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Biochemical CuSO₄ Toxicity in *Drosophila melanogaster* Depends on Sex and Developmental Stage of Exposure

Paula Tais Halmenschelager[1] · João Batista Teixeira da Rocha[1]

Abstract

Copper is a transition metal that exists in different chemical forms (e.g., Cu^{2+}, Cu^+, and Cu^0) and at high concentrations it is toxic. Here, we investigated the Cu^{2+}-induced toxicity in *Drosophila melanogaster*, evaluating the survival, locomotion, and the activity of acetylcholinesterase (AChE) and glutathione *S*-transferase (GST) enzymes. Flies were exposed to Cu^{2+} (0.1–1 mmol $CuSO_4$/kg of diet or approximately 0.1–1 mM Cu^{2+}) and allowed to mate during 24 h. GST and AChE enzymes were evaluated in the larvae and in the head and the body (thorax + abdomen) of the adult male and females flies. The total number of adult females (0.4–1 mM) and males (0.75 and 1 mM) was decreased by $CuSO_4$. The climbing ability was hampered in flies exposed to 1 mM Cu^{2+}. In larvae, Cu^{2+} (0.4–1 mM) increased AChE activity ($P < 0.002$). In males' heads, 0.4 mM Cu^{2+} increased the AChE activity ($P < 0.01$). In adults' bodies, Cu^{2+} inhibited the activity in both sexes, but with greater effectiveness in males (0.1 to 1 mM) than in females (1 mM). Regarding GST activity, 0.1 mM Cu^{2+} increased, but 1 mM decrease GST in larvae. In the head of flies, Cu^{2+} decreased the GST activity at intermediate (0.4 mM) and increased GST at the highest concentration (1 mM) in males. In the bodies, the effect of Cu^{2+} was similar. In conclusion, Cu^{2+} exposure in *D. melanogaster* disrupted locomotion and enzymatic parameters that can be related to changes in AChE and in the detoxifying GST enzyme.

Keywords Copper · *Drosophila melanogaster* · Acetylcholinesterase · Glutathione *S*-transferase

Introduction

Copper is a transition metal that exists in different ionic forms (e.g., Cu^{2+} and Cu^+) and is essential to all living organisms from bacteria to humans [1]. Indeed, at low concentrations, copper is required for proper cellular metabolism; but at high concentrations, copper can be toxic because of its redox-active properties [2–7]. The concentrations of copper in water and soil are approximately 7 and 50 ppm, respectively; and in the atmosphere, Cu ranges from 5 to 200 ng/m³. In humans, the allowed upper limit of the copper concentration is approximately 1.5 mg/L of serum [8]. The majority of copper in healthy cells is found in the prosthetic groups of enzymes or bound to proteins, for instance, cytochrome c oxidase, copper-zinc-superoxide dismutase (Cu, Zn-SOD), and ceruloplasmin [9, 10].

In contrast to the protein-associated Cu, the free copper is potentially toxic and accumulated in the liver and brain [11–13]. Of particular importance, copper has been implicated in the pathogenesis of neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases [14–16]. The presence of free copper in the living cells can accelerate the ROS formation via Fenton or Haber-Weiss reactions that can induce protein and DNA damage, lipid peroxidation, mitochondrial swelling, and cell death [17–19].

Copper homeostasis is tightly coordinated by copper chaperones that ensure the suitable distribution of copper to the cells and copper-requiring proteins [20, 21]. Bacteria, yeast (*Saccharomyces cerevisiae*), and mammalians have homologs of these proteins, which indicate a conserved metabolism of copper in these organisms [22]. In general, the eukaryotes have three different copper chaperones: Atx1p/Atox1 which delivers copper to ATP7 copper-requiring protein transporters,

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Cox17 which takes copper to the mitochondrial cytochrome c oxidase, and CCS, a copper chaperone that supplies copper to superoxide dismutase 1 [23–25].

Cu toxicity can occur after accidental, occupational, or environmental exposure [26]. In addition, human exposure is frequently associated with the use of copper in agriculture [27], by water consumption [28], and in the industrial production of electric conductors [29–31]. Toxicological endpoints of copper exposure have been reported for many model organisms, including bacteria [32, 33], yeast [34, 35], nematodes [36, 37], amphibians [38], and human cell lines [39–41].

The fruit flies *Drosophila melanogaster* is an important non-mammalian experimental organism that can be used to assess macroscopic dysfunctions (lifespan, fertility, phenotypic aberrations) induce by metals as well as the molecular pathways that can be involved in the toxic response to the metals [42, 43]. Regarding copper metabolism, *D. melanogaster* has been exploited as an excellent model to unravel the molecular mechanisms involved in Cu physiology [44, 45]. Of particular importance, *D. melanogaster* synthesizes five Cu-thionin isoforms (i.e., MtnA, MtnB, MtnC, MtnD, and MtnE) [46] and has numerous genes and detoxification mechanisms analogous to humans [47]. In this view, the activity of the acetylcholinesterase enzyme has been shown to be an efficient and widely used mechanism to evaluate toxicological endpoints involving the nervous system [48, 49].

Since only a few studies have studied comparatively the copper toxicity at different developmental stages in flies [50–54], here we evaluated the toxicity of Cu²⁺ during the entire life cycle of *D. melanogaster* (i.e., from larvae to adults), using morphological, behavioral, and biochemical endpoints of toxicity. Particularly, with the demonstration that inbred female flies were more sensitive to Cu²⁺ than males [7], we have hypothesized and tested here that the exposure during the entire development stages of flies could indicate differences in the biochemical and behavioral responses of male and female adult flies to copper. In fact, the fruit fly *D. melanogaster* is a holometabolous insect and its development from egg to the adult stage has high energy costs [55]. Furthermore, the sensitivity of developing flies to toxicants can be expected to be higher than that of adults. In this view, previous studies have indicated that CuSO₄ (≈ 0.6 to 5 mM) delayed the development of the fruit flies, but the authors have not investigated the potential influence of sex on the morphological and biochemical endpoints of toxicity [56]. As cited above, copper homeostasis in *D. melanogaster* seems to be modulated by X-linked genes [7], particularly the expression metallothionein-related genes, which are more expressed in male than in female flies [52]. Thus, one would be expected a higher vulnerability of females to copper. However, a similar [52] or higher negative impact of copper in males than females have been reported [52, 53]. In view of these incongruences, it is necessary to further study the influence of sex differences in copper susceptibility in *D. melanogaster*.

Material and Methods

Chemicals

Copper sulfate pentahydrate (CuSO₄·5H₂O), 1-chloro-2, 4-dinitrobenzene (CDNB), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and acetylthiocholine iodide were purchased from Sigma (USA). All other reagents were commercial products of the highest purity grade available.

Drosophila melanogaster Stock and Culture

Diet preparation was performed following Adedara and collaborators' [57] method with some adaptations. *D. melanogaster* wild-type was obtained from the National Species Stock Center (Bowling Green, OH, USA). The flies were maintained on cornmeal medium (1% corn flour, 2% *w/v* brewer's yeast, 1% *w/v* sucrose, 1% *w/v* powdered milk, 1% *w/v* agar, and 0.08% *v/w* nipagin and 93.92% distilled water) at constant temperature and humidity (22–24 °C; 60–70% relative humidity) under 12-h dark/light cycle conditions.

Cu²⁺ Exposure

Four different copper concentrations were chosen to perform the present study: a non-toxic concentration (0.1 mM), a low toxic (0.4 mM), a middle-toxic (0.75 mM), and a high toxic (1 mM). The doses were selected according to both to the literature data and to pilot studies (data not shown) using the survival pattern observed. Indeed, the decrease in survival rate responses was dose-dependent for all the phases analyzed. Here, we have to emphasize that 0.1 mM did not decrease the survival rates in all the developmental stages studied. Another study from our laboratory, for instance, in an acute exposure of 4 days to 0.5, 1, 3, 5, 10, and 20 mM Cu²⁺, has found that the concentrations of 3 mM cause the death of 50% of the flies and 1 mM were already toxic [58].

In relation to Cu²⁺ exposure, basically, a mother solution of CuSO4 (150 mmol/L) was prepared in distilled water and kept on the refrigerator (4–6 °C). This solution was diluted to (in mmol of CuSO4/L) 10.1, 40.4, 75.75, and 101, and then 0.3 ml of these solutions were mixed with 30 g of still hot and liquid diet medium to give the final concentrations of approximately 0.1, 0.4, 0.75, and 1 mmol of Cu²⁺/kg of diet (0.1–1 mM of CuSO₄). The mixture was stirred manually until homogenous solutions were obtained. The 4-day-old adult flies (30 per vial) from the five experimental groups were allowed to mate for 24 h. Then, they were removed, and the development of larvae, pupae, and adults was analyzed for 5, 9, and 13 days of exposure, respectively. Six independent replicates were made for each group.

Drosophila melanogaster Development

The *D. melanogaster* development was analyzed according to Rand and collaborators [59] with some adaptations. The animals were counted on the fifth (larvae stage), ninth (pupae stage), and thirteenth (adults) days after mating to determine the number of animals that survived. At the adult stage, the males and females were counted separately and expressed in percentage.

Negative Geotaxis

The locomotor activity of copper exposure was assessed using the negative geotaxis assay perform according to Ali and collaborators [60] with some adaptations. The potential neurotoxicity of $CuSO_4$ was assessed using the negative geotaxis assay (climbing test). Ten 4-day-old adult male or female flies from each group were immobilized under mild ice anesthesia for 10 min. Then, they were placed in a vertical glass column (height 10 cm, diameter 1.5 cm) marked with a line in the height corresponding to 6 cm. The flies were allowed to recover anesthesia for 20 min. After that, the animals were tapped to the bottom of the column and, after 6 s, the number of flies that crossed the 6-cm mark was recorded.

Preparation of Larvae Samples

Twenty larvae from each group were anesthetized on ice for 10 min and weighed. The larvae were homogenized in 1 mL potassium phosphate buffer (0.1 M pH 7.4) and centrifuged at $10,000 \times g$ for 10 min at 4 °C. The supernatant was used for the biochemical analysis, and protein concentrations were carried out at 280 nm absorbance in NanoDrop 2000 spectrophotometer, using bovine serum albumin (BSA) as a standard. The samples were diluted to 1 mg/ml before the determination of acetylcholinesterase and glutathione *S*-transferase activities.

Preparation of Adult Fly Samples

Four-day-old adult male or female flies (groups of 20 flies) were anesthetized in ice for 10 min. Then, their heads were separated from the thorax and abdomen (body). The tissues were weighed and homogenized in 1 mL potassium phosphate buffer (0.1 M, pH 7.4) and centrifuged at $10,000g$ for 10 min at 4 °C. The supernatant was separated from the pellet and protein contents were assess by Lowry (1951) [61]. The samples were diluted to 1 mg/ml before the determination of acetylcholinesterase and glutathione *S*-transferase activity in both tissues, bodies (thorax and abdomen), and heads, separately.

Head, Total Body (Entire Fly), and Body (Thorax + Abdomen) Weight and Head-to-Total Body Weight Ratio

The head and body (thorax + abdomen) weights of groups of 20 flies were determined in a digital balance with 0.01 mg precision (SHIMADZU AUW220D, Japan). The total body weight was determined by summing the head + the body weights. The head-to-total body weight ratio was calculated as an endpoint of toxicity. The use of this parameter in toxicology can give an idea about the toxicity of a given chemical to specific organs in different species [62, 63].

Determination of Acetylcholinesterase Activity

Determination of AChE activity was carried out according to Ellman (1961) [64]. The reaction mixture contained 20 μL of potassium phosphate buffer (0.1 M, pH 7.4), 20 μL of 25 mM, 5,5′-dithiobisnitrobenzoic acid (DTNB), and 60 μL sample (the total protein content in each well was 60 μg). The reaction was initiated by adding 20 μL of 8 mM acetylthiocholine solution. The reaction was monitored for 60 min (15-s intervals) at 415 nm with a BioRad iMark microplate reader. The enzyme activity was calculated as ηmol of acetylthiocholine hydrolyzed per mg protein per minute, using the cysteine as the source of thiol in the standard curve [57].

Determination of Glutathione *S*-Transferase Activity

Glutathione *S*-transferase activity was determined according to Habig (1974) [65] in which the conjugation of 1-chloro-2,4 dinitrobenzene (CDNB) with glutathione (GSH) is determined. The reaction was performed in 120 μL of GST buffer (0.25 M potassium phosphate buffer, pH 7.0, containing 2.5 mM ethylenediaminetetraacetic acid (EDTA), 60 μL of sample (the total protein content in each well was 60 μg), 10 μL of 100 mM GSH, and 10 μL of 25 mM CDNB. The thioether formation was monitored for 8 min (30-s intervals) at 340 nm (25 °C) in a SpectraMax plate reader (molecular devices). The activity was expressed as specific activity (delta A340 nm/min/mg of protein). Here, it was not possible to use the absorption molar coefficient of CDNB-GSH conjugate because the path length of light in the wells of ELISA reader plate is not 1 cm. Here, it was not possible to use the absorption molar coefficient of CDNB-GSH conjugate because the path length of light in the wells of ELISA reader plate is not 1 cm.

Statistical Analysis

All data were expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed by one- or two-way analysis of variance (ANOVA) followed by the

Newman-Keulls post hoc test, using the GraphPad Prism5 software. Differences were considered statistically significant among groups when $P < 0.05$. The details of the two-way ANOVA analysis are available in supplementary files.

Results

Development of Flies Exposed to Cu²⁺

Copper exposure caused a dose-dependent decrease in the development of larvae, pupae, and adult flies (Fig. 1a–c). In larvae, $F_{(4,25)} = 56.03$ ($P < 0.0001$). The means $\pm$ SEM of the number of larvae (after 5 days of exposure) were 100.2 ± 6.06,

82.0 ± 0.73, 67.0 ± 2.20, 40.5 ± 4.94, and 33.0 ± 1.93 for the control, 0.1, 0.4, 0.75, and 1 mM CuSO₄ groups, respectively. Similar tendency was observed for pupae ($F_{(4,25)} = 15.20$ ($P < 0.0001$)) after 9 days of treatment. The means were 96.33 ± 8.08, 69.33 ± 1.11, 66 ± 5.87, 41.33 ± 7.86, and 42.33 ± 2.48 for the control, 0.1, 0.4, 0.75, and 1 mM CuSO₄ groups, respectively. The total number of adult flies (male + female) after 13 days of exposure to copper $F_{(4,25)} = 23.60$ ($P < 0.0001$) was also reduced in a dose-dependent manner: 87.66 ± 7.59 (control), 67.83 ± 2.78 (0.1 mM CuSO₄), 57.0 ± 4.84 (0.4 mM CuSO₄), 26.66 ± 6.22 (0.75 mM CuSO₄), and 34.33 ± 1.72 (1 mM CuSO₄).

In accordance with the results described in the previous paragraph (one-way ANOVA for the total number of adult

Fig. 1 Number of larvae (**a**), pupae (**b**), adults (**c**), and males and females (**d**) after exposure to Cu²⁺. Larvae, pupae, and adults were determined after 5, 9, and 13 days of treatment, respectively. Data are expressed as the means $\pm$ SEM ($N = 6$) analyzed by one-way ANOVA (**a**, **b**, **c**) or two-way ANOVA (**d**) followed by Newman-Keuls post hoc test. Asterisk indicates significant differences compared to control ($P < 0.05$)
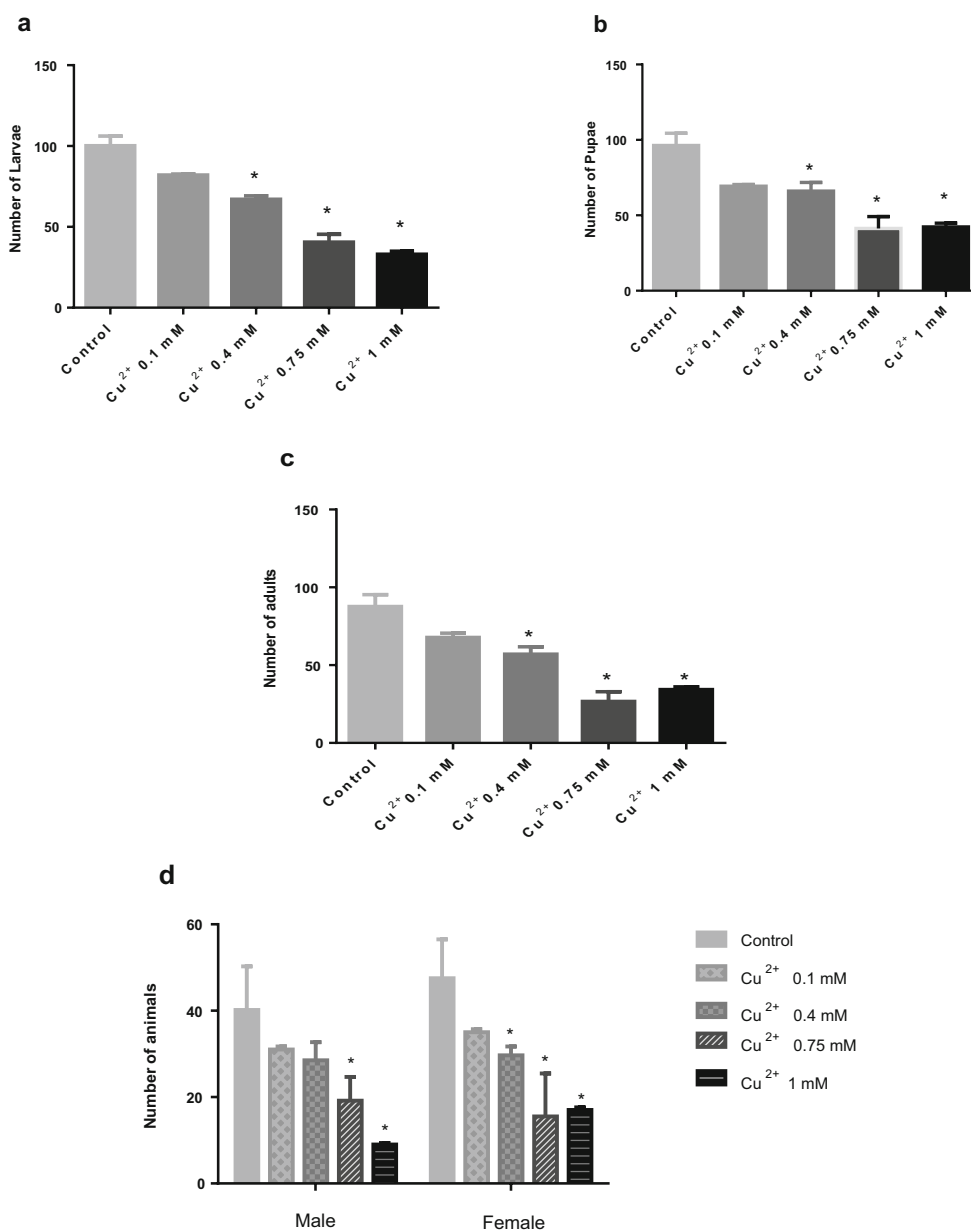
Table 1 Eclosion rate of male and female flies after 13 days of exposure to Cu^{2+} ($N = 6$). The number in parentheses represents the percentage of flies

Concentration (mM)	Absolute number of male and female adults and percentage (%)		
	Males	Females	Total
Control	40 (46% ± 1.06)	47 (54% ± 1.06)	87
0.1	31 (47% ± 0.57)	35 (53% ± 0.57)	66
0.4	28 (49% ± 2.88)	29 (51% ± 2.88)	57
0.75	19 (56% ± 6.73)	15 (44% ± 6.73)	34
1	9 (35% ± 0.43)	17 (65% ± 0.43)	26

flies), two-way ANOVA yielded a significant main effect of copper ($F(4,50) = 23.60$; $P < 0.0001$). Post hoc comparisons indicated that the number of eclosions in females (0.4–1 mM) and males (0.75 and 1 mM) was decreased by $CuSO_4$ (Fig. 1d). In contrast, the main effect of sex was not significant ($P = 0.596$), which was a consequence of the similar proportion of male and female hatchlings in all groups (Fig. 1d and Table 1). The means ± SEM of the groups was 40.16 ± 4.11 (males) and 40.47 ± 3.69 (females) in control flies, 31.0 ± 0.73 (males) and 35 ± 0.73 (females) in 0.1 mM $CuSO_4$ group, 28.5 ± 4.24 (males) and 29.6 ± 2.07 (females) in 0.4 mM $CuSO_4$ group, 19.16 ± 2.24 (males) and 15.5 ± 4.08 (females) in 0.75 mM $CuSO_4$ group, and 9.00 ± 0.36 (males) and 17.0 ± 0.63 (females) in 1 mM $CuSO_4$ group.

Weight of Larvae Exposed to Cu^{2+}

The means (± SEM) of the weight (in mg) of the larvae were 3.34 ± 0.09 (control), 3.10 ± 0.04 (0.1 mM $CuSO_4$), 2.89 ± 0.03 (0.4 mM $CuSO_4$), 2.20 ± 0.05 (0.75 mM $CuSO_4$), and 1.88 ± 0.12 (1.0 mM). Statistical analysis indicated a significant reduction in the larvae weight in the animals exposed to Cu^{2+} (0.4–1 mM) ($F(4,25) = 60.62$, $P < 0.0001$), when compared to control group (Fig.2).
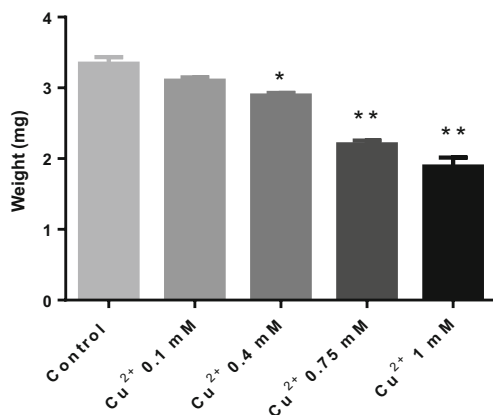
Acetylcholinesterase Activity in the Larvae Treated with Cu^{2+}

One-way ANOVA indicated a significant effect of copper ($F(4,25) = 9.11$; $P = 0.0001$) in the activity of larval AChE. The means (± SEM) of AChE activity in the larvae exposed to 0.4 (3.15 ± 0.31), 0.75 (3.23 ± 0.14), and 1 mM Cu^{2+} (3.05 ± 0.55) were significantly higher than in the control group (1.90 ± 0.10). The concentration of 0.1 mM Cu^{2+} (1.23 ± 0.10) did not show significant differences when compared to the control group (Fig. 3).

Glutathione S-Transferase Activity in the Larvae Treated with Cu^{2+}

One-way ANOVA has shown a significant effect of copper ($F(4,25) = 38.52$; $P < 0.001$) in larvae GST. The GST activity was increased significantly in the group exposed to 0.1 mM Cu^{2+} (0.47 ± 0.01) but exhibited a decrease in the group exposed to 1 mM Cu^{2+} (0.20 ± 0.01) when compared to the control group (0.28 ± 0.01) (Fig. 4). However, the groups exposed to 0.4 mM Cu^2 (0.25 ± 0.02) and 0.75 mM Cu^2 (0.207 ± 0.014) did not show significant differences compared to the control.

Fig. 2 Total body weight of the larvae after 5 days of treatment with $CuSO_4$. Data represent the mean ± SEM ($N = 6$) analyzed by one-way ANOVA followed by Newman-Keuls post hoc test. Single asterisk indicates significant differences among the groups when $P < 0.05$ and double asterisk for $P < 0.01$
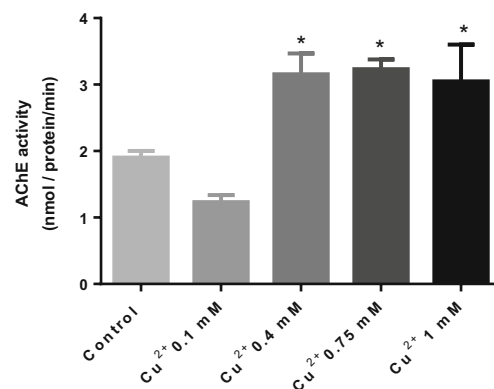

Fig. 3 Larval AChE activity after 5 days of exposure to Cu^{2+}. Data represent means ± SEM ($N = 6$) analyzed by one-way ANOVA followed by Newman-Keuls post hoc test. Asterisk indicates significant differences compared to control ($P < 0.05$)
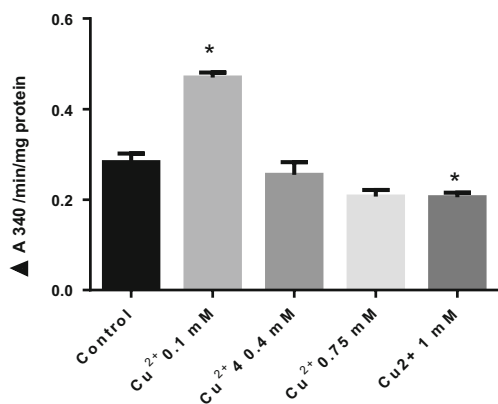
Fig. 4 Glutathione *S*-transferase activity in the larvae treated with Cu^{2+} for 5 days. Data represent means ± SEM ($N = 6$) analyzed by one-way ANOVA followed by Newman-Keuls post hoc test. Asterisk indicates significant difference compared to control ($P < 0.05$)

Weight of the Adult Flies Exposed to Cu^{2+}

In adult flies, two-way ANOVA yielded a significant main effect of copper in heads ($F(4,50) = 16.09$; $P < 0.0001$; Fig.5a) in both males and females. In the flies exposed to 0.75 (0.57 ± 0.02 for males and 0.61 ± 0.02 for females) and at 1 mM of Cu^{2+} (0.59 ± 0.024 for males and 0.57 ± 0.003 for females), there was a decrease in the mean of the head weight when compared to the control group (1.07 ± 0.11 in males and 1.09 ± 0.04 in females).

A similar tendency was observed for the total body weight (Fig. 5b). The results obtained in the two-way ANOVA exhibited a main effect of copper ($F(4,50) = 4.21$; $P < 0.0001$). In accordance, the flies exposed to 0.75 (1.59 ± 0.07 in males and 1.49 ± 0.005 in females) and at 1 mM of Cu^{2+} (1.55 ± 0.05 in males and 1.53 ± 0.05 in females) have a reduction in the body weight means when compared to control means (2.75 ± 0.13 in males and 2.79 ± 0.19 in females).
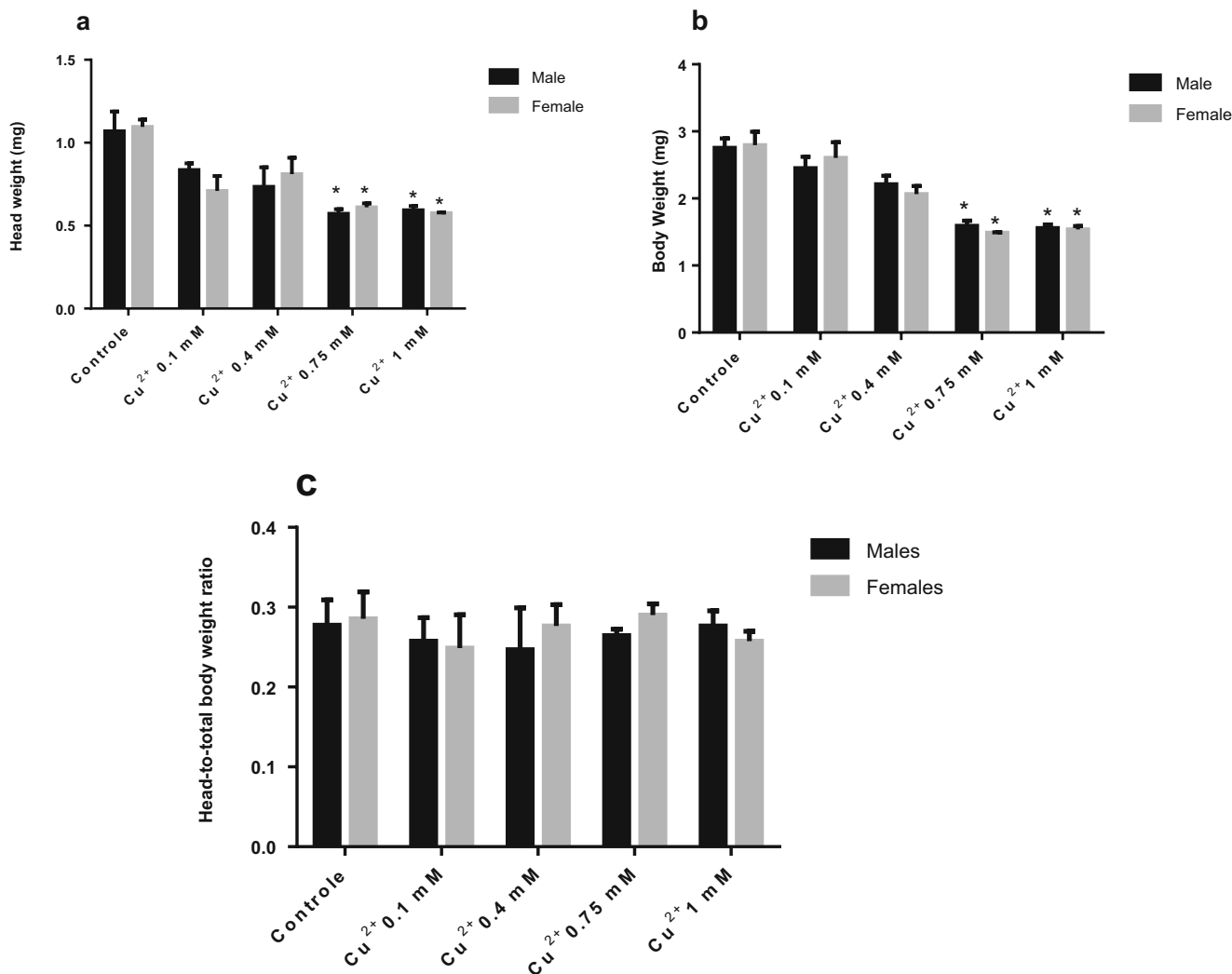

Fig. 5 Weights of the heads (in mg) (**a**), bodies (thorax + abdomen; in mg) (**b**), and the head-to-body weight ratio (**c**) of 4-day-old adult flies after 13 days of Cu^{2+} exposure. Data represent the mean ± SEM ($N = 6$) analyzed by two-way ANOVA followed by Newman-Keuls post hoc test. Asterisk indicates significant differences compared to control ($P < 0.05$)
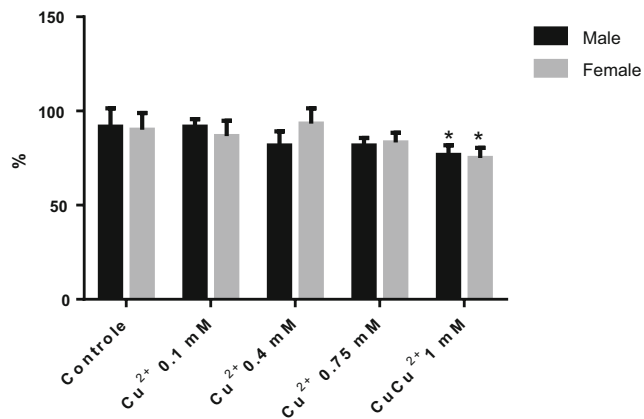
Fig. 6 Climbing ability of 4-day-old adult flies after 13 days of Cu^{2+}
exposure. Data represent the mean ± SEM ($N = 6$). Data were analyzed
by two-way ANOVA followed by Newman-Keuls post hoc test. Asterisk
indicates significant differences compared to control ($P < 0.05$)

There were no significant differences between male and
females weights. The head-to-body weight ratio is being
shown in Fig. 5c.

## Climbing Behavior (Negative Geotaxis)

Two-way ANOVA yielded a significant main effect of copper
($F(4,50) = 9.17$; $P < 0.001$). Flies of both sexes exposed to

1 mM of Cu^{2+} (76.66 ± 2.10 in males and 75.0 ± 2.23 in fe-
males; data expressed as mean ± SEM of the percentage of
flies that reached the 6-cm line within 6 s) exhibited a reduc-
tion in climbing ability when compared to the control group
(91.66 ± 4.01 in males and 90 ± 3.65 in females (Fig. 6).

Acetylcholinesterase Activity in the Adult Flies Treated with Cu^{2+}

In the adult flies, the AChE activity was measured separately
in the head and body (thorax + abdomen). In the heads
(Fig. 7a), two-way ANOVA yielded a significant main effect
of copper ($F(4,50) = 60.23$; $P < 0.0001$), of sex ($F(4,50) =$
7.503; $P < 0.0001$), and copper × sex interaction ($F(4,50) =$
7.816; $P < 0.0001$). The head AChE activity was higher in
females than in males ($P < 0.0001$) and cooper did not modify
the AChE in females. However, in the head of males, 0.4 mM
Cu^{2+} (48.37 ± 3.37) increased the enzyme activity when com-
pared to the control group (35.56 ± 3.30). The means ± SEM
of the head AChE activity in ηmol acetylthiocholine hydro-
lyzed/min/mg protein were 35.56 ± 3.30 (males) and $53.96 \pm$
2.55 (females) for the control group, 33.17 ± 1.71 (males) and
47.05 ± 1.32 (females) in 0.1 mM of Cu^{2+}, 48.37 ± 3.37
(males) and 44.81 ± 2.21 (females) in 0.4 mM of Cu^{2+},

Fig. 7 AChE activity of 4-day-old adult flies after 13 days of Cu^{2+} exposure in the heads (a) and bodies (thorax + abdomen) (b) of 4-day-old adult flies. Data represent means ± SEM ($N = 6$) analyzed by two-way ANOVA followed by Newman-Keuls post hoc test. Asterisk indicates significant differences compared to control ($P < 0.05$)
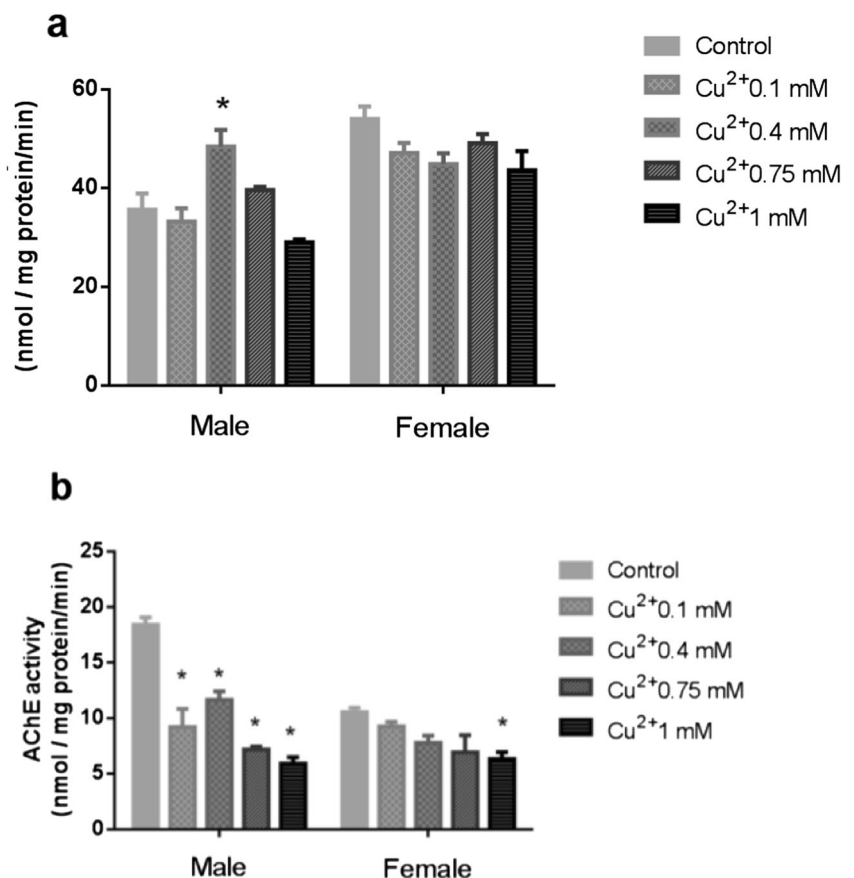
39.59 ± 0.41 (males) and 49.08 ± 1.16 (females), and 29.03 ± 0.36 (males) and 43.57 ± 2.47 (females).

In the bodies, two-way ANOVA yielded a significant main effect of sex ($F_{(4,50)} = 38.72$; $P < 0.0001$), of copper ($F_{(4,50)} = 61.92$; $P < 0.0001$), and copper × sex interaction ($F_{(4,50)} = 18.63$; $P < 0.0001$). In contrast to the head, the AChE in the body of males was higher than in females ($P < 0.0001$). Copper inhibited the activity both in females and males, but with greater effectiveness in males than in females (Fig. 7b). The means (± SEM) of the AChE activity (in ηmol/min/mg protein) were 18.40 ± 0.66 (males) and 10.54 ± 0.41 (females) for the control group, 9.19 ± 1.64 (males) and 9.26 ± 0.41 (females) in the groups treated with 0.1 mM of Cu^{2+}, 11.66 ± 0.76 (males) and 7.79 ± 0.64 (females) in 0.4 mM of Cu^{2+}, 7.17 ± 0.27 (males) and 6.93 ± 1.53 (females) in the groups treated with 0.75 mM of Cu^{2+}, and 5.90 ± 0.59 (males) and 6.33 ± 0.62 (females) in the groups treated with 1 mM Cu^{2+}.

Glutathione *S*-Transferase Activity in Adult Flies Treated with Cu^{2+}

In the heads (Fig. 8a), two-way ANOVA yielded significant main effects of Cu^{+2} ($F_{(4,50)} = 5.16$; $P = 0.0015$), sex ($F_{(4,50)} = 6.97$; $P = 0.011$), and Cu^{+2} × sex interaction

($F_{(4,50)} = 20.86$; $P < 0.0001$). In both sexes, CuSO$_4$ decreased the GST activity at intermediate concentrations (0.4 mM); and at the highest concentration (1 mM), Cu^{2+} increased the activity in the heads of males. The means (± SEM) of the GST activity (in delta absorbance at 340 nm or deltaA340/min/mg protein) were 0.095 ± 0.006 (males) and 0.088 ± 0.01 (females) for the control group, 0.107 ± 0.009 (males) and 0.074 ± 0.01 (females) in the flies treated with 0.1 mM of Cu^{2+}, 0.05 ± 0.001 (males) and 0.065 ± 0.006 (females) in the flies treated with 0.4 mM of Cu^{2+}, 0.085 ± 0.009 (males) and 0.09 ± 0.0009 (females) in the flies treated with 0.75 mM of Cu^{2+}, and 0.144 ± 0.09 (males) and 0.105 ± 0.004 (females) in the flies treated with 1 mM of Cu^{2+}.

In the bodies (thorax + abdomen) (Fig. 8b), two-way ANOVA revealed a significant main effects of copper ($F_{(4,50)} = 23$, 57, $P < 0.0001$), sex ($F_{(4,50)} = 64.08$; $P < 0.0001$), and copper × sex interaction ($F_{(4,50)} = 4.045$; $P = 0.0064$). As observed in the heads, low and intermediate concentrations of Cu^{2+} decreased the GST in males and females bodies (though the effect was more pronounced in males). In addition, the highest concentration increased the body GST activity in females and males (but the effect tended to be robust in females). The means (± SEM) of the GST activity (delta A340/min/mg protein) were 0.201 ± 0.009 (males) and

Fig. 8 Glutathione *S*-transferase activity of 4-day-old adult flies after 13 days of Cu^2 exposure in the heads (**a**) and bodies (thorax + abdomen) (**b**). Data represent means ± SEM ($N = 6$) analyzed by two-way ANOVA followed by Newman-Keuls post hoc test. Asterisk indicates significant differences compared to control ($P < 0.05$)
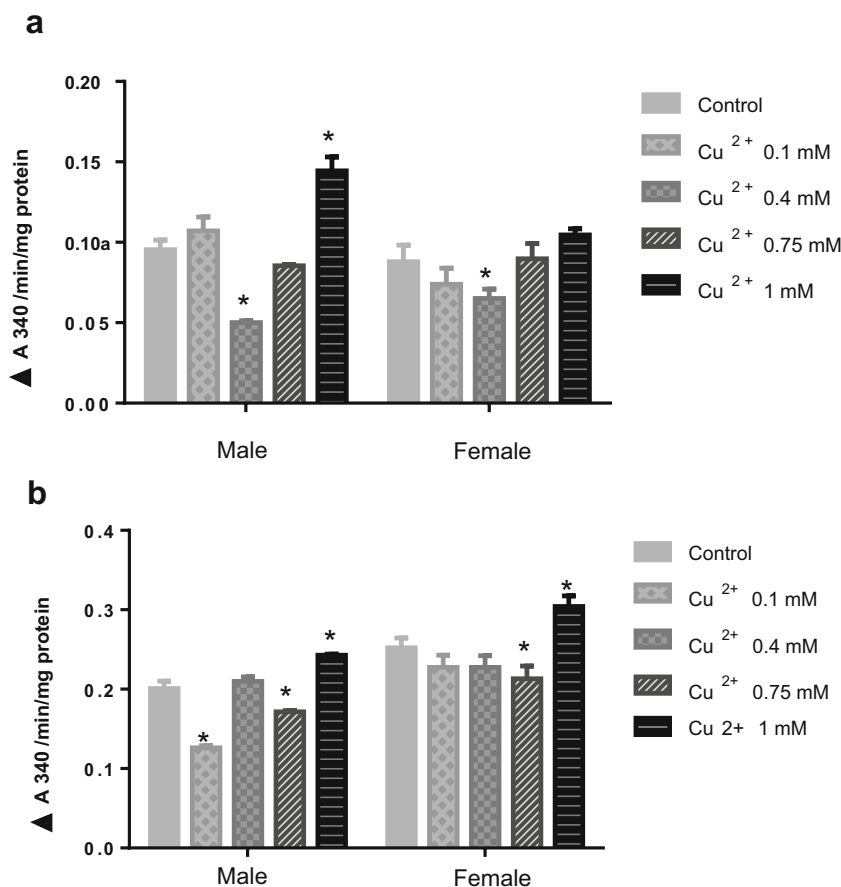
0.252 ± 0.01 (females) for the control group, 0.126 ± 0.003 (males) and 0.228 ± 0.015 (females) in flies treated with 0.1 mM of Cu^{2+}, 0.210 ± 0.006 (males) and 0.227 ± 0.015 (females) in flies treated with 0.4 mM of Cu^{2+}, 0.171 ± 0.002 (males) and 0.213 ± 0.016 (females) in flies treated with 0.75 mM of Cu^{2+}, and 0.243 ± 0.001 (males) and 0.304 ± 0.013 (females) in flies treated with 1 mM of Cu^{2+}.

Discussion

The fruit fly *D. melanogaster* has been widely used as an alternative model to study copper metabolism and its dyshomeostasis [23, 25, 52, 53, 57, 66, 67]. In accordance with other studies [7, 51, 55, 57, 68], here, we have observed that copper decreases the development and the survival rate of larvae, pupae, and adults of *D. melanogaster.* From 0.4 to 1 mM, Cu^{2+} caused a concentration-dependent decrease in the number of larvae, pupae, and adults, whereas the dose of 0.1 mM Cu^{2+} did not cause overt toxicity. Literature data have indicated that exposure of *D. melanogaster* to 50 μg/mL ($\approx$ 0.8 mM) copper sulfate exhibited around 20% developmental loss in the larval stage and delayed the time to pupation by 10–18 h. In fact, the effect of copper was dependent on the concentration and exposure to concentrations higher than 2.5 mM for long periods (from egg to adult period) caused a drastic reduction in survival [7, 55].

Adequate food intake is essential for life and regulated by complex metabolic mechanisms and specific neural circuits [68, 69]. Our results indicated a severe effect of Cu^{2+} (0.4 to 1 mM) in larvae weight gain when compared to the control group. However, in the adults, only the two highest doses (0.75 and 1 mM of Cu^{2+}) caused a decrease in body weight. The loss of weight may be associated with an aversion to the diets containing a high content of copper or to a direct toxic effect of accumulated copper in critical metabolic processes [57, 70].

Copper toxicity can cause locomotor dysfunctions [57, 71–73] and the negative geotaxis can be used to determine locomotor deficits in flies [57, 74, 75]. The deleterious effects of Cu^{2+} in the locomotor ability of flies can be explained by copper-induced dysfunctions in the nervous system as observed in vertebrates [76–78]. The behavioral results obtained here are in agreement with previous studies from our laboratory with *D. melanogaster*, where exposure to 1 and 3 mM of $CuSO_4$ for 4 days caused a significant inhibition of total AChE activity and an impairment in the climbing ability (negative geotaxis) of adult flies. Here, we observed that both male and female flies exposed to 1 mM of $CuSO_4$ for 13 days had an impairment in climbing ability and inhibition of body AChE (abdomen + thorax), but not in the head. Since in the previous study [57], the activity was determined in the entire flies, we may suggest that the effects of Cu^{2+} in AChE in the

thorax + abdomen was more related to AChE inhibition in the ganglia than in the brain (head). The abdominal ganglia mediate the larval locomotion and are intrinsically associated with aminergic and cholinergic transmission [79]; consequently, an increase or a decrease in AChE can disrupt the locomotion and other behaviors of larvae and adult flies.

As discussed above, previous studies have indicated that climbing behavior can be impaired in flies exposed to 0.5–15 mM of copper [57, 80]. Here, in the adult flies, the effect of Cu^{2+} in the activity of AChE varied depending on the region used (head or bodies (thorax + abdomen)), on the concentration of $CuSO_4$, and on the sex of the flies. As a role, the intermediate concentrations of $CuSO_4$ increase the AChE activity in males' head and did not modify the head AChE activity in the females. Furthermore, the AChE activity in the females' head was higher than that of males. In contrast, the body (thorax + abdomen) AChE activity was higher in males than in females, and the enzyme was inhibited in both sexes, but with higher potency in males. The sexual and body region differences in enzyme activities may be related to sexually dimorphic responses to endogenous or xenobiotics stressors in flies [81–89], which have been linked to complex neurochemical, metabolic, and hormonal differences in male and female flies [81, 82, 86, 89].

The inhibition of AChE activity might be explained by copper-induced protein unfolding and, subsequently, wrong aggregation. In addition, metals have a capacity to bind to functional groups of proteins, such as imidazole, sulfhydryl, and carboxyl groups, leading to a decrease in AChE activity. Although the method of Elmann's is not appropriate to determine the in vitro effect of metals that can oxidized thiol groups [90], Cu^{2+} can interact and inhibit AChE directly [90]. Thus, the protein-metal interaction can compromise the AChE catalytic activity and, consequently, can cause stable enzyme dysfunction. However, the concentration of copper required to inhibit the AChE or ChE in vitro is high (10 and 20 mmol/L) and in vivo, the exposure to low concentrations (0.06 mg/L or $\approx$ 1 μM) of Cu^{2+} for 2 days caused an increase in AChE activity in fish [91]. Thus, after in vivo exposure to Cu^{2+}, the effects observed here in larvae and flies, as well as in previous studies with adult flies [57], were possibly indirect.

In relation to GST, the activity increased in larvae exposed to 0.1 mM Cu^{2+} but decrease in Cu^{2+} 1 mM. The inhibition of GST by metals may have occurred via the production of ROS that can interact directly with the enzyme, or can indirectly deplete its substrate (glutathione—GSH), and/or downregulation the expression of GST genes [92]. However, the GST activity increased in the heads of adult male flies, and in the bodies of males and females flies exposed to 1 mM Cu^{2+}, when compared to the control. In addition, female flies presented a higher activity in bodies GST when compared to males. Thus, in adults, copper may have induced the expression of GST genes. Similarly, studies performed in fish have

shown that glutathione *S*-transferase activity was significantly increased in the kidney, liver, and gills when exposed to copper nanoparticles (100 μg/L or ≈ 1.6 μM) when compared to control [93, 94]. In this view, *D. melanogaster*, such as humans, has orthologue glutaredoxins enzymes that regulate the redox activity on chaperones copper bound sites. The glutaredoxins enzymes, in accordance with our results, have shown a dose-dependent increase in activity and expression when exposed to copper [95].

Conclusion

In conclusion, the exposure of *D. melanogaster* to Cu^{2+} from the egg to the adult period disrupted the locomotor behavior of adults and enzymatic parameters in larvae and adults. In fact, the development was delayed in a dose-dependent manner. The weight of larvae and adult flies was decreased at the two highest doses of CuSO₄, and the locomotor activity was negatively affected by the highest dose tested. These effects may be related to the changes observed in AChE and GST activities. In short, further studies are necessary to investigate the mechanisms involved in copper-induced toxicity at specific stages of *D. melanogaster* development.

Compliance with Ethical Standards

Conflict of Interest   The authors declare that they have no conflict of interest.

Ethical Approval   All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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