Ecotoxicology (2021) 30:351–360
https://doi.org/10.1007/s10646-021-02358-w

Effect of long-term exposure to copper on survival and development of two successive generations of *Culex pipiens* (Diptera, Culicidae)

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Accepted: 25 January 2021 / Published online: 10 February 2021

Abstract

Aquatic invertebrates can be exposed to copper from various sources, including agricultural applications. For example, concentrations up to 1000 µg L^{-1} are found within rice fields, where copper-containing formulations are used as fungicides and algaecides. We conducted toxicity tests to study lethal and sublethal effects of copper sulfate pentahydrate on all immature stages across two generations of *Culex pipiens* mosquitoes as our model organism. Mortality was dose-dependent at concentrations of 500 µg L^{-1} and above in the first generation, and 125 µg L^{-1} and above in the second generation. The median lethal concentrations (LC_{50}) of copper sulfate pentahydrate for larval *Cx. pipiens* were 476 ± 30.60 µg L^{-1} and 348.67 ± 23.20 µg L^{-1} for the first and second generations, respectively. Generation one pupation decreased from 96% in controls to 48% at 500 µg L^{-1}, while the second-generation pupation decreased from 96% in controls to 17.5% at 500 µg L^{-1}. Mortality during the pupal stage varied from 2 to 10% at 500 µg L^{-1} of first and second generations, respectively. Higher levels also delayed development to adulthood in both generations. The duration of the immature period was 14.8 days in controls in both generations, but when exposed at 500 µg L^{-1} it increased to 18.8 days in the first generation and to 20.5 days in the second generation. The chronic, multi-generation exposures in this study showed greater toxicity than reported for shorter exposures of *Cx. pipiens*, and confamilial taxa like *Culex hortensis* and *Anopheles hispaniola*.

Keywords Aquatic insects · Chronic effects · Copper sulfate · Cross-generation effects · Mosquito

Introduction

All over the world, natural ecosystems have been adversely affected by anthropogenic activities (Dirilgen, 2001; Rajaganapathy et al., 2011; Benson et al., 2016). Industrialization, modern farming and increased vehicular use have led to elevated concentrations of heavy metals such as copper, cadmium, chromium, lead, nickel, and zinc throughout the biosphere (Farombi et al., 2007; Atafar et al., 2010; Rajaganapathy et al., 2011). These potentially toxic heavy metals become part of the biogeochemical cycle when they contaminate soil, air and water regularly (Lee et al., 2006; Byrne et al., 2012). Numerous researchers have examined effects of heavy metals on aquatic ecosystems (Dirilgen, 2001; El-Sheikh et al., 2010; Malaj et al., 2012), species diversity (Yan et al., 1996; Dutta et al., 2010), community productivity (Hill et al., 1997; Farombi et al., 2007) and animal behavior (Bonnard et al., 2009; Mogren & Trumble, 2010; Thomas et al., 2016). Despite many studies concerning the effect of metal pollutants and their physiological costs, there are gaps in knowledge about the sublethal and lethal effects of metal pollutants. For example, long-term and cross-generational studies are rare (Vedamanikam & Shazilli, 2008; Brix et al., 2011; Marinković et al., 2012; Ellis et al., 2020).

Insects are popularly used as bioindicators because the Class Insecta is the most diverse group of animals found in fresh water (Hare, 1992; El-Shenawy et al., 2010). Insects can accumulate metals depending on chemical speciation and free metal ion activity in which concentrations of metal in insects is often correlated with that of the environment (De Jonge et al., 2014). Heavy metals have great acute and chronic effects on various insects, such as morphological

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changes, growth inhibition, developmental abnormalities, decreased hatchability and reduced reproduction (e.g., Sildanchandra & Crane, 2000; El-Sheikh et al., 2010; Perez & Noriega, 2014; Goswami et al., 2019; Mebane et al., 2020). We focused on copper because it has played a significant role in reducing the abundance and altering the community composition of aquatic insects (Brix et al., 2011, Clements et al., 2013).

We chose the mosquito *Culex pipiens* (Culicidae: Diptera) as our test organism for two reasons. First, mosquitoes are one of few aquatic insects that can be cultured in the laboratory and so they are useful as indicator species. It is essential to determine the susceptibility to metal pollutants of insects relative to other taxa (Brix et al., 2011). *Cx. pipiens*, is distributed worldwide except for Australia and Antarctica (Farajollahi et al., 2011). It is an essential vector for periodic lymphatic filariasis (Farid et al., 2001), West Nile virus (Turell et al., 2001), St. Louis encephalitis (Mackenzie et al., 2002), Western equine encephalitis, Japanese encephalitis, and Rift Valley fever (Kramer & Ebel, 2003; Farajollahi et al., 2011). It frequently inhabits very small containers as well as larger habitats (Vinogradova, 2000; Vezzani & Albicocco, 2009).

Copper is one of many metals that *Cx. pipiens* are exposed to in their environment. Copper is one of the trace metals that are important for functions of many enzymes like hemocyanin, cytochrome oxidase and monoamine oxidase but it could cause environmental impacts when it used in excessive amounts (Scheiber et al., 2014). Copper may enter water when used as a biocide in antifouling paint formulations or from agricultural and urban application sites. It is widely used as a fungicide, as an algaecide and as a molluscicide to control leeches and tadpole shrimps in rice farming, and for root treatment of various crops (Bishop et al., 2014; Wagner et al., 2017; Lusk & Chapman, 2020). Copper sulfate is often used as algaecide with the rate of 1000–1500 µg L^{-1} in freshwater systems (Houmann, 2015). Copper is used in both conventional and organic agriculture, and poses a risk to ecosystems (Goswami et al., 2019). Copper affects the nervous systems of aquatic organisms (Viarengo & Nicotera, 1991; Tilton et al., 2011; Thomas et al., 2016), insect metabolism (Bagatto & Shorthouse, 1996; Joachim et al., 2017; Ilahi et al., 2020) and it impacts non-target organisms, resulting in environmental concerns (Bishop et al., 2014; Rothmeier et al., 2020). Additionally, copper can be used to clarify main ecotoxicological concepts like biodynamics, lethality and sublethal effects (Luoma & Rainbow, 2005; Clements et al., 2013; Goswami et al., 2019).

This study aims to investigate long-term effects of copper exposure on instar development and adult emergence of *Cx. pipiens* across two successive generations. Due to copper's known toxicity, we hypothesized that long-term elevated copper exposure would decrease larval survival, growth rate, and development to pupal and adult stages. In addition, we hypothesized a lower sensitivity of *Cx. pipiens* in subsequent generations, due to the loss of susceptible individuals and genetic adaptation.

Materials and methods

Insect collection

We obtained *Cx. pipiens* larvae from the Sacramento-Yolo Mosquito and Vector Control District (SYMVCD; www.fightthebitenet), and reared them through adulthood to produce first instar larvae for exposures. This laboratory population was initially obtained from Merced, CA., sometime in the 1950s, and has been maintained as a "susceptible" strain, unexposed to toxicants. While inbreeding is likely, the insects appear robust and normal. Such strains are used routinely in environmental toxicology as a precautionary approach. A domesticated strain was also the best choice for a two generations study because field populations may not breed in the lab. Field caught populations have additional issues associated with them such as epigenetic modifications and potential localized pesticide resistance. Moreover, standard mosquito strains are used in many laboratories in the world because they are more experimentally reproducible than field strains (Bonizzoni et al., 2012; Costa-Da-Silva et al., 2017). The strain we used is maintained by several California Mosquito and Vector Control Districts, which rear the insects in large colonies of hundreds to thousands. Districts exchange specimens every few years to maintain colony vigor. Due to the ubiquity of pesticide-resistance genes in wild mosquito populations outcrossing with wild mosquitoes has not been possible in many years (Personal communication, Deborah Dritz, Sacramento-Yolo Mosquito and Vector Abatement District, Elk Grove, CA). SYMVCD reared larvae and adults in an environmental chamber at 25 ± 0.5 °C and ~80% humidity, with 18:6 h day/night cycle. We followed SYMVCD's general rearing technique. Larvae were reared in enamel trays (40 × 25 cm) filled with ~1 L reconstituted deionized water (RDiW). The reconstituted deionized water consisted of 0.096 g NaHCO3, 0.06 g $MgSO_4$, 0.004 g KCl, and 0.06 g $CaSO_4.2H_2O$ dissolved in 1 L deionized water mixed according to (Weber, 1991). Larval diet stock proportions consisted of 5 g ground fish food (Tetramin®), 5 g ground and sifted alfalfa, ½ g of nutritional yeast and ½ g of ground beef liver. About 80% of rearing water was renewed once a week. We transferred pupae into cups filled with RDiW water and kept them in cages with dimensions of (30 × 30 × 30 cm) until adult emergence. Each cage was provided with a piece of sponge soaked in 10% sugar solution. After 4 or

5 days, heparinized sheep blood meal (Hemostat Laboratories, Dixon, CA) was made available to females to feed from via a small beaker covered with thin parafilm, inverted and placed on the top of the cage. We provided oviposition cups filled with RDiW water in each cage to receive F1 egg rafts.

Copper solution

Copper sulfate pentahydrate ($CuSO_4.5H_2O$) was obtained from Sigma Aldrich, CA; purity: 99.9%. We prepared a stock solution of $10\,g\,L^{-1}$ by weighing 0.1 g of $CuSO_4.5H_2O$, putting it in a volumetric flask, and adding RDiW water to a volume of 10 mL and mixing well. From this stock solution, we prepared four test concentrations: 125, 250, 500 and $1000\,\mu g\,L^{-1}$ in RDiW in addition to controls. We chose these concentrations to be under the human safe limit of copper ($1300\,\mu g\,L^{-1}$) in fresh water (Fitzgerald, 1998; Potera, 2004). On the first day of the experiment, 30 mL of newly prepared control water and each concentration were saved in plastic Falcon© tubes at 4 °C for chemical analysis. Nominal concentrations of $CuSO_4.5H_2O$ were designed to provide equivalent concentrations of Cu^{+2} of 0, 31, 63, 127, $254\,\mu g\,L^{-1}$ which were measured and confirmed using an ICP-MS instrument (Interdisciplinary Center for Plasma Mass Spectrometry, UC Davis) to be 0 ± 0.001, 28 ± 0.002, 69 ± 0.008, 129 ± 0.013 and $335 \pm 0.031\,\mu g\,L^{-1}$ respectively under the following liquid sample digestion procedure. A volume of 120 µL concentration trace metals grade HNO_3 was added to 1.5 mL of each sample (suffix-D), then samples were introduced to a hot block at 95 °C for ½ h. A volume of 75 µL 30 % H_2O_2 was added incrementally to hot samples (25 and 50 µL was added over 5 min) and heating was continued for an additional 1 h, before removal from the hot block. Samples were allowed to cool, capped, centrifuged and brought up to a final volume (FV) = 1.5 mL with MQW. Final matrix = 8% HNO_3.

Effects of copper exposure on *Cx. pipiens* development and emergence

We conducted toxicity tests according to (El-Sheikh et al., 2010) under laboratory controlled conditions of 25 ± 2 °C and a 18:6 light: dark photoperiod, as described for rearing conditions. The study consisted of five replicates for controls and each concentration of the first generation, while the second generation consisted of five replicates only for controls and $125\,\mu g\,L^{-1}$, and only four replicates for higher concentrations, due to fewer animals available. Each replicate was a polyethylene plastic cup containing 100 mL of copper solution and ten first instar larvae (<24 h); plastic is recommended for such tests because most metals do not bind to it (Nollet et al., 2000). We initially added 0.002 g of larval food to each container, increasing the amount to 0.004 g as larvae grew. We renewed 90% of the solution every 48 h. Each day we recorded development (instar/stage), at which time mortality was also recorded and any dead organisms removed from the vessels (Hasenbein et al., 2015). Emerged adults from the first generation were kept in cages with dimensions of ($30 \times 30 \times 30$ cm; described above) to continue with the second-generation toxicity experiment. We combined the adults from the replicates within treatments, for one cage per treatment. Sponges soaked in 10% sugar solution were provided in each cage. After 4 or 5 days, females were allowed to take heparinized sheep blood meal and we provided oviposition cups. Egg rafts resulting from control and each concentration except $1000\,\mu g\,L^{-1}$ were collected separately and transferred to rearing trays without tracking of their productivity. Each tray contained not more than three egg rafts. Hatchling larvae were then chosen randomly from each tray and used in the second-generation experiment, conducted as described.

Statistics

Response variables were larval mortality, accumulated mortality through adult emergence, and complete immature period (days). Histograms and QQ-plots were used to check the normality of the data. Almost none of the response variables met the normality assumption. We could not find any transformation that normalized the data after trying several transformations. We conducted Kruskal–Wallis one-way analysis of variance (ANOVA) tests on larval mortality, accumulated mortality through adult emergence and development time. For post-hoc tests we used Dunn's Test as a non-parametric multiple comparison method using rank sums, which controls error rate for multiple comparisons within response variables. While this does not adjust the experiment-wise alpha level, it strikes a balance between Type I and II error rates and is further justified because all tests were based on the a priori hypotheses that increasing copper sulfate exposures would have negative effects on mosquito growth and development. We used probit analysis using Minitab program to determine median lethal concentrations (LC_{50}) of the total larval period for each *Cx. pipiens* generation with 95% confidence intervals. Lifetime survival was estimated using the non-parametric Kaplan-Meier survival curve. All analyses were carried out using Minitab program.

Results

Expected and measured Cu^{2+} concentrations were usually very similar (Table 1). Therefore, for simplicity we will

Table 1 Nominal, expected and measured copper (Cu) concentrations in different CuSO4.5H2O concentrations

Nominal $CuSO_4.5H_2O$ ($\mu g\ L^{-1}$)	Expected dissolved Cu (Nominal, $\mu g\ L^{-1}$)	Measured dissolved Cu ($\mu g\ L^{-1}$)
0	0	1.2 ± 0.000
125	31.8	28.4 ± 0.002
250	63.6	69.8 ± 0.008
500	127	129.3 ± 0.013
1000	254	335.8 ± 0.031

present results in terms of the nominal concentrations of $CuSO_4.5H_2O$.

The first generation showed a sharp increase in larval mortality ($H(\chi^2) = 19.90$, $p = 0.001$*, df = 4) from an average of 4% in controls to 100% at the highest concentration of 1000 µg L^{-1} (Fig. 1). Mortality at 1000 µg L^{-1} was significantly greater than all treatments except 500 µg L^{-1} (Table 2). We calculated the LC_{50} of the first generation's larvae to be 476 ± 30.60 µg L^{-1} (421.99–546.06 µg L^{-1}). The accumulated mortality through adult emergence increased significantly very similar to larval mortality ($H(\chi^2) = 19.90$, $p = 0.001$*, df = 4) from 94% in controls to zero at 1000 µg L^{-1} and 54% at 500 µg L^{-1} (Fig. 1). There was a non-significant pupal mortality of 2% at 125 and 500 µg L^{-1} of this generation. The effect of concentration of 500 µg L^{-1} was significantly different from all other treatments except for 250 µg L^{-1} (Table 3). Copper also delayed development ($H(\chi^2) = 11.68$, $p = 0.0128$*, df = 4): surviving controls all emerged by day 16 whereas at least some larvae exposed to 1000 µg L^{-1} were still in the larval stage by days 20–22, although none of these pupated (Fig. 1). The effect of concentrations 250 and 500 µg L^{-1} also showed non-significant trends toward developmental delay (both p ~0.10; Fig. 1 and Table 4).

The second generation showed notably larger changes in larval mortality ($H(\chi^2) = 13.61$, $p = 0.003$*, df = 3) with copper exposure. Control and 125 µg L^{-1} mortalities were again about 4% but rose to 82.5% at 500 µg L^{-1} (Table 2 and Fig. 2). The LC_{50} level was substantially lower than in the first generation at 348.67 ± 23.20 µg L^{-1} (306.52–400.85 µg L^{-1}). Both controls started to pupate at day 8. The second-generation cumulative mortality through adult emergence decreased significantly ($H(\chi^2) = 13.96$, $p = 0.003$*, df = 3) from 96% in controls to 7.5% at 500 µg L^{-1}. There was a non-significant pupal mortality of 10% at 500 µg L^{-1} of this generation. The development delays of the second generation were higher than that of the first (Fig. 1 and 2). There were significant increases ($H(\chi^2) = 13.31$, $p = 0.0029$*, df = 3) in the immature period at all concentrations tested, of up to ~4–5 days compared to controls (Table 4 and Fig. 2). We caution that two false positives might be expected among the post-hoc contrasts presented above. However, 16 contrasts were significant, and all were in expected directions.

Discussion

Multi-generation toxicology studies are valuable in revealing whether tolerance increases or decreases across generations (Vedamanikam & Shazilli, 2008; Marinković et al., 2012; Jegede et al., 2019; Ellis et al., 2020) depending on the nature of maternal effects or selection. However, our study could not distinguish between selection and maternal effects, and selection on a laboratory strain might not reflect what could happen with field strains (Marinković et al., 2012). In contrast to our hypothesis that effects would decrease across generations due to genetic adaptation and loss of susceptible individuals, we found increased sensitivity effects across generations. In the first generation, 48% of larvae survived at 500 µg L^{-1}. In the second-generation bred from parents that were exposed to copper during their immature stages, only 17.5% of larvae survived a 500 µg L^{-1} exposure. Reza & Ilmiawati (2020) also demonstrated the susceptibility of *Cx. pipiens* to copper in a one generation study. This might be due to copper bioaccumulation within insect tissue. They argued these findings were due to the impact of copper on the commensal bacteria in the larval midgut. In our study, the increase in sensitivity of the second generation may be due to longer-term exposure of the immature stages at the second generation than those of the first one. Phenotypic plasticity is another mechanism that could change sensitivity across generations (Marinković et al., 2012). Reza & Ilmiawati (2020) postulated the susceptibility was due to intestinal dysfunction (Matsuoka et al., 2015) that reduced the larva's ability to produce energy to complete its development. To our knowledge, this is the first study quantifying the effects of copper on the survival and development of the widespread mosquito *Cx. pipiens* across two successive generations.

Chronic exposure to copper at 1000 µg L^{-1} resulted in 100% mortality of developing, first generation, *Cx. pipiens* mosquitoes. This is an approved application rate for controlling algae in rice fields (Epstein & Bassein, 2003; Stevens et al., 2014). This suggests that mosquito control may not be necessary immediately after algaecide treatment. However, further field tests are advisable because sensitivities of wild mosquitoes may differ. Reported sensitivity to pollutants, including copper exposure, varies across mosquito species. Our study species, *Cx. pipiens*, is considered to be somewhat tolerant of poor water conditions (Vinogradova, 2000). Members of its species complex can occur in waters contaminated with copper from industrial, domestic, and agricultural sources, and this has the potential to affect breeding success (Ilahi et al., 2020). *Cx. pipiens* are

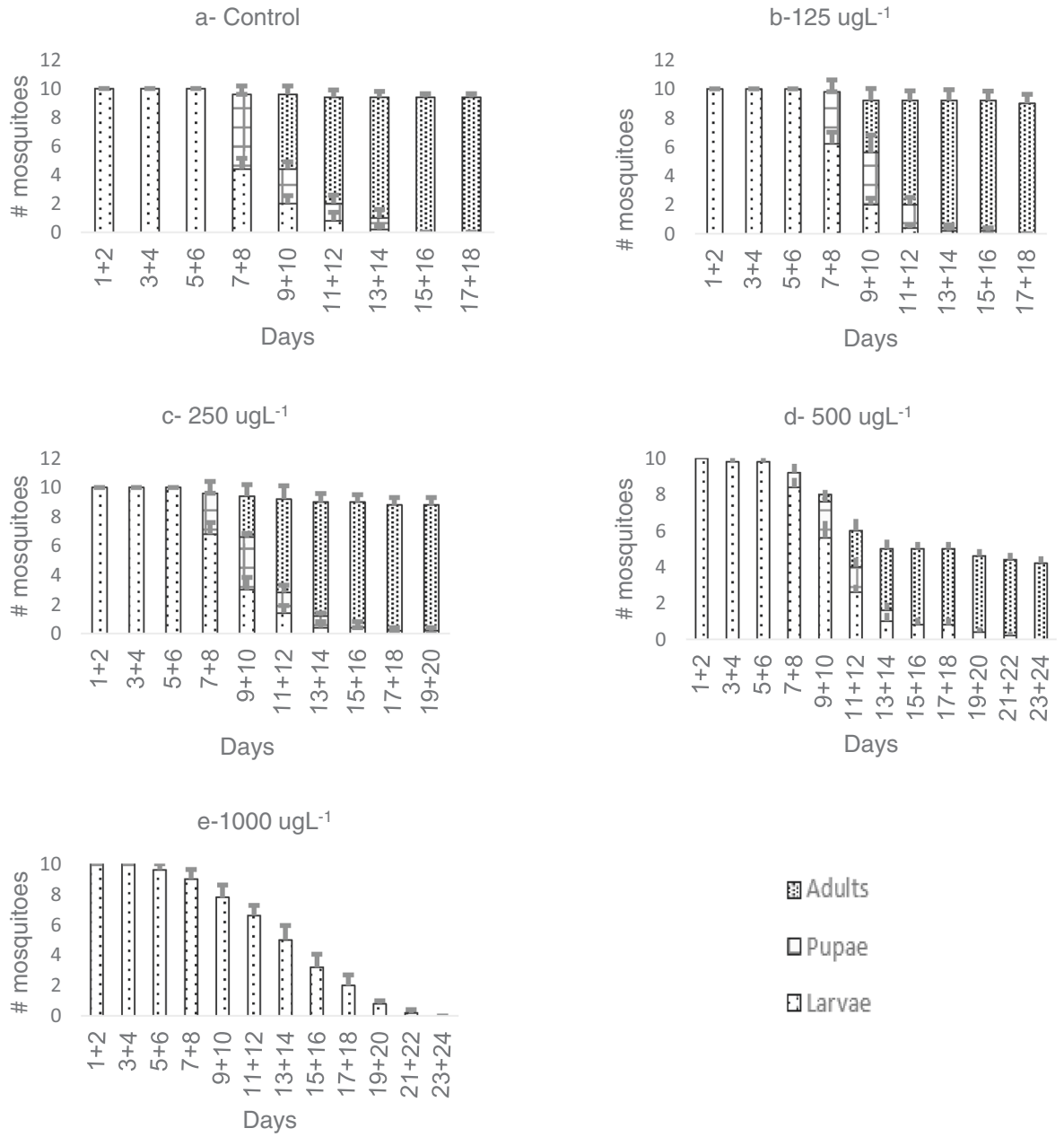

Fig. 1 Effect of long-term exposure to different concentrations of copper sulfate pentahydrate ($CuSO_4.5H_2O$) on the larval and pupal development time, and adult emergence of the first generation of *C. pipiens* (mean ± SE). **a** Control, **b** 125 μg L^{-1}, **c** 250 μg L^{-1}, **d** 500 μg L^{-1} and **e** 1000 μg L^{-1}

Table 2 *p* value of multiple comparisons—Dunn's test: treatment effects of different concentrations of $CuSO_4.5H_2O$ (unexposed control, 125, 250, 500 and 1000 μg L^{-1}) on percentage larval mortality during two successive generations of *C. pipiens* mosquitoes in laboratory tests

First generation: % larval mortality					Second generation: % larval mortality			
μg L^{-1}	0	125	250	500	μg L^{-1}	0	125	250
125	0.7290				125	1.0000		
250	0.4051	0.4051			250	0.0699	0.699	
500	0.0097[a]	0.0254[a]	0.0796		500	0.0018[a]	0.0018[a]	0.2194
1000	0.0002[a]	0.0008[a]	0.0044[a]	0.2733				

[a]Meets $p \leq 0.05$ significance level

Table 3 p value of multiple comparisons—Dunn's test: treatment effects of different concentrations of $CuSO_4.5H_2O$ (unexposed control, 125, 250, 500 and 1000 µg L^{-1}) on total mortality through adult emergence during two successive generations of *C. pipiens* mosquitoes in laboratory tests

First generation: mortality through adult emergence					Second generation: mortality through adult emergence			
µg L^{-1}	0	125	250	500	µg L^{-1}	0	125	250
125	0.6621				125	0.8556		
250	0.4982	0.8101			250	0.0468[a]	0.0694	
500	0.0105[a]	0.0340[a]	0.6026		500	0.0013[a]	0.0024[a]	0.2489
1000	0.0002[a]	0.0013[a]	0.0029[a]	0.2731				

[a]Meets $p \le 0.05$ significance level

Table 4 p value of multiple comparisons—Dunn's test: treatment effects of different concentrations of $CuSO_4.5H_2O$ (unexposed control, 125, 250, 500 and 1000 µg L^{-1}) on complete immature period during two successive generations of *C. pipiens* mosquitoes in laboratory tests

First generation: complete immature period					Second generation: complete immature period			
µg L^{-1}	0	125	250	500	µg L^{-1}	0	125	250
125	0.5801				125	0.0059[a]		
250	0.1016	0.2783			250	0.0254[a]	0.7186	
500	0.0730	0.2153	0.8769		500	0.0031[a]	0.7186[a]	0.4941
1000	0.0040[a]	0.0201[a]	0.2153	0.2783				

[a]Meets $p \le 0.05$ significance level

relatively resistant to insecticides such as deltamethrin, lambda-cyhalothrin, beta-cyfluthrin and bifenthrin (Al-Sarar, 2010; Ghorbani et al., 2018), so negative effects on this taxon are worrying for other taxa as well.

The LC_{50} levels determined in this study were 476 ± 30.60 µg L^{-1} (421.99–546.06 µg L^{-1}) and 348.67 ± 23.20 µg L^{-1} (306.52–400.85 µg L^{-1}) for the first and second generations respectively in which larvae have been exposed from the first instar. Another unigenerational study estimated the LC_{50} of *Cx. pipiens* toward copper to be 300 µg L^{-1} (Reza & Ilmiawati, 2020), which might be lower because they observed larval mortality until day 22 only. This is lower than previously reported in this and other mosquito species, however, most other exposure tests were shorter (24–96 h) (e.g., Bouallam & Nejmeddine, 2001; Mireji et al., 2010; Perez & Noriega, 2014). In our study most mortality occurred after 96 h. Although 24–96 h tests are standard, total mortality throughout the immature stages is also of interest.

The contrast in sensitivities to copper exposure across semiaquatic invertebrates is broad (Bouallam & Nejmeddine, 2001). Although there are few exposure tests lasting most of larval development, some tests conducted longer than 24 h also show lower effect concentrations. Since our larvae have been exposed from the first instar, they are expected to be more sensitive than those exposed at later stages. In a study determining LC_{50} levels of $CuSO_4$ for chironomid fly larvae *Chironomus ramosus*, third instars were exposed to $CuSO_4$ for 24, 48, 72 and 96 h which resulted in LC_{50} of 3280,1073, 780 and 1830 µg L^{-1} respectively (Majumdar & Gupta, 2012). A study on *Cx. pipiens* collected from the field and reared for several generations in the lab indicated that the LC_{50} of larval stage (starting from the second instar) for $CuSO_4$ was 5090 µg L^{-1} (El-Sheikh et al., 2010). Bouallam & Nejmeddine (2001) studied the sensitivity of *Cx. pipiens*, *Cx. hortensis* and *Anopheles hispaniola* to $CuSO_4$ in an experimental wastewater lagoon using 24 h exposure of the fourth larval stage, finding LC_{50} of 5910, 5020 and 3780 µg L^{-1} respectively. Another mosquito, *Aedes aegypti*, had an intermediate LC50 of 2060 µg L^{-1} in 24 h trials in the third instar (Rockefeller strain; Perez & Noriega, 2014). However, that species' response is variable; Rayms-Keller et al. (1998) found an LC_{50} for 24 h of the third instar of the Puerto Rico strain of *Aedes aegypti* of 33000 µg L^{-1}. Differences between studies could also be due to stage, source localities or to the number of generations that the organisms were maintained in the laboratory

To put these dipteran studies in a broader context, a review showed that among aquatic insects, the insect orders most tolerant to copper may be Trichoptera and the suborder Zygoptera, while the most sensitive orders may be Ephemeroptera, Diptera and the suborder Heteroptera (Malaj et al., 2012). However, considerable variation may be found at the species level. Similarly, Brix et al. (2011) found that Trichoptera and Plecoptera were less sensitive than Ephemeroptera and Diptera (mainly chironomids). Studies concerning the effect of Cu on non-dipteran aquatic insects, including field studies (Schmidt et al., 2010) and microcosm experiments (Clark & Clements, 2006; Clements, 2004) showed greater sensitivities of mayflies. Brinkman & Johnston (2008) found that the mayfly *Rhithrogena hageni* had an LC_{50} of waterborne Cu at 96 h of 137 µg L^{-1}. Although a review by Iwasaki & Ormerod

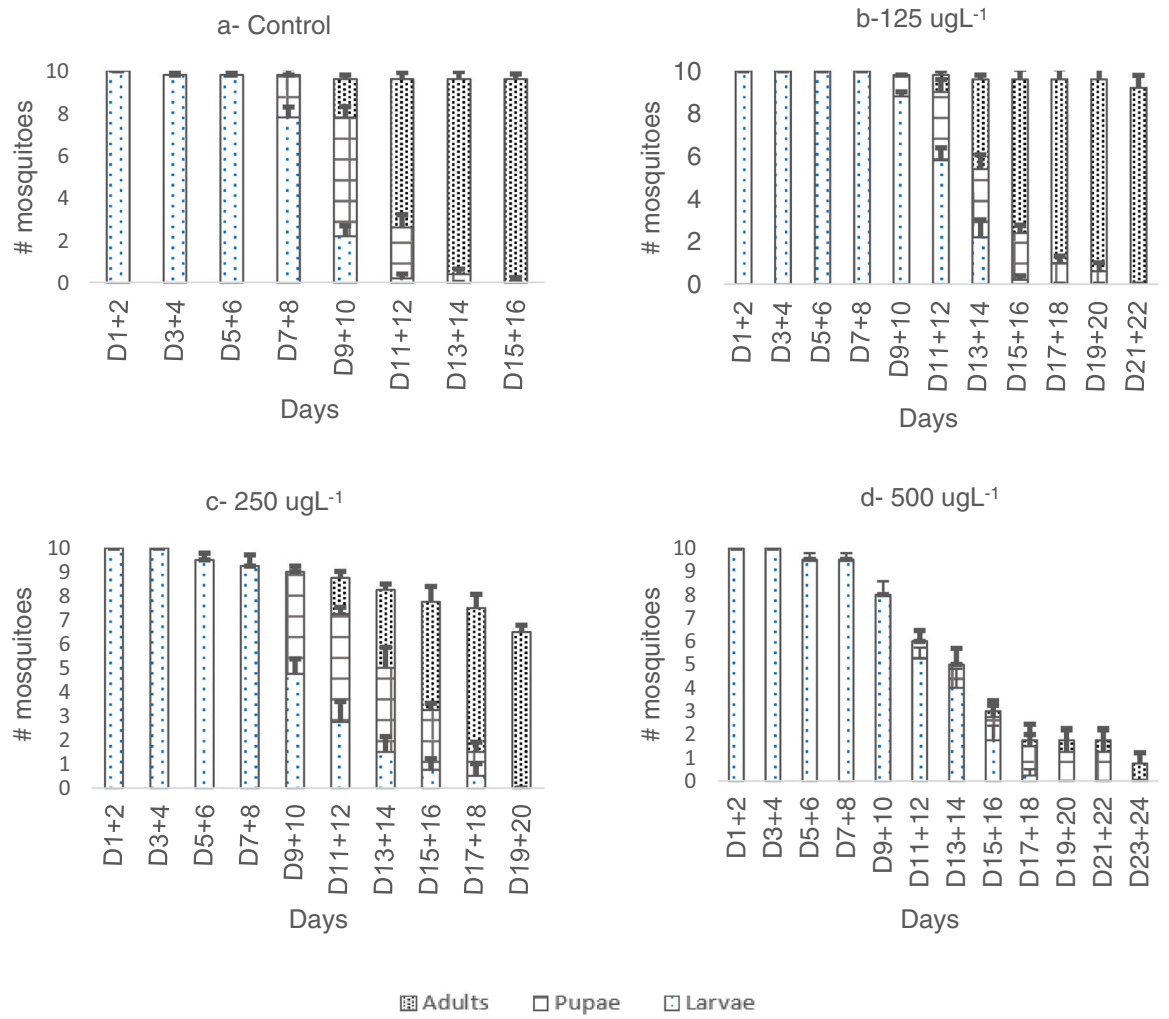

Fig. 2 Effect of long-term exposure to different concentrations of copper sulfate pentahydrate ($CuSO_4.5H_2O$) on the larval and pupal development time, and adult emergence of the second-generation *C. pipiens* (mean ± SE). **a** Control, **b** 125 µg L^{-1}, **c** 250 µg L^{-1} and **d** 500 µg L^{-1}

(2012) suggested that 6.6 µg L^{-1} was generally safe, it is useful to know sensitivities of different groups to inform how food webs might change with greater exposures.

In addition to causing larval mortality, copper delayed mosquito development and reduced adult emergence, both of which are relevant to mosquito abatement. Developmental delay exposes immature mosquitoes to other sources of mortality such as predation (if predators are not similarly impacted by copper), and pathogen transmission could be reduced if there are fewer and smaller generations of adults. In the first generation almost all control mosquitoes had pupated by day 14, but at concentration of 500 µg L^{-1} and above at least some larvae remained as larvae past day 20 (Fig. 1d). Generation two larvae were more sensitive to copper exposure, with higher developmental delays and much greater losses in emerged adults (Fig. 2). Less than 10% emerged as adults at the highest concentration 500 µg L^{-1} of the second generation (Fig. 2d). Other studies have also found developmental delays in dipterans. In a study of the effect of copper sulfate pentahydrates on *Aedes aegypti*, larval development period was 17.38 days versus 5.95 days for controls (Perez & Noriega, 2014). Another study on *Culex pipiens*, *Aedes aegyptae* and *Anopheles stephensi* demonstrated that copper prolonged pupation time and adult emergence (Reza & Ilmiawati, 2020). Also at 1.8 µg L^{-1} $CuSO_4$ concentration, pupation of the *Chironomus ramosus* fly was delayed (Majumdar & Gupta, 2012). In contrast, there were few and variable sublethal effect of copper on nine generations of *Chironomus riparius* in which copper concentrations were 10–30 mg Cu/kg dw of sediment (Marinković et al., 2012).

We conducted this study using copper sulfate pentahydrate due to its broad use. Copper is one of the highly impactful heavy metals to aquatic organisms and ecosystems. Sensitive insects will be affected at exposures less than some current application rates, which could affect the

equilibrium and the composition of the aquatic community. Impacts on mosquito numbers and development rates may be beneficial to mosquito abatement. However, when viewed as a model organism, the high toxicity of copper to a relatively pollution-tolerant mosquito is worrying. This study filled an information gap concerning long-term exposure and cumulative effects across generations. Our results revealed that current rates of application for some purposes may not be protective of aquatic insects. In addition, we found higher sensitivity of the second generation of *Cx. pipiens* relative to the first one, and also revealed that copper caused a developmental delay.

Acknowledgements We thank the biostatistical consultant team Xingmei Lou, Zihan Xiao, and Jiahui Li for their help in conducting an initial statistical analysis under the supervision of Wang Jane-Ling and Cody Carroll. We are also very grateful to Deborah Dritz, Sacramento-Yolo Mosquito, and Vector Abatement District for providing us with mosquito larvae. We would like to express our special thanks to Austin Cole, UC Davis Interdisciplinary Center for Plasma Mass Spectrometry (UCD/ICPMS) who conducted the chemical analysis and to trained N. Raffat in his lab.

Funding This research was supported by the Egyptian Cultural and Educational Bureau, Washington DC.

Author contributions All authors contributed to the study conception and design. Material preparation and data collection were performed by NRA. NRA, SPL, and REC contributed to data analysis. The first draft of the manuscript was written by NRA. All authors reviewed at least one draft of the manuscript and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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