THE DIRECT AND INDIRECT EFFECTS OF COPPER ON VECTOR-BORNE DISEASE DYNAMICS

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Abstract

Metal pollution is a growing concern that affects the health of humans and animals globally. Copper is an essential insect micronutrient required for respiration, pigmentation and oxidative stress protection but can also act as a potentially toxic trace element. While several studies have focused on the negative fitness effects of copper on the aquatic larvae of mosquitoes, the effects of larval copper exposure on adult mosquito fitness (i.e., survival and fecundity) and their ability to transmit parasites (i.e., vector competence) remains unclear. Here, using a well-studied model vector-parasite system, the mosquito *Aedes aegypti* and parasite *Dirofilaria immitis*, we show that sublethal copper exposure in larval mosquitoes alters adult female fecundity and vector competence. Specifically, mosquitoes exposed to copper had a hormetic fecundity response and mosquitoes exposed to 600 µg/L of copper had significantly fewer infective parasite larvae than control mosquitoes not exposed to copper. Thus, exposure of mosquito larvae to copper levels far below EPA-mandated safe drinking water limits (1300 µg/L) can impact vector-borne disease dynamics not only by reducing mosquito abundance (through increased larval mortality), but also by reducing parasite transmission risk. Our results also demonstrated that larval copper is retained through metamorphosis to adulthood in mosquitoes, indicating that these insects could

transfer copper from aquatic to terrestrial foodwebs, especially in urban areas where they are abundant. To our knowledge this is the first study to directly link metal exposure with vector competence (i.e., ability to transmit parasites) in any vector-parasite system. Additionally, it also demonstrates unequivocally that mosquitoes can transfer contaminants from aquatic to terrestrial ecosystems. These results have broad implications for public health because they directly linking contaminants and vector-borne disease dynamics, as well as linking mosquitoes and contaminant dynamics.

Keywords: *Aedes aegypti*; *Dirofilaria immitis*; Copper; Vectorial capacity; Vector competence

Introduction

Metal pollution is an important issue at local and global scales (Driscoll et al., 2013; Islam et al., 2015). Metals can be released into the environment through natural (e.g., geological activity and erosion; Driscoll et al., 2013) or through anthropogenic activity (e.g., industrial; Runck, 2007). Anthropogenic sources are of grave concern due to the release of metal pollutants in more toxic (e.g., Copper(II) Oxide Nanoparticles; Angelé-Martinez et al., 2017) and mobile forms (e.g., Cu (II) complexes; Stohs and Bagchi, 1995). These metal pollutants can have negative consequences at the individual (e.g., behavior, survival, fecundity; Rayms-Keller et al., 1998; Neff et al., 2019) and ecosystem (e.g., aquatic-terrestrial productivity; Kraus, 2019) levels. Aquatic ecosystems are especially vulnerable to metal pollution due to industrial and urban runoff, as well as domestic wastewater and effluent discharge (Sarkar et al., 2004; Barrera et al., 2008; Mireji et al., 2008; Ruchter and Sures, 2015; Schertzinger et al., 2018).

Metal pollution in aquatic systems can have important negative consequences at various levels of biological organization. At the ecosystem scale metal pollution can negatively impact both biodiversity and biomass (Carlisle and Clements, 2003; Kraus et al., 2014a). Alternatively, at the individual scale exposure to metals during aquatic development could have lethal (e.g., increase larval mortality; Rayms-Keller et al., 1998; Sheikh et al., 2010; Reza and Ilmiawati, 2020) and sublethal consequences to organisms (e.g., reduced reproduction; Mireji et al., 2010; Perez and Noriega, 2012; Neff et al., 2019) at the adult stage. Aquatic insects have been long recognized as good bioindicators for metal pollution because of their relatively short life span and ease of sampling (Crampton et al., 1997; Batzer et al., 1999; Day, 2016; Erasmus et al., 2020). Research on aquatic insects has primarily focused on the effects of contaminants on aquatic life-history stages (e.g., development rates) (Marinković et al., 2011; Perez and Noriega,

2014; Debecker et al., 2017; Oliver and Brooke, 2018). However, many insects – such as mosquitoes – have semi-aquatic life cycles with aquatic juvenile stages and terrestrial adult stages, and such life history transitions could affect contaminant cycling in natural food webs (Kraus et al., 2014a; Kraus et al., 2014b; Kraus, 2019). For example, aquatic and terrestrial invertebrates play a substantial role of energetic provision to insectivores in streams and riparian zones (Baxter et al., 2005), and emerging adult insects could transfer contaminants from aquatic to terrestrial systems (Sullivan and Rodewald, 2012).

More than 80% of the world is at risk of vector-borne disease (Golding et al., 2015), and mosquitoes are recognized as a global public health threat because they are an important contributor to vector-borne disease transmission globally (WHO, 2017; Leta et al., 2018). Mosquitoes can also be convenient bioindicators of metal contamination because early development is completely aquatic (Day, 2016), they are ubiquitous and easy to sample especially in urban areas (Frankie and Ehler, 1978; Little et al., 2017) , and have a short generation time (14-21 days; Crampton et al., 1997). Previous research has revealed exposure to metals during aquatic development stages can affect adult mosquito fitness directly (e.g., reduced fecundity; Mireji et al., 2010; Perez and Noriega, 2014) and indirectly (e.g., altered behavior; Neff et al., 2019). However, the effects of exposure to metals, such as copper, on vector-borne disease transmission dynamics of diseases remains unknown (Rivera-Perez et al., 2017). We expect that metal exposure could affect mosquito-borne disease transmission dynamics by impacting two critical aspects affecting vectorial capacity (the ability of mosquitoes to transmit parasites): the likelihood of the vector surviving to the extrinsic incubation period (i.e., until the parasite becomes infective) and the likelihood of an immature parasite developing to its infective stage (vector competence) (Kartman, 1954).

79 The overarching goal of this study was to elucidate how exposure of mosquito larvae to
80 sublethal metal concentrations affect mosquito metal accumulation, affect adult mosquito fitness
81 (mortality and reproduction), and vectorial capacity. Copper is an essential micronutrient in
82 insects, and was used for this study due to its long use as an effective algicide, fungicide, and
83 insecticide (Bellini et al., 1998; Romi et al., 2000) and its key role as a component of enzymes
84 associated with respiration (Muttkowski, 1921), pigmentation (Sugumaran and Barek, 2016),
85 immunity (Christensen et al., 2005; Lu et al., 2014), metal homeostasis and detoxification (e.g.,
86 metallothionein; Amiard et al., 2006; Perez and Noriega, 2014; Rivera-Perez et al., 2017).
87 Additionally, copper physiology can also serve as an effective model for other metals that are
88 also detoxified via metallothionein binding (e.g., mercury, silver, zinc and cadmium) (Rivera-
89 Perez et al., 2017) and other metals that tend to be co-correlated in aquatic environments (Sarkar
90 et al., 2004; Ruchter and Sures, 2015).

91 We used the yellow fever mosquito (*Aedes aegypti*) and the dog heartworm (*Dirofilaria*
92 *immitis*) as the model vector-parasite system. *Aedes aegypti,* a common urban mosquito (Jeffrey
93 and Walter, 2013), is responsible for vectoring numerous human pathogens such as Zika,
94 Dengue, Chikungunya Viruses (Marchette et al., 1969; Alto et al., 2008; Weaver et al., 2018) and
95 a natural vector of *D. immitis* (Bowman and Atkins, 2009). This system is ideal to study the
96 interactive effects of metals and parasites on mosquito fitness because *D. immitis* exerts a strong
97 selective pressure on the fitness of *Aedes* mosquitoes (i.e. parasite-mediated mortality; Serrão et
98 al., 2001 Ledesma and Harrington, 2015; Dharmarajan et al., 2019). The major objectives of this
99 study were to: (1) Test the effects of copper on larval development; (2) Test if copper
100 accumulated during larval development is retained through metamorphosis to adults; (3) Test the

effects of sublethal copper exposure during larval development on adult female survival and fecundity; (4) Test if copper exposure impacts mosquito vector competence.

Materials and Methods

Study system

The mosquito used for investigation was the *A. aegypti,* Liverpool Blackeye strain, a model system for *Aedes* sp. research (Buxton and Mullen, 1981; Dharmarajan et al., 2019). These lab strain mosquitoes are a well-established vector system, wherein infection dynamics have been well characterized (Palmer et al., 1986; Mary et al., 2005; Ariani et al., 2015) and allow easy experimental repeatability (Perez and Noriega, 2012; Perez and Noriega, 2014). *Aedes aegypti* females become infected with dog heartworm, *D. immitis*, when an uninfected mosquito acquires a blood meal from an infected canine. The microfilaria (mf) ingested by the mosquito during a blood meal (Bowman and Atkins, 2009; Evans, 2011) enter the mosquito midgut, and subsequently migrate to the Malpighian tubules. Within the cells of the Malpighian tubules the mf develops into first stage larvae (L1) and will molt two additional times to reach their infective L3 stage. The L3 emerge from the Malpighian tubules, and travel to the head and proboscis of the mosquito, and are thus able to continue their lifecycle if the mosquito bites a susceptible vertebrate host. The development time in the mosquito for dog heartworm is the extrinsic incubation period (EIP), which averages 14 days post-infection in an optimal environment (McCall, 1981).

Mosquito maintenance

Aedes aegypti acquired from the Filariasis Research Reagent Resource Center (FR3) (Michalski et al., 2011), were lab reared in an environmental chamber with a 12:12-hour light diurnal cycle with conditions set to 27 °C and 80 $\pm$ 5% relative humidity. The *A. aegypti* life cycle takes eight

days to develop from egg to adult (Crampton et al., 1997). Mosquito eggs were hatched from egg laden paper towels in a pan (35.6 cm × 28.3 cm × 21.9 cm) of deionized water (2 L) and 500 mg of mosquito food. Mosquito food consisted of a thoroughly blended combination of equal parts rodent chow (500g added as PicoLab Irradiated Rodent Diet; Stewarts Feed, St. Louis, MO), lactalbumin (500g added as Lactalbumin; Sigma-Aldrich, St. Louis, MO), and brewer's yeast (500g added as Yeast, Brewer's Powder; Carolina Biological Supply, Burlington, NC). After a day had passed to allow egg hatch, two hundred larvae were allocated into individual 400 mL jars. These larval containers were filled with 300 mL of deionized water and then given 50 mg of mosquito food. The following days post-hatching each container was provided with 100, 300, 400, and 500 mg of mosquito food on days two, three, four, and five respectively, as per standard protocols (FR3, 2018). Seven days post-egg hatch, pupae were transferred into a cup with deionized water and these cups were moved into adult mosquito housing containers (henceforth referred to as a "cage") to allow for adult eclosion. Each cage was a 2 L plastic container with a mesh top and a transfer opening covered with a latex dental dam. Post-adult eclosion, mosquitoes were provided water and sugar. Five days post-adult eclosion, which allowed mosquitoes to mate, females were prepared to blood feed.

Mosquito Blood Feeding

Mosquitoes were blood fed using a two-chamber inverted glass jacketed feeder (Glass Mosquito Feeder, CG-1835-70; Chemglass Life Sciences; Vineland, NJ). Acquired from FR3, uninfected canine blood and *D. immitis m*icrofilariae in dog blood were heparinized, to prevent coagulation, for infection protocols. Adult female mosquitoes were removed from each cage, per respective protocol below, and placed into a new blood feeding cage. The blood feeding cages had their sugar removed the day prior and water removed at least 4 hours prior to blood feeding. A blood

147 feeder was assembled by placing parafilm over the open mouth of each feeder and then placed on
148 top of each blood feeding cage, one per cage. After placement, each blood feeder was connected
149 by tubing and into a hot water bath, set to 40°C. The connected blood feeders were connected to
150 a water pump to allow 40°C water to pump through the external chamber of the glass jacketed
151 feeders. Each blood feeder was affixed to each blood feeding cage with a rubber band to
152 efficiently secure the feeding apparatus and press the mouth of the feeder into the top of the
153 mesh. After the feeders were set, each feeder was allocated 200 uL of blood and allowed
154 mosquitoes to feed for approximately two hours or until repletion.

155 ***Copper Treatment***

156 A stock solution was prepared by adding Copper (II) Sulfate Pentahydrate (Sigma-Aldrich, St.
157 Louis, MO, USA) to milli-Q water brought to a copper concentration of 10 mg/mL. For each
158 treatment during the experiment, serial dilutions were made from the same stock solution in
159 order to add 1mL of serial dilution to the final dilution per larval container, a final volume of 300
160 mL per larval container. Larval container copper concentration of the water was measured and
161 acidified before larval development and after mosquito pupation by inductively coupled plasma-
162 mass spectroscopy (ICP-MS) (Nexlon 300X ICP-MS, Perkin Elmer, Norwalk, CT). Scandium 45
163 and Indium 115 were used as internal standards. All samples were acidified using trace metal
164 grade nitric acid (TMG HNO_3).

165 ***Effects of Copper on Larval Development***

166 Mosquito larvae were exposed to 0, 300, 600, 1200, 2400, and 4800 µg/L concentrations of
167 copper. Each treatment had three replicates consisting of an initial count of 200 larvae per jar.
168 These concentrations were based upon previous LC50 copper experiments on mosquitoes
169 (Rayms-Keller et al., 1998; Romi et al., 2000; Mireji et al., 2010). Each larval container was

filled with 299 mL of deionized water and 1 mL of a serially diluted stock solution respective to treatment. Feeding protocols followed *Mosquito Maintenance* and treatment followed *Copper Treatment* protocols. Mosquito pupae were collected every day after the first day of pupation and the experiment continued for a total of ten days.

Copper Accumulation

Three replicates of three copper concentrations (0, 300, and 600 µg/L) were set up using *Copper Treatment* and *Mosquito Maintenance* protocols. We did not consider using concentrations over 600 because these concentrations led to a greater than 2-fold increase in larval mortality compared to uncontaminated controls (see Results). Each replicate had three jars, which were pooled for data collection. Prior to Day 1 (Pre) mosquito feeding, and on Day 8 (Post), a sample of water was collected per replicate and treatment by pooling 3 mL of sample from each jar. Water was collected for quality analysis and control for copper treatments. On Day 5, 30 larvae were collected per replicate and treatment by pooling 10 larvae from each jar. On Day 7, 30 pupae were collected per replicate and treatment by pooling 10 pupae from each jar. On Day 9 thru 11, 75 adult males and females were collected separately per replicate and treatment by pooling 25 females and 25 males from each cage. Male and female pupae were assessed in the same samples because sex differentiation for mosquito pupae is determined by size and sex contaminated samples were incredibly likely. On the same days that adults were collected; 150 pupal husks were collected per replicate and treatment by pooling 50 husks from each jar. All samples collected were placed into pre-weighed 15 mL metal free conical tubes (VWR, Suwanee, GA). Additionally, we also collected nine mL of pre-experiment (i.e., before the addition of mosquito larvae) and post-experiment (i.e., after mosquito pupation) water samples for metal analyses, and these samples were acidified to 0.5% acid by adding 0.045 mL of TMG

193 HNO_3.Wet weights of the mosquito tissue samples were obtained for each replicate and
194 treatment once mosquito life stage was fully collected. Samples were frozen and then placed
195 onto a freeze dryer for 24 hours, and then weighed. The dried samples were digested using a hot-
196 block digestion with TMG HNO_3 and diluted with Milli-Q water, due to <0.2 g sample mass
197 whole samples were digested and not ground and homogenized. For each sample, HNO_3 was
198 added based upon what stage the mosquito had reached: Larvae 1.5 mL, Pupae 1.5 mL, Pupal
199 Husk 0.5 mL, Adult 2.5 mL. The amount of acid added to each sample was to ensure the entire
200 sample had been fully digested. Afterwards, the samples were diluted 1:5 prior to ICP-MS
201 analysis (as described earlier). Blanks and a certified reference material were used for quality
202 control. The reference material used was TORT-3 lobster hepatopancreas tissues (National
203 Research Council, Ottawa, On, Canada), and averaged a 90% recovery during analyses.
204 ***Effects of Copper on Adult Mortality***
205 Three exposure levels of copper were used to determine the effects of copper on adult mosquito
206 mortality: 0, 300, and 600 µg/L (see details above). The concentrations were based upon the
207 *Effects of Copper Exposure on Larval Development* experiments (see Results). Briefly, nine
208 larval containers were set up per copper concentration to allow the enough adult female mosquito
209 eclosion to be utilized for experimentation, and the entire experiment was replicated twice. The
210 metal exposure and mosquito rearing protocols followed those outlined earlier (see *Copper*
211 *Treatment* and *Mosquito Maintenance* sections above, respectively).
212 To ensure similarly aged mosquitoes, pupae were only collected during the first three
213 days of pupation (at which point ~85% of mosquito larvae had pupated). Mosquitoes were
214 moved into pupal cups and placed into cages according to treatment and jar number. After adult
215 mosquitoes emerged, female mosquitoes were pooled by copper exposure and equally allocated

into nine containers: 3 for uninfected blood fed (1 per copper dose) and 6 for infected blood fed (2 per copper dose) mosquitoes. We used more containers for infected mosquitoes because we expected higher mortality in these treatments (Ledesma and Harrington, 2015). Five days post-eclosion, females were fed a total of 400 uL of blood per cage using either uninfected blood or infected blood (at a microfilarial dose of 4,500 mf/mL) depending on the infection treatment the cage was assigned to (see *Mosquito Blood Feeding* protocols described earlier for details), unfed mosquitoes were removed. Mosquito mortality was monitored daily for 17 days post-blood feeding.

Effects of Copper on Fecundity

Three days post-blood feeding, 33 uninfected and 46 infected female mosquitoes per copper concentration were moved into individual 50 mL conical tubes containing wet paper towels and filled with 7.5 mL of deionized water for mosquito oviposition. Each 50 mL conical tube was covered with three layers of mesh to prevent escape and were supplied with a sugar cube which was replaced daily. Five days after mosquito allocation to the 50 mL conical tubes, mosquitoes were removed, and paper towels were dried prior to egg quantification.

Effects of Copper on Vector Competence

The same day post-blood feeding, unfed mosquitoes were removed and five fed female mosquitoes per infected container were collected for dissection to determine initial infection (zero hour). Subsequently, on day 15, 16, and 17 mosquitoes were collected, killed, dissected, and investigated underneath a compound microscope for each infected treatment and copper dose to determine individual filarial count and development.

Data Analyses

238 We used the statistical program R version 4.0.0 (R Foundation for statistical computing, Vienna,
239 Austria) for all the analyses. Analyses were carried out using three main analytical approaches:
240 (a) Cox proportional hazard mixed effects models (CMM) for survival data; (b) linear mixed
241 effects regression (LMER) for normally distributed dependent variables; (c) generalized mixed-
242 effects regression (GLMER) for dependent variables that had other than normal (e.g., Binomial
243 or Negative binomial) distributions. The CMM analyses were implemented in the R package
244 SURVIVAL, while LMER and GLMER analyses were implemented in and the R package LME4 (Bates
245 et al., 2015). The lowest Akaike Information Criterion (AIC; Burnham and Anderson, 2004)
246 scores was used to select the best fit model, though we retained any variable that was a primary
247 focus of the analysis. The least square means, implemented in the R package EMMEANS (Lenth,
248 2016), were used to visualize regression model results. Details of specific analyses used in this
249 study include: *(a) Effects of Copper on Larval Development:* We first tested for the effects of
250 copper concentration on larval development. We used a CMM to model the cumulative
251 proportion of larvae developing to pupae per day. Any larva that had not pupated after 10 days
252 was considered to be (right) censored data for the purpose of this analysis. We used copper
253 concentration (i.e., zero – 4800 µg/L) as the independent variable and Replicate as a random
254 effect. To test for the effects of copper on the overall probability of pupation we used a GLMER
255 with a binomial error distribution (and log link). We used the number of pupae observed
256 (“successes”) given the initial number of larvae (“trials”) as our dependent variable, copper
257 concentration (i.e., zero – 4800 µg/L) as the independent variable and Replicate as a random
258 effect. *(b) Copper Accumulation:* To test for the effects of copper concentration on levels of
259 copper accumulation we used a LMER. Briefly, we used the estimated copper concentrations (in
260 g/kg) in mosquito tissues as the dependent variable, and copper concentrations in the treatments

(i.e., 0, 300 and 600 µg/L) and tissue type (i.e., larvae, pupae, pupal husk, and adult) as independent variables. All models included Replicate as a random effect; *(c) Effects of Copper on Adult Mortality:* We tested the combined effects of larval copper exposure and infection on adult mosquito survival using CMM (Therneau and Grambsch, 2000). Replicate was treated as a random factor. Escapees and/or accidental death was treated as censored data; *(d) Effects of Copper on Fecundity:* We tested the combined effects of larval copper exposure and infection on fecundity (number of eggs in each tube) using a GLMER (Bates et al., 2015) with a Poisson error distribution. We used Replicate, as random effects; *(e) Effects of Copper on Vector Competence:* Vector efficiency was measured in terms of the proportion of L3 developed mosquitoes and total number of parasites surviving to the extrinsic incubation period using zero-hour data. We used a GLMER (Bates et al., 2015) negative binomial error distribution (and log link) to model the total number of L3s as a linear effect of concentration (0, 300, and 600 µg/L). All models included Replicate and Status (i.e. Died or Censored) as random factors.

Results and Discussion

Effects of Copper on Larval Development

Our data show copper concentrations $\geq$600 µg/L reduced both development rate (Table S2) and the proportion of *A. aegypti* pupating (N_{GROUP} = 18; $N_{INDIV/GROUP}$ = 200, Copper: χ^2 (DF) = 194.989 (5); $P < 0.001$; Figure 1; Table S1). Concentrations above 600 µg/L showed similar negative impacts on survival to pupation as reported in other studies (Rayms-Keller et al., 1998; Sheikh et al., 2010). Previous studies on chronic exposure of metals on *A. aegypti* found delays of adult eclosion for copper concentrations $\geq$ 3200 µg/L (Rayms-Keller et al., 1998) and longer larval development period at 1000 µg/L (Perez and Noriega, 2012). In other mosquito studies, copper exposure prolonged pupation for *A. aegypti, A. albopictus*, *Anopheles stephensi,* and

Culex pipiens (Perez and Noriega, 2012; Reza and Ilmiawati, 2020) and reduced pupation on day of maximal pupation for *Anopheles arabiensis* (Oliver and Brooke, 2018). Furthermore, negative effects of copper on the aquatic larval stages observed decrease in larval survival for *Chironomus riparius* (Marinković et al., 2011) and Odonata (Tollett et al., 2009).

Copper Accumulation

There were significant main and interactive effects between mosquito life-history stage/tissue (material) and copper dose [No. of groups (N_{GROUP}) = 3; Mean sample size/group ($N_{MEAN/GROUP}$) = 15; Copper: $\chi 2$ (DF) = 209.527 (2); $P < 0.001$; Material: $\chi 2$ (DF) = 130.117 (4); $P < 0.001$; Copper × Material: χ^2 (DF) = 78.508 (8); $P < 0.001$]. There was no significant decline in copper concentrations in water during the experiment (N_{GROUP} = 9; $N_{MEAN/GROUP}$ = 2; Copper: $\chi 2$ (DF) = 818.530 (2); $P < 0.001$; Material $\chi 2$ (DF) = 2.929 (1); $P = 0.087$; Copper × Material: χ^2 (DF) = 13.186 (2); $P = 0.001$; Figure S1). This is likely due to the large volume of waters vs. total copper consumed by the larvae. The highest level of copper contamination (Figure 2; Table S3) can be seen during the larval stages of development, which was expected due to rapid nutrient acquisition during this stage (Merritt et al., 1992). During the pupal and adult stages of the mosquito there were sequential decreases in copper burden. Similarly, patterns of metal accumulation have been observed in *Lymantria dispar L.* life stages (Gintenreiter et al., 1993), in mayfly larvae and subimagoes, *Baetis tricaudatus*, exposed to zinc (Wesner et al., 2017), in *A. albopictus* larvae and adults exposed to Chromium and Selenium during larval development (Zhou et al., 2020) . A possible mechanism of copper reduction in adult mosquitoes is the excretion of copper in the pupal exuviae (Figure 2). In other studies, metal loss has been observed in the exuvium of Chironomids (Timmermans and Walker, 1989), mayflys (Kim et al., 2012), and other invertebrates (Kraus et al., 2014b).

307 However, not all copper is excreted into the exuvia of the mosquitoes (Figure 2) and a
308 substantial portion is carried into the adult stage. Interestingly, adult female mosquitoes had
309 higher copper concentrations compared to adult male mosquitoes and female mosquitoes had a
310 seemingly proportional copper accumulation respective to copper dose. Previous studies have
311 shown that copper carried to the adult stage could impact adult longevity positively (e.g.,
312 *Anohpelese arabiensis* and *A. aegypti*; Perez and Noriega, 2014; Oliver and Brooke, 2018) or
313 negatively (e.g., *Ischnura elegans*; Debecker et al., 2017), and negatively affect egg viability
314 (Mireji et al., 2010).

315 ***Effects of Copper on Adult Mortality***

316 We found a significant negative effect of parasite infection on adult mortality (N_{GROUP} = 6;
317 $N_{MEAN/GROUP}$ = 383, χ^2 (DF) = 208.599 (1); $P < 0.001$; Figure 3; Table S4), as shown in
318 investigations of *A. aegypti* (Ledesma and Harrington, 2015), *Aedes albopictus* (Lai et al., 2000;
319 Dharmarajan et al., 2019), and *Culex quinquefasciatus* (Lai et al., 2000). However, the main
320 effect of copper exposure and interaction between copper exposure and infection were
321 nonsignificant, indicating that infection had a stronger selective pressure on mortality hazard
322 than larval copper exposure.

323 ***Effects of Copper on Fecundity***

324 We found significant main and interactive effects between copper exposure and infection
325 (N_{GROUP} = 6; $N_{MEAN/GROUP}$ = 79; Copper: χ^2 (DF) = 14.732 (2); $P < 0.001$; Infection: χ^2 (DF) =
326 325.873 (1); $P < 0.001$; Copper × Infection: χ^2 (DF) = 53.117 (2); $P < 0.001$; Table S5).
327 Fecundity in infected treatments expressed a hormetic copper exposure effect (Figure 4).
328 Hormesis is a phenomenon where low levels of potentially toxic agents causes stimulatory
329 effects (Stebbing, 1982; Calabrese and Baldwin, 2003; Calabrese and Blain, 2004; Sinclair,

2005; Agathokleous et al., 2020). Further experimentation with additional copper concentrations are needed to determine if there is a true effect of hormesis.

However, when mosquitoes are infected fecundity was significantly reduced across all copper exposures. Similarly, when *A. aegypti* were infected by ≥3 *Brugia malayi* there was significant reduction of eggs laid (Gleave et al., 2016). Although, egg production increased in copper exposed mosquitoes there could be indirect consequences. Another study exposing *A. aegypti* to sublethal levels of copper found metal stress can result in compromised adult performance such as lowered adult body mass, neutral storage lipids at emergence, starvation tolerance, fecundity and starvation tolerance of offspring (Perez and Noriega, 2014). However, the same study found larval metal stress had a hormetic effect of increased longevity in female mosquito adults. Which suggests exposed mosquitoes could result in different levels of fitness due to larval metal stress and could result in reduced fitness for subsequent mosquito generations. Although mosquito fecundity increased, further experimentation would be required to assess copper exposed mosquito egg fertility because an increase in fecundity could be offset by reduction in egg fertility. Additionally, higher fecundity for copper exposed mosquitoes could be a way to offset or excrete copper contamination carried over from the larval stage. In *A. aegypti*, iron is ingested as hemoglobin and ferric-transferrin from vertebrate host blood, which is either excreted as waste, retained in the body, or allocated to the eggs (Zhou et al., 2007). Additionally, pre- and postpartum *Centroptilum triangulifer* adults were analyzed after juvenile zinc exposure and found prepartum adults had significantly higher levels of zinc than postpartum adults (Kim et al., 2012). Thus, it is possible that contaminated mosquitoes could offset negative consequences of copper through maternal copper transfer to eggs. Further experimentation of pre-and postpartum mosquito adults will have to be analyzed for maternal transfer of copper and

to elucidate possible side effects of copper contamination on offspring fitness. Also, experiments could be conducted to determine if egg fertility offsets increased egg production as an indirect tradeoff of maternal copper transfer to eggs.

Effects of Copper on Vector Competence

We found no significant difference between zero-hour infections of copper exposure treatments (

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Perturbations to the environment from anthropogenic disturbance can negatively affect ecosystem health (Costanza and Mageau, 1999). For example, metal pollution can negatively affect species richness and diversity (Blanar et al., 2009). Numerous studies have shown that parasites are effective indicators of ecosystem structure and function because they are sensitive to perturbations, including anthropogenic effects on ecological systems, that affect host community structure, species interaction dynamics and/or food web topology (Hudson et al., 2006; Marcogliese et al., 2010; Whiles et al., 2013; Preston et al., 2016; Sures et al., 2017a; Sures et al., 2017b; Vannatta and Minchella, 2018). There is strong evidence that metal pollution can significantly impact parasite species richness as well as infection intensity (Sures et al., 2017a; Sures et al., 2017b; Vannatta and Minchella, 2018). Additionally, parasites that actively feed on host tissue (as in the case of *D. immitis*) occupy a trophic level similar to predators (Nachev et al., 2017), and are thus likely to be exposed to higher concentrations of metal contaminants through bioaccumulation and/or biomagnification pathways (Ryman et al., 2008; Olivero-Verbel and Caballero-Gallardo, 2013). Interestingly, to our knowledge, no previous

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411 study has investigated the direct and indirect effects of larval metal exposure on infective
412 parasite burden and mortality rates of insect vectors. These parameters are critical because they
413 jointly affect vectorial capacity and hence control vector-borne disease transmission dynamics.
414 Our study indicates that female mosquitoes exposed to 600 µg/L of copper had a similar
415 mortality hazard to unexposed mosquitoes and significantly reduced *D. immitis* parasite load.
416 This reduction in parasite load likely is driven by importance of copper-containing enzymes
417 (e.g., Laccase 2) on melanization (Dittmer et al., 2004; Dittmer and Kanost, 2010; Sugumaran
418 and Barek, 2016), which is the main immune mechanism mosquitoes use to defend against
419 macroparasites like *D. immitis* (Christensen et al., 2005). Indeed, the suppression of Laccase 2
420 has been shown to reduce resistance to parasites in mosquitoes (Du et al., 2017).
421 Previous studies have also shown that insects play an important role in ecosystem health
422 by affecting contaminant cycling. Most studies on contaminant cycling focus on the transfer of
423 contaminants from terrestrial to aquatic systems (e.g., urban/industrial runoff and erosion; Mason
424 et al., 1994; Schroeder and Munthe, 1998; Driscoll et al., 2013; Rumrill et al., 2018). However,
425 there is increasing interest in energetic pathways that can transfer contaminants from aquatic to
426 terrestrial environments (Sullivan and Rodewald, 2012). Insects with aquatic life history stages
427 are particularly important for contaminant transfer from aquatic to terrestrial systems through the
428 food chain (Menzie, 1980; Walters et al., 2008). Mosquitoes constitute an important taxon of
429 insects, especially in urban areas. However, the potential role of mosquitoes in contaminant
430 transfer from aquatic to terrestrial systems remains unclear. Many organisms have evolved to
431 handle metal stress by offloading contaminants through excretion (e.g., exuvium; Timmermans
432 and Walker, 1989; Kraus et al., 2014b) or through maternal transfer (e.g., eggs; Kim et al.,

433 2012). We found significant copper excretion in the exuvium of the mosquito but retained
434 significant copper concentrations to adulthood at 300-600 µg/L of copper (Figure 2).
435 Copper concentrations in water are highly variable, ranging from 100-69,000 µg/L due to
436 mining drainage, abandoned mines, industrial discharge, and power plant effluent ranging
437 (Dorsey et al., 2004), and from 5-30,000 µg/L in drinking water (WHO, 2004). However, the
438 results from our experiments are of ecological relevance because the levels of copper exposure
439 we focus on (0-600 µg/L) are within the range of concentrations found in natural mosquito
440 breeding habitats (50-2880 µg/L) (Sarkar et al., 2004; Mireji et al., 2008) and well below the US
441 Environmental Protection Agency mandated limits for copper in drinking water (1300 µg/L;
442 EPA, 2020). We feel it is also important to recognize that copper is strongly associated with
443 other metals (e.g., zinc and cadmium) in the aquatic environments (Sarkar et al., 2004; Ruchter
444 and Sures, 2015), and many of these metals also share similar detoxification mechanisms
445 (Rivera-Perez et al., 2017). Consequently, our results could also point to broader connections
446 between vector-borne diseases and the cumulative heavy metal load in natural and anthropogenic
447 environments. Future directions of this research can investigate natural populations of *Aedes* sp.
448 of mosquitoes to acquire data further representative of wild type populations. In conjunction to
449 testing wild type populations, surveying breeding sites in nature to help quantify levels of metal
450 exposure and copper analyses of mosquito larvae and adults to determine aquatic-terrestrial
451 contaminant transfer. Furthermore, predation experiments could elucidate the impact transfer
452 from aquatic larvae to terrestrial adults could impact copper cycling.
453 To conclude, our study indicates that metal contamination can negatively affect vector-
454 borne disease transmission dynamics by reducing adult mosquito recruitment (through increased
455 larval mortality) and vector competence (through reduced parasite load). Our study also shows

456 that mosquitoes could be an important link in transferring metal contaminants from aquatic to
457 terrestrial systems, especially in urban areas where they are common.
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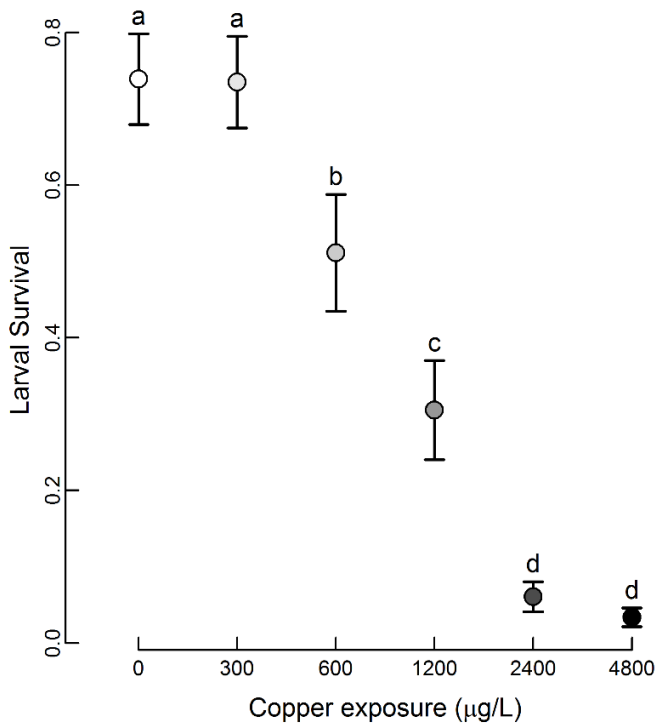


Figure 1. Effects of copper exposure on mosquito larval survival. The data indicates the mean proportion of larvae developing to pupae at each level of larval copper exposure (0, 300, 600, 1200, 2400, and 4800 µg/L). Means followed by a common letter are not significantly different at $\alpha = 0.05$, and error bars represent the standard error of mean.

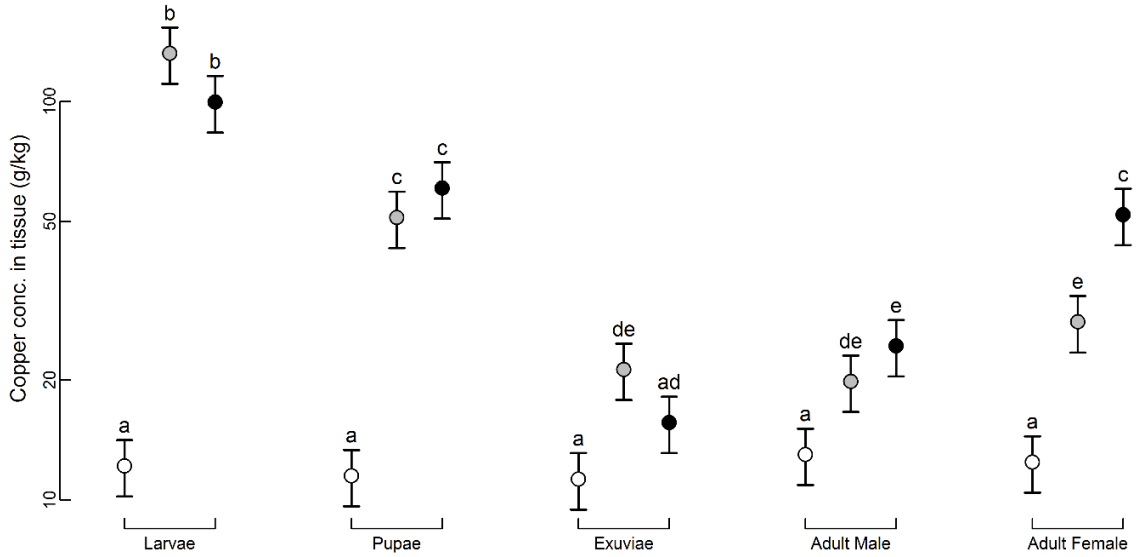
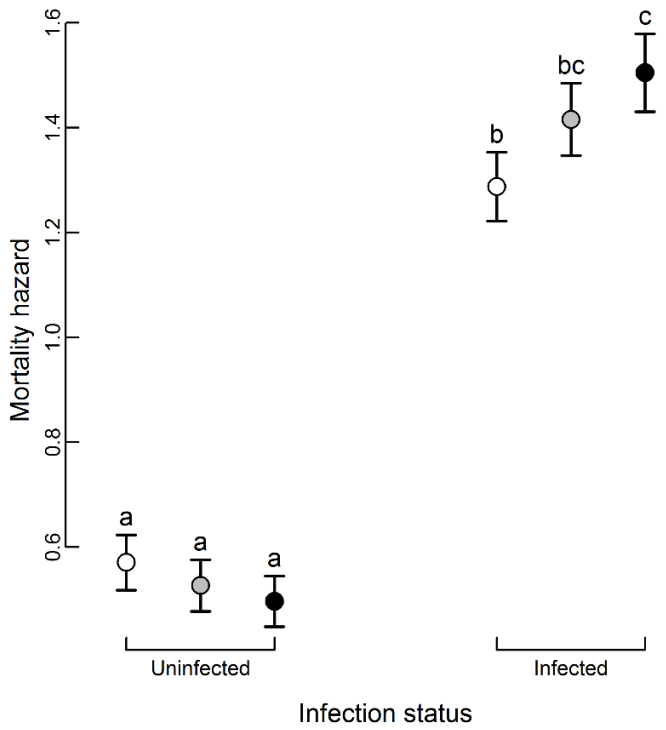


Figure 2. Effects of copper exposure on copper concentration in different tissues collected across the mosquito life cycle. The data indicates the mean copper concentration (g/kg) in mosquito tissues at three levels of larval copper exposure: 0 (white symbols), 300 (gray symbols) and 600 (black symbols) µg/L. Means followed by a common letter are not significantly different at $\alpha = 0.05$, and error bars represent the standard error of mean.



750

751 **Figure 3.** Effects of *Dirofilaria immitis* infection and copper exposure on mosquito mortality.
752 Data represents the daily mortality hazard of uninfected or infected female mosquitoes raised
753 from larvae exposed to three levels copper: 0 (white symbols), 300 (gray symbols) and 600
754 (black symbols) µg/L. Means followed by a common letter are not significantly different at α =
755 0.05, and error bars represent the standard error of mean.

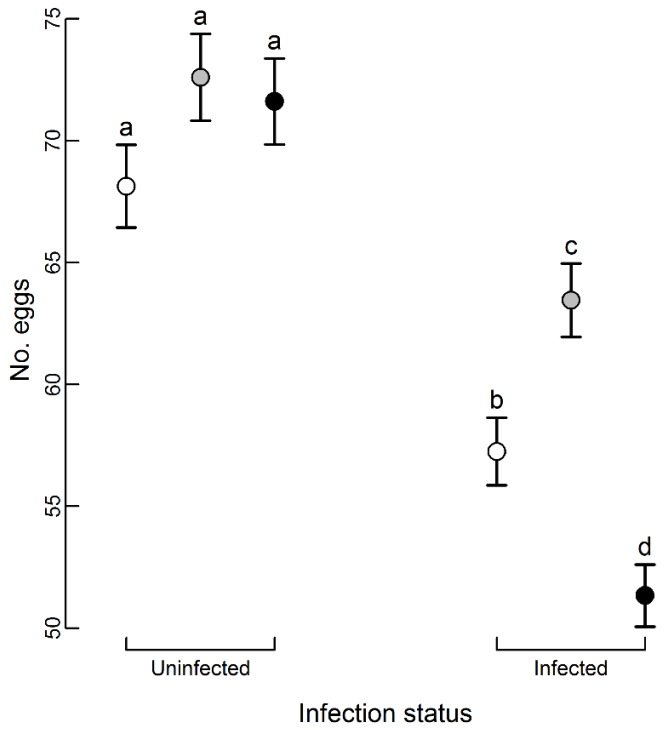
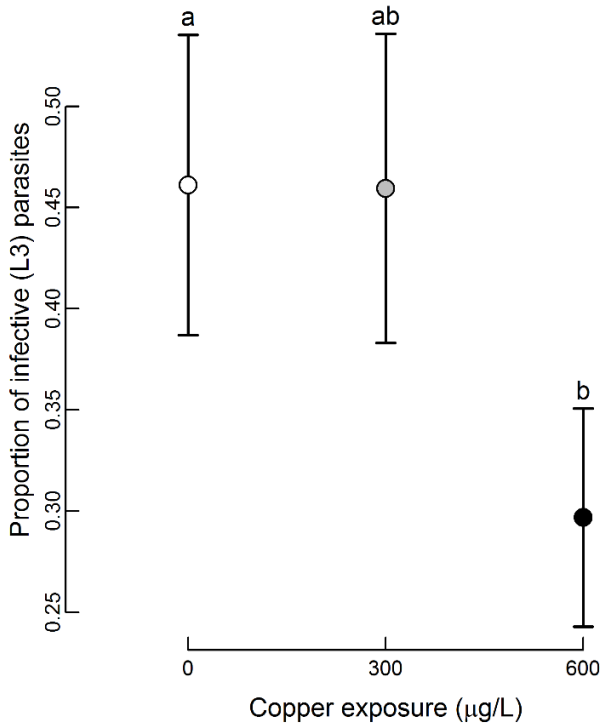


Figure 4. Effects of *Dirofilaria immitis* infection and copper exposure on mosquito fecundity. Data represents the number of eggs oviposited by uninfected or infected female mosquitoes raised from larvae exposed to three levels copper: 0 (white symbols), 300 (gray symbols) and 600 (black symbols) µg/L. Means followed by a common letter are not significantly different at $\alpha = 0.05$, and error bars represent the standard error of mean.



762

763 **Figure 5.** Effects of copper exposure on the vector efficiency of mosquitoes infected with
764 *Dirofilaria immitis*. Data represents the proportion of *D. immitis* parasites developing to infective
765 larvae (L3) in female mosquitoes raised from larvae exposed to three levels copper: 0 (white
766 symbols), 300 (gray symbols) and 600 (black symbols) µg/L. Means followed by a common
767 letter are not significantly different at $\alpha = 0.05$, and error bars represent the standard error of
768 mean

769