss in flies. Similarly, experiments conducted by Mongi et al. (2011) reported weight loss in rats treated with the pesticide Deltamethrin. Two hypotheses could contribute to the weight of loss, firstly, could be a possible decrease in the food consumption by flies, and a second hypothesis could be a lower absorption of nutrients caused by potential injuries to the

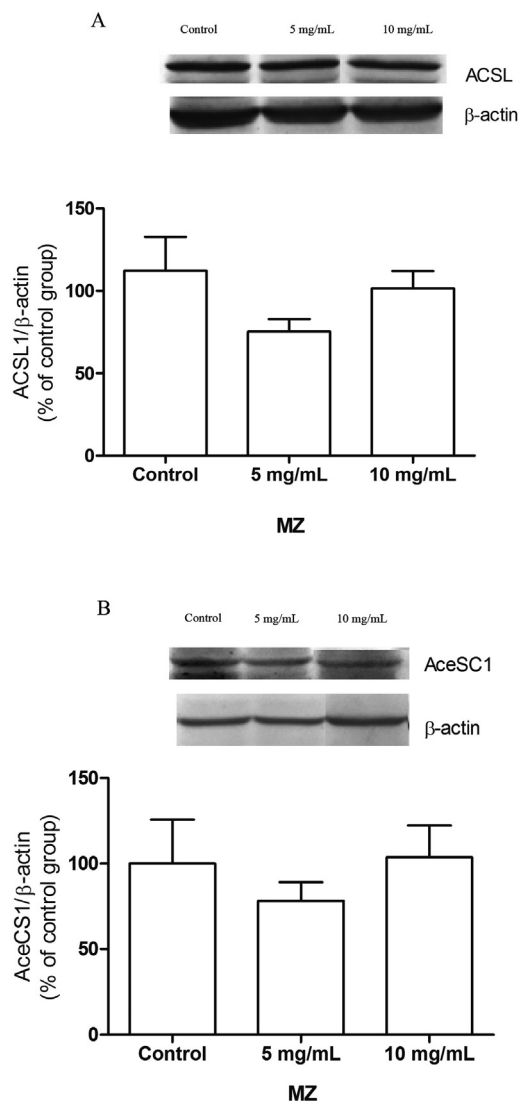


Figure 2. ACSL1 and ACeCS1 content in response to the treatment of *D. melanogaster* with MZ. After treatments, flies were homogenized, and the proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. The total content of proteins was quantified using specific antibodies. The upper panel is a Western Blot showing expression levels of Acyl-Coenzyme Synthetase (ACSL1) adjusted with the respective contents of β -actin (A). The graph shows the ratio of immunoreactive bands of ACeCS1/ β -actin and represents an average \pm S.E.M. Three sets of experiments were conducted. (B) shows representative blotting of the total content of Acetyl-CoA Synthetase (ACeCS1) and β -actin content and a graph of the data obtained by quantification of the bands adjusted with β -actin content. Statistical difference between groups was evaluated by one-way ANOVA, followed by Bonferroni's posthoc test. *** indicates a significant difference between the control group and the MZ exposition ($P < 0.0005$).

gastrointestinal tract impacting the levels of glucose, glycogen and disaccharide trehalose in the fly body (Yasugi et al., 2017).

In glycolysis, the glucose is converted into two pyruvate molecules, which have different fates; one fate is entering the citric acid cycle within the mitochondrial matrix and undergoing oxidative phosphorylation (Melkonian and Schury, 2019). Chronic hypoglycemia, a state of low glucose levels for a prolonged period, led to a decreasing in state 3 of respiration and RCR (respiratory control ratio) (Rehni and Dave, 2018). In accordance, the lower levels of glucose observed in this study's results could contribute to the reduced mitochondrial respiration rate due to the low availability of energetic substrates.

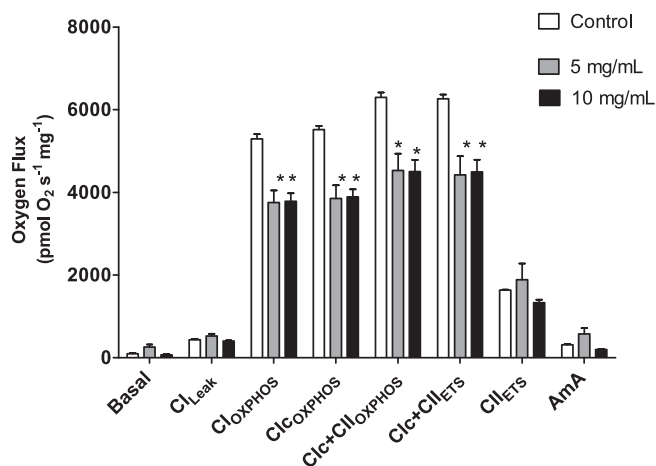


Figure 3. Mitochondrial activity of complexes by High-Resolution Respirometry (HRR) using Oxígrafo-2k (O2k, OROBOROS Instruments, Innsbruck, Austria) in response to MZ. The abbreviation(s) of the complex(es) is followed by the state of respiration measured in presence of L-proline + pyruvate + malate (CI_{LEAK}), + ADP (CI_{OXPHOS}), + cytochrome c (CI_{cOXPHOS}), + succinate (CI_c + CI_{OXPHOS}), + FCCP (CI_c + CI_{ETS}), + rotenone (CI_{ETS}), + antimycin A (Ama). Results are presented as means ± SD, from 4 different preparations. Statistical difference between groups was evaluated by one-way ANOVA, followed by Bonferroni's posthoc test. **p* < 0.05.

In a condition of low availability of glucose, triglycerides can supply energy for the cells releasing glycerol and fatty acids, which are oxidized by β -oxidation reactions into acetyl CoA, which is used in the citric acid cycle to generate ATP by aerobic respiration, in the same mode that Acetyl CoA derived from pyruvate (Gordon Betts et al., 2013). The augmented triglycerides induced by MZ exposure could be associated with a lower demand for Acetyl CoA attributed to the inhibition of mitochondrial complexes and lower respiration rate. A possible induction of lipid biosynthesis by MZ was argued and investigated by analysis of cytoplasmic acetyl-Coa Synthetase (AceCS1) and acyl CoA synthetase (ACSL) levels. The first catalyzes the conversion of acetate and CoA to acetyl-CoA, which is used in fatty acid and lipid biosynthesis (Ikeda et al., 2001), the second catalyzes fatty acids with chain lengths from 12 to 20 carbon atoms to form acyl-CoAs, which are lipid metabolic intermediates (Yan et al., 2015). The expression of those enzymes was unchanged by MZ, indicating that the augmented lipids levels were not attributed to these enzymes' altered levels. Mitochondrial function is essential for aerobic organisms' survival, and mitochondrial bioenergetics represents a potential target of pesticides (Gao et al., 2017; Leung and Meyer, 2019). Dysfunction in mitochondrial machinery is associated with several dysfunctions, like cancer and neurodegenerative disorders like Parkinson's and Alzheimer's diseases (Clarke, 2007; Prauchner, 2017; Uttara et al., 2009). It was demonstrated that MZ inhibited complex I and ATP levels and induces H₂O₂ generation in brain tissue, which was not observed for the MZ metabolite Etilenothiorueia (ETU) or MZ organic backbone without the presence of Manganese ion in nervous cell cultures (Domico

Table 1. Effects of different concentrations of Mancozeb in *D. melanogaster*, evaluating the respiratory control ratios and cytochrome c effects.

Treatments	RCR CI	Cyt C
Control	12.29 ± 0.67	1.04 ± 0.02
5 mg/mL	7.77 ± 1.77 *	1.01 ± 0.02
10 mg/mL	10.40 ± 1.70#	1.03 ± 0.01

The respiratory control ratio for Complex I CI (RCR = CIOXPPOS/CI LEAK) and the cytochrome c effect. Values were expressed as a mean and standard deviation. The ANOVA one-way were carried out, followed by Bonferroni's post hoc test, **P* < 0,05) comparing to control, #*P* < 0.05 comparing to 5 mg/mL.

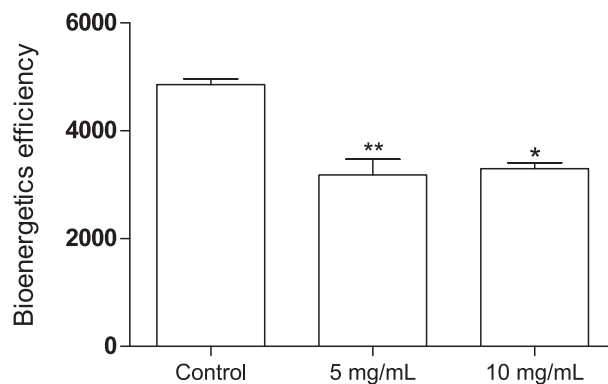


Figure 4. Analysis of Bioenergetics capacity of *D. melanogaster* exposed MZ different concentrations. Results are presented as means ± S.E.M, from 3 different preparations. Statistical difference between groups was evaluated by one-way ANOVA, followed by Bonferroni's posthoc test. **P* < 0.05* and ***P* < 0.005.

et al., 2006; Srivastava et al., 2016). Damage to this organelle by environmental contaminants can impair cellular bioenergetics and induce ROS generation. The authors have previously demonstrated an oxidant effect of MZ on flies causing lipoperoxidation reflected by augmented MDA levels and DCFDA fluorescence and altered antioxidant enzyme activity (Saraiva et al., 2018). Thus a possible deficit in mitochondrial activity could contribute to oxidative stress and associated damage in MZ response. The present work demonstrated that exposure of *D. melanogaster* to MZ impairs the OXPPOS in CIOXPPOS and CIc + CIOXPPOS. Additionally, it was observed the disruption in ETS at CIc+CIcETS but not CIcETS. Impairment of ETS and OXPPOS capacity lead to a deficit in the mitochondrial bioenergetics rate. Thus, this research showed that MZ caused deregulation of mitochondrial respiration and energetic substrates critical for cellular function maintenance.

6. Conclusion

MZ exposure disrupts the mitochondrial respiration compromising bioenergetics capacity, resulting in reduced ATP necessary for sustaining essential cellular activities. This fact may contribute to augmented ROS generation corroborating with previous results when oxidative damage was confirmed. In parallel, MZ leads to a decay in energetic substrates followed by increasing in triglycerides. MZ unaltered the levels of enzymes involved in triglycerides biosynthesis. This study also shows that similarly to vertebrates, *Drosophila's* mitochondria are targeted by MZ, and the amount of energetic substrates is altered by these compounds, which could compromise the maintenance of essential cellular functions.

Declarations

Author contribution statement

M. A. Saraiva: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

N. R. Carvalho: Performed the experiments; Analyzed and interpreted the data.

I. K. Martins; G. E. Macedo; N. R. Rodrigues; K. K. Gomes; C. C. Ziech: Performed the experiments.

P. B. Vieira: Conceived and designed the experiments; Performed the experiments.

M. Prigo; J. L. Franco: Contributed reagents, materials, analysis tools or data.

T. Posser: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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