The effect of metal pollution on the life history and insecticide resistance phenotype of the major malaria vector *Anopheles arabiensis* (Diptera: Culicidae)

Shüné V. Oliver[1,2]*, **Basil D. Brooke**[1,2]

1 Centre for Emerging Zoonotic and Parasitic Diseases, National Institute for Communicable Diseases, Johannesburg, South Africa, **2** Wits Research Institute for Malaria, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

* shuneo@nicd.ac.za

Abstract

Metal exposure is one of the commonest anthropogenic pollutants mosquito larvae are exposed to, both in agricultural and urban settings. As members of the *Anopheles gambiae* complex, which contains several major malaria vector species including *An. arabiensis*, are increasingly adapting to polluted environments, this study examined the effects of larval metal exposure on various life history traits of epidemiological importance. Two laboratory strains of *An. arabiensis*, SENN (insecticide susceptible) and SENN DDT (insecticide resistant), were reared in maximum acceptable toxicity concentrations, (MATC—the highest legally accepted concentration) of cadmium chloride, lead nitrate and copper nitrate. Following these exposures, time to pupation, adult size and longevity were determined. Larvae reared in double the MATC were assessed for changes in malathion and deltamethrin tolerance, measured by lethal time bottle bioassay, as well as changes in detoxification enzyme activity. As defence against oxidative stress has previously been demonstrated to affect the expression of insecticide resistance, catalase, glutathione peroxidase and superoxide dismutase activity was assessed. The relative metal toxicity to metal naïve larvae was also assessed. SENN DDT larvae were more tolerant of metal pollution than SENN larvae. Pupation in SENN larvae was significantly reduced by metal exposure, while adult longevity was not affected. SENN DDT showed decreased adult size after larval metal exposure. Adult insecticide tolerance was increased after larval metal exposure, and this effect appeared to be mediated by increased β-esterase, cytochrome P450 and superoxide dismutase activity. These data suggest an enzyme-mediated positive link between tolerance to metal pollutants and insecticide resistance in adult mosquitoes. Furthermore, exposure of larvae to metal pollutants may have operational consequences under an insecticide-based vector control scenario by increasing the expression of insecticide resistance in adults.

Introduction

Human activity has led to a large-scale increase in various environmental pollutants. Pollution in rural areas accrues primarily from agrochemicals such as fertilizer and pesticides, but also from domestic refuse, sewage and livestock excrement [1]. Urban pollutants arise from domestic waste, traffic and industry [2]. In South Africa, like many other African countries, vital economic activities such as artisanal gold mining and coal power stations are a major source of pollution [3]. Pollution in rural and urban environments typically leads to an increase in heavy metal contamination.

Mining is a source of urban metal pollution that has the potential to cause widespread contamination by runoff and rainwater seepage into water systems [4]. Increased ground transportation also results in increased levels of water soluble heavy metals (reviewed in [5]). Although rural areas are not commonly associated with metal pollution, the use of phosphate fertilizers can contribute to the increase of hazardous heavy metal trace elements [6]. In general, metal pollution is highly pervasive wherever human activity occurs. Importantly, these heavy metal contaminants have a tendency to pollute water sources [4] and this has numerous environmental and public health consequences.

Members of the *Anopheles gambiae* species complex typically prefer to breed in open, sunlit temporary bodies of water that are primarily unpolluted [7]. Therefore, reports of members of this complex breeding in polluted water represent a significant biological shift in this species [8–10]. Furthermore, this shift is not only reported in *An. gambiae sensu stricto*, but other members of this species complex including *An. arabiensis* [11, 12]. As non-pesticidal residues have been demonstrated to modulate detoxification enzyme capacity [13–15], as well as insecticide resistance phenotypes [16], this adaption could inadvertently affect biological attributes of epidemiological importance, such as insecticide susceptibility.

The importance of environmental pollution on pyrethroid resistance has been reviewed [2], and metal pollution has been named as one of the most important effectors of resistance phenotype in urban settings. The effect of metal pollution has been examined in *An. gambiae* and *Aedes aegypti*. Adapting to the presence of environmental metal pollutants incurs a fitness cost to the larvae. Heavy metals arrest egg hatching and compromise the integrity of the larval peritrophic matrix in *Ae. aegypti* larvae when naïve populations are exposed [17]. It is possible to select for metal tolerance in *An. gambiae* relatively rapidly under laboratory conditions [10]. However, this adaption incurs a significant biological cost, including reduced egg viability, immature survivorship and emergence as well as reduced reproductive capacity [18].

At the molecular level, the development of tolerance to metal pollutants involves protein induction shifts [10]. For example, the stress response genes coding glutathione S-transferase [19], metallothionein [20] as well as mucin and α-tubulin have been associated with metal tolerance [17, 21, 22]. Several cytochrome P450 genes, particularly in the CYP6 class, have also been associated with metal tolerance. The induction of these genes is sex-specific and although the relevant genes are expressed at their lowest levels in females, the gene induction was female specific, with males incurring a significant reduction in the expression of CYP6M2, CYP6P3 and CYP6Z1 [23].

Although adaptation to metal pollution has also been reported in *An. arabiensis* [11, 12], very little work has been done on this species' molecular response to pollution [2]. *Anopheles arabiensis* is a dominant malaria vector species in southern Africa, including South Africa [24]. The variable feeding and resting behaviours of this species presents a challenge to vector control methods that are based on indoor applications of insecticide [25, 26]. This is because outdoor biting and resting can lead to sustained low-level residual transmission in a control setting [27], which threatens South Africa's malaria elimination agenda.

This aim of this study was to understand the effects of heavy metal pollution on various life history traits as well as the expression of insecticide resistance in *An. arabiensis*.

Methods

Materials

All experiments were performed in the Botha de Meillon insectary, National Institute for Communicable Diseases, Johannesburg. Basic *Anopheles* colony rearing and feeding conditions were as described in [28]. The baseline strain used in this study was SENN, which was colonised from Sennar, Sudan, in 1980. This strain is mostly insecticide susceptible, with low-level permethrin resistance [29]. The SENN DDT strain was selected from the SENN strain and has been continuously exposed to DDT since 1995. It currently displays resistance to DDT, permethrin, deltamethrin, λ-cyhalothrin and malathion [29, 30]. Resistance in this strain is due to elevated detoxification enzymes as well as the L1014F mutation [31, 32] Elevated oxidative stress enzymes have also been implicated in the construction of the resistant phenotype [33].

Comparative metal-induced lethality in insecticide resistant and susceptible *An. arabiensis*

Three representative metals were used in this study, based on previous research [10]. Ten percent stock solutions of cadmium chloride, copper nitrate and lead nitrate were prepared. The minimum lethal concentrations of these metal salts were determined by using standard WHO larviciding protocols [34]. As lead was significantly less toxic, a slight variation in concentration range was used to determine the lethal dose of lead nitrate, with working concentrations of 100–16000 ppm. The specific concentrations used for these experiments are given as supplementary materials. Twenty-five fourth instar larvae were used per concentration, and 4 replicates were performed as per WHO recommendations. Lethal concentrations inducing 50% mortality (LC_{50}) were determined by Probit analysis [35], using IBM SPSS v21(IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.).

The effect of metal pollution on larval development

The development of SENN and SENN DDT larvae when exposed to metal pollutants was assessed. Newly emerged larvae, less than 24 hours old, were exposed to the maximum acceptable toxicant concentration (MATC) of the relevant metal salts. Larvae reared in untreated water served as a control. MATCs for the representative metal salts were defined in [22] (0.36μg/L for cadmium chloride, 1.86μg/L for copper nitrate and 4.39μg/L for lead nitrate). All larvae were fed the same amount of food. Larvae were monitored until pupation, and the percentage of pupation on the day of maximal pupation (the day the most pupae emerged; in this study—day 9) was determined. This study was replicated three times from three different egg batches, resulting in a total of 9 replicates, with a concurrent control (rearing in untreated water) with each replicate.

The effect of larval metal pollution on adult longevity

To assess the effect of larval metal pollution on subsequent adult longevity, 100 24-hour-old SENN and SENN DDT 1st instar larvae were exposed to the MATC of cadmium chloride, copper nitrate or lead nitrate, with corresponding larvae in clean water serving as a control. The larvae were fed the same amount of food as described in [31]. Adults that were obtained from these treatments were used for longevity assays. Thirty males and 30 females were assessed for

longevity. Cadavers were removed daily, and the adults were allowed *ad libitum* access to 10% sucrose, but never allowed a blood meal for the course of their lifetime. Survival was monitored until all individuals were dead. This experiment was replicated 3 times from 3 different cohorts. Longevity was assessed using the Kaplan-Meier estimator with the log-rank test used as a measure of significance.

The effect of larval metal exposure on adult size

Fifty 24-hour SENN and SENN DDT 1st instar larvae were exposed to the MATC of either cadmium chloride, copper nitrate and lead nitrate or clean water (control). The larvae were fed the same amount of food until emergence as adults. The adults were harvested and cold-killed. The wings were removed and affixed to a microscope slide. Wing-length was used as a proxy for size, with the wing measured from the wing tip to the allula [36]. Wing-length was measured at 200x magnification. This experiment was replicated three times from three different cohorts, and means were compared by 1-way analysis of variance.

The effect of larval metal exposure on adult insecticide tolerance

SENN and SENN DDT larvae were used in this experiment. As SENN is significantly more susceptible to insecticides an accurate lethal dose could not be determined. As such a lethal time approach was adopted. Due to the aforementioned difference in resistance phenotype, different timescales were employed to determine the lethal times. Two hundred and fifty 24-hour old 1st instar larvae were reared in the MATC of either cadmium chloride, copper nitrate or lead nitrate, with control larvae reared in untreated water. The larvae were fed the same amount of food until pupation. Adults were harvested at 3 days, and females were not allowed to blood feed. The adults were then subjected to CDC bottle bioassay insecticide exposures to determine lethal time to 50% mortality (LT50). SENN adult females were exposed to either 0.001% malathion or deltamethrin for 2,4, 8, 16 or 32 minutes. SENN DDT adults were exposed to 0.01% malathion or deltamethrin for 10, 20, 40 or 80 minutes. This experiment was replicated three times from three different cohorts. Differences in LT50 between adult cohorts arising from pollutant treatments were compared by 1-way ANOVA.

The effect of larval metal exposure on adult enzyme activity

SENN and SENN DDT larvae were prepared as for the adult insecticide tolerance experiments. At 3 days old, non-blood fed adult females were cold-killed. Adults were homogenised in either PCR grade water or 0.1M Potassium phosphate pH 7.0. Haeme peroxidase, glutathione S-transferase (GST) and α- and β-esterase activity levels were assessed in order to determine detoxification enzyme activities in association with exposure to heavy metals. Superoxide dismutase, glutathione peroxidase and catalase activities were determined as representative of oxidative stress defence enzyme activity. Enzyme activity by treatment was determined as described in [37]. In brief, all assays employed a calorimetric assessment of enzyme activity. Haeme perxoxidase activity was determined by tetramethyl benzidine peroxidation, GST activity by rate of 1-Chloro 2–4 Dinitrobenzene conjugation with reduced glutathione, and esterase activity by quantification of the hydrolosis of α and β-napthyl acetate to α and β-napthol. These products were spectrophotometrically quantified at 650, 340 and 575 nm respectively. Superoxide dismutase activity was determined using a commercial kit (Sigma Aldrich: 19160) as per manufacturers' instructions. Glutathione peroxidase activity was determined as function of Nicotinamide adenine dinucleotide phosphate (NADPH) consumption measured at340nm and catalase activity determined as a function of hydrogen peroxide
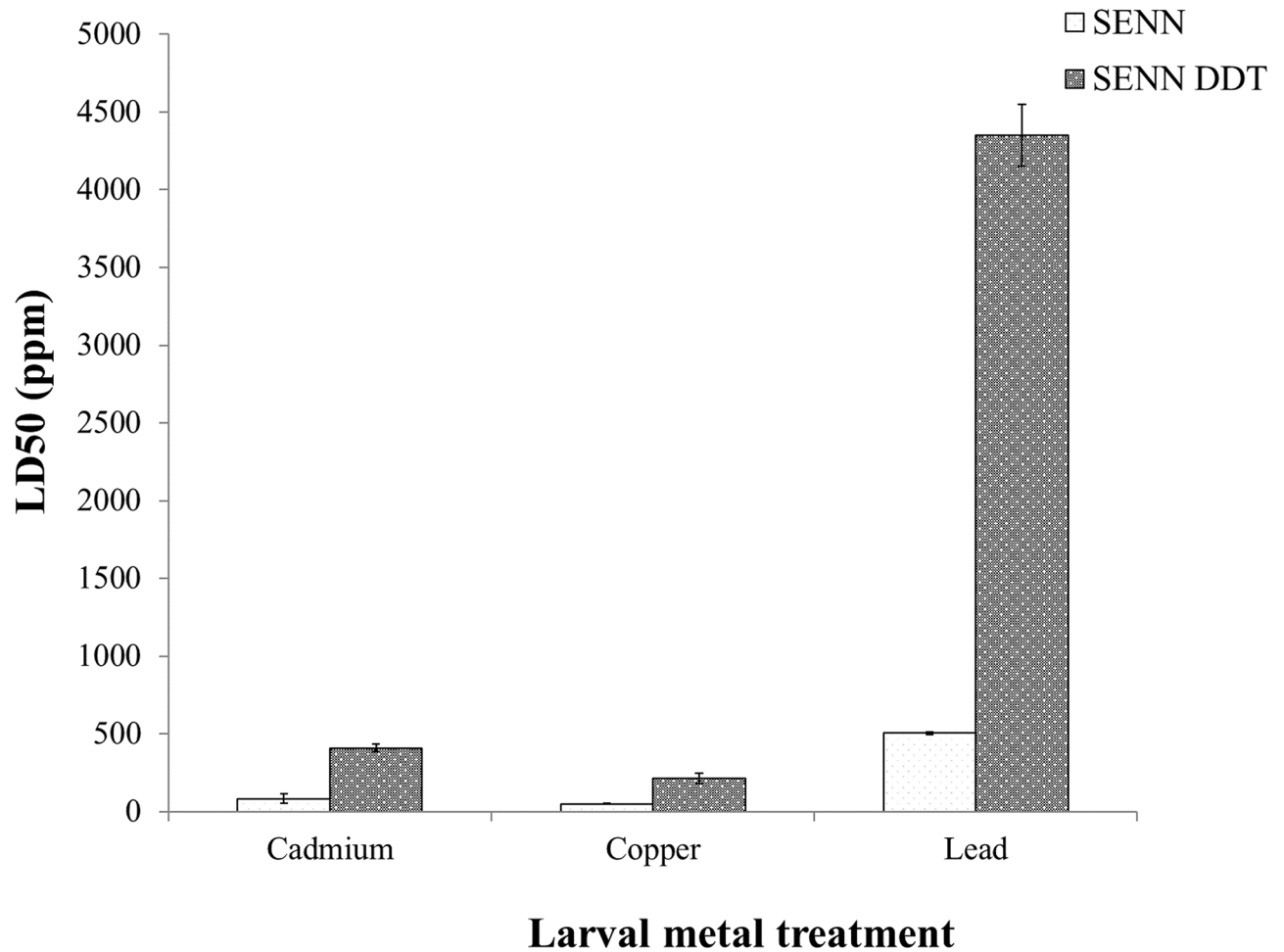
Fig 1. Relative toxicity of heavy metals on insecticide resistant and susceptible *Anopheles arabiensis* **laboratory strains.** The insecticide resistant *An. arabiensis* strain SENN DDT showed a significantly higher tolerance for metal exposure than the insecticide susceptible SENN strain, based on LD50s. Copper nitrate was the most toxic metal to both strains, while both strains showed a high tolerance to lead nitrate. Significant differences (p<0.05) are indicated by an asterisk (*).

consumption measured at 570nm. All calorimetric analysis was performed on performed on a Multiskan Ascent 96 well plate reader (Thermo Scientific).

Results

Insecticide resistant phenotype and comparative metal lethality

SENN DDT larvae showed significantly higher tolerance to metals, as measured by differences in lethality. The respective LC50s of SENN DDT were 409.41, 212.87 and 4349.10 parts per million (ppm) for cadmium chloride, copper nitrate and lead nitrate respectively as opposed to 82.93, 50.53 and 504.36ppm respectively for SENN. SENN DDT LD50 values are significantly higher than those of SENN (1-way ANOVA: p<0.01; df = 5; F = 401) (Fig 1).

The effect of metal pollution on larval development

For both strains, maximal pupation was reached on day 9, regardless of treatment. For SENN DDT, the mean percentage of larvae that pupated on day 9 did not differ between treatments;
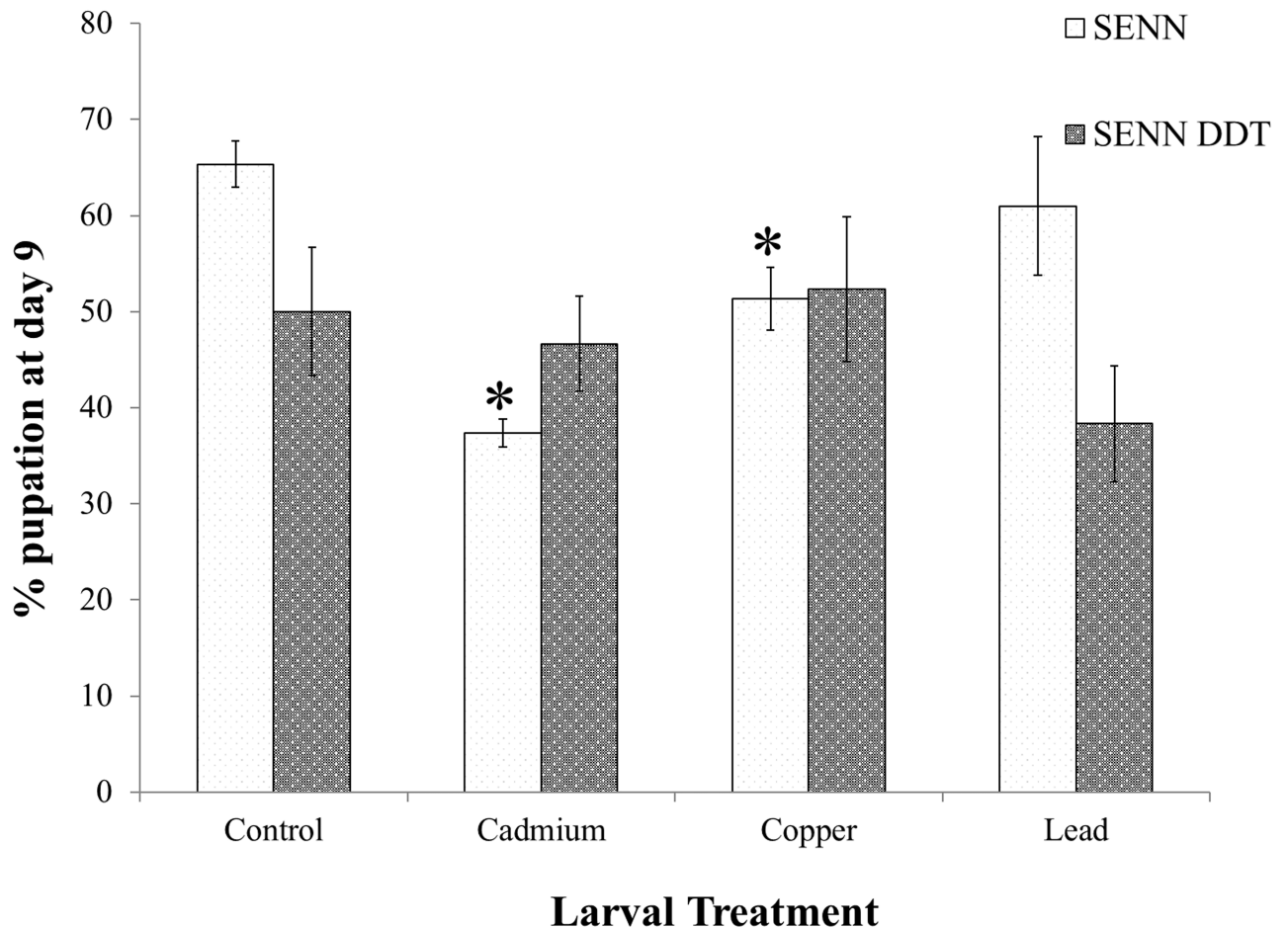
Fig 2. The effect of metal pollution on larval development in insecticide susceptible and resistant *Anopheles arabiensis*. SENN and SENN DDT larvae were reared in the maximum acceptable toxicity concentration (MATC) of cadmium chloride, copper nitrate and lead nitrate. Both strains had the highest number of pupae emerging on day 9, regardless of whether they were reared in metal polluted or untreated control water. There was variation in the total number that pupated on day 9. Larval cadmium chloride and copper nitrate treatment significantly reduced the percentage that pupated on day 9 in the insecticide susceptible SENN strain, but not the resistant SENN DDT strain. Lead nitrate treatment did not affect pupation in either strain.

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control 50%, cadmium chloride 46.7%, copper nitrate 52.3% and lead nitrate 38.3% (1-way ANOVA: p = 0.47; df = 3; F = 0.93). In contrast, while lead nitrate did not result in a significant decrease in pupation rate compared to the control (control: 65.3%; lead nitrate: 61.0%) (2-sample t-test: p = 0.62; df = 2.4; T = 0.57), copper nitrate (47.5%) and cadmium chloride (37.3%) treatments did result in a significant decrease in mean percentage pupation at day 9 (copper nitrate—2-sample t-test: p = 0.03, df = 3, T = 3.90; cadmium chloride– 2-sample t-test: p<0.01, df = 3.3, T = 9.97) (Fig 2).

The effect of metal pollution at the larval stage on adult longevity

Larval metal exposure did not have a marked effect on adult longevity. No larval metal treatments affected adult longevity in SENN males (Log-rank test: p = 0.70; χ^2 = 1.42; df = 3) or SENN females (Log-rank test: p = 0.35; χ^2 = 3.30; df = 3) compared to untreated controls. Similarly, SENN DDT females were not affected (Log-rank test: p = 0.51; χ^2 = 2.31; df = 3). However, adult longevity in male SENN DDT was affected (Log-rank test: p<0.01 χ^2 = 17.28;
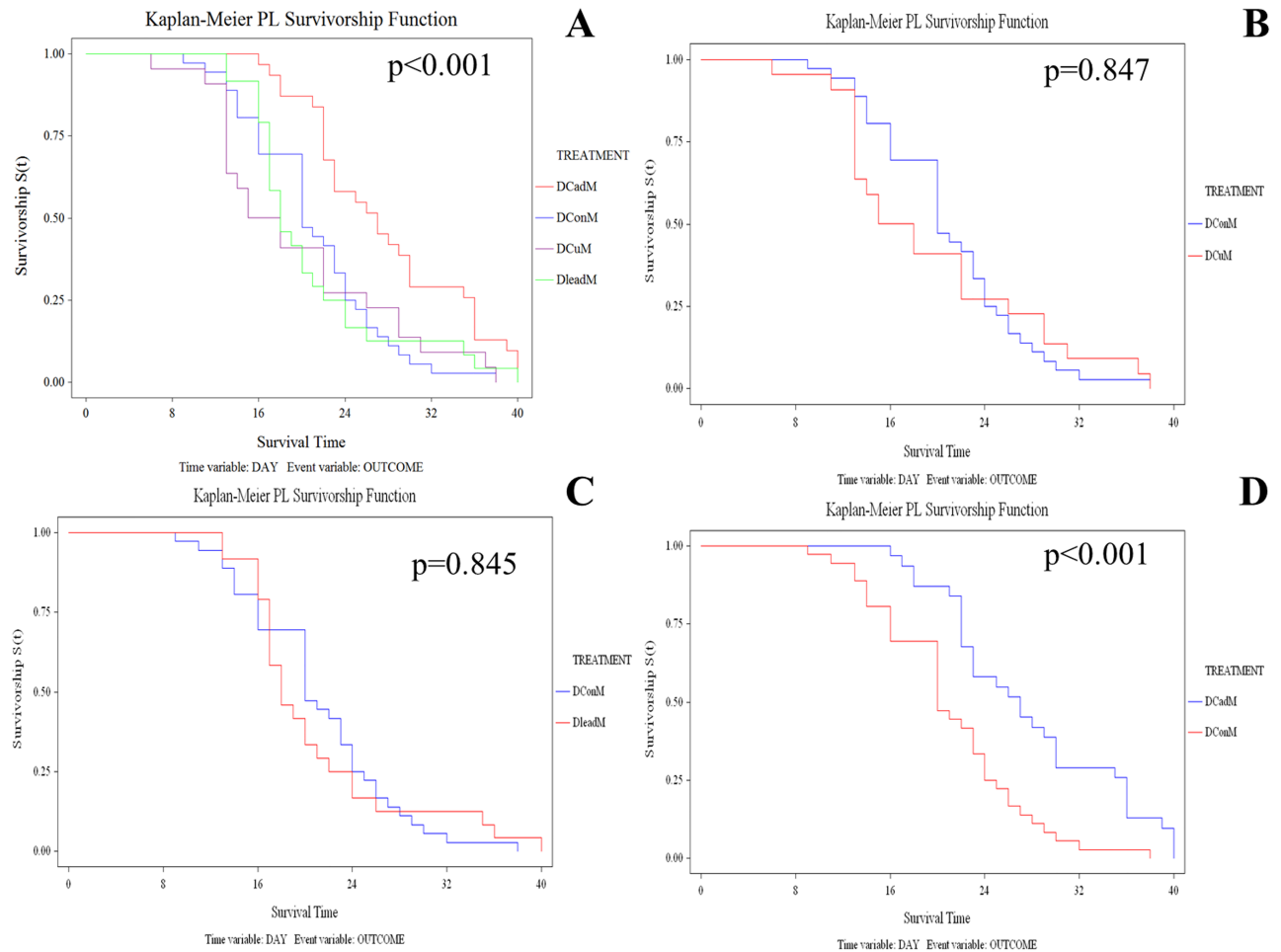
Fig 3. The effect of larval metal exposure on adult longevity in insecticide susceptible and resistant *Anopheles arabiensis*. Larval metal exposure did not affect adult longevity in insecticide susceptible SENN males or females, and did not affect the longevity of insecticide resistant SENN DDT females. SENN DDT males, however, did show an increase in longevity after larval metal treatment (A). That change was not due to copper nitrate (B) or lead nitrate (C) treatment, but was due to larval cadmium treatment (D).

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df = 3) (Fig 3A). When examining each treatment individually it was found that this effect was not due to larval copper treatment (Log-rank test: p = 0.85; χ^2 = 0.04; df = 1) (Fig 3B) or lead treatment (Log-rank test: p = 0.85; χ^2 = 0.04; df = 1) (Fig 3C). The significant change was an increase in male longevity after cadmium treatment (Log-rank test: p<0.01; χ^2 = 11.97; df = 1) (Fig 3D).

The effect of metal pollution at the larval stage on adult size

Larval metal pollution had no significant effect on the size of SENN adults, regardless of gender (1-way ANOVA: males: p = 0.85, df = 3, F = 0.26; females: p = 0.03, df = 3, F = 3.59, Tukey's post hoc test, no significant pairwise difference, critical Q = 3.87). In contrast, SENN DDT adult size was affected by larval metal pollution, although it differed by gender. SENN DDT males were not affected (1-way ANOVA: p = 0.07, df = 3, F = 2.38). Copper nitrate and cadmium chloride larval exposure significantly decreased adult size in females (1-way ANOVA: p = 0.01, df = 2, F = 4.94) (Fig 4).
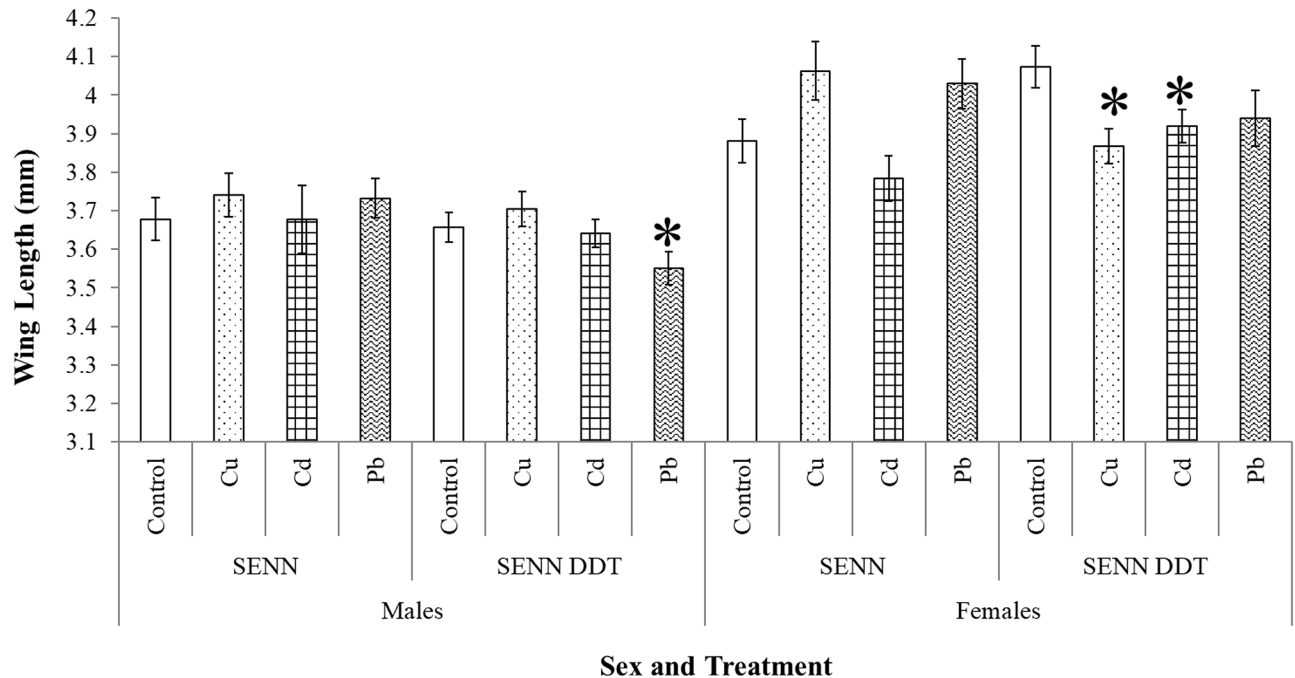
Fig 4. The effect of larval metal exposure on adult size in insecticide susceptible and resistant *Anopheles arabiensis*. Although larval metal treatment had no significant effect on adult size in the insecticide susceptible SENN strain, it did result in significant changes in the size of adults of the resistant SENN DDT strain. SENN DDT males that emerged from larvae exposed to lead nitrate were significantly smaller than those reared in untreated water. Similarly, SENN DDT females that eclosed from larvae that were exposed to copper nitrate and cadmium chloride were significantly smaller than adult females reared in untreated water. Significant differences from the control (p<0.05) are indicated by an asterisk (*).

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The effect of metal exposure at the larval stage on adult insecticide tolerance

Exposure to metal pollution at the larval stage had a marked effect on subsequent insecticide tolerance in adult mosquitoes. In the SENN DDT strain, exposure of larvae to all three metal treatments significantly increased adult insecticide tolerance. For malathion, lead treatment resulted in a 2.2 fold increase in LT50 (2 sample t-test: p<0.01, df = 3.8, T = -17.11), copper treatment in a 4.0 fold increase (2 sample t-test: p<0.01, df = 2.1, T = -14.87) and cadmium in a 5.5 fold increase (2 sample t-test: p<0.01, df = 4, T = -9.30). Similarly, for deltamethrin lead treatment resulted in a 2.5 fold increase in LT50 (2 sample t-test: p = 0.04, df = 3.8, T = 3.96), copper treatment in a 1.4 fold increase (2 sample t-test: p = 004, df = 5, T = -2.72) and cadmium in a 5.5 fold increase (2 sample t-test: p<0.01, df = 3.8, T = -6.10) (Fig 5A).

For the SENN strain, although exposure to copper nitrate did not result in a significant increase in adult LT50 (2-sample t-test: deltamethrin- p = 0.21, df = 2.4, T = -1.71; malathion: p = 0.13, df = 3.5, T = -1.97), cadmium chloride did result in a significant increase in LT50 (2-sample t-test: deltamethrin-p = 0.0.1, df = 5.0, T = -4.45; malathion: p = 0.02, df = 5.0, T = -3.44), as did lead nitrate (2-sample t-test: deltamethrin- p = 0.02, df = 6.0, T = -3.19; malathion: p = 0.02, df = 5.5, T = -3.49) (Fig 5B).

The effect of larval metal treatment on adult detoxification enzyme activity

The effect of larval metal treatment on subsequent adult detoxification enzyme activity was varied. For GST activity, larval metal exposure had no effect on SENN females (2 sample t-test: cadmium- p = 0.12, df = 43.4, T = -1.56; copper- p = 0.50, df = 45.7, T = 0.68; lead- p = 0.19,
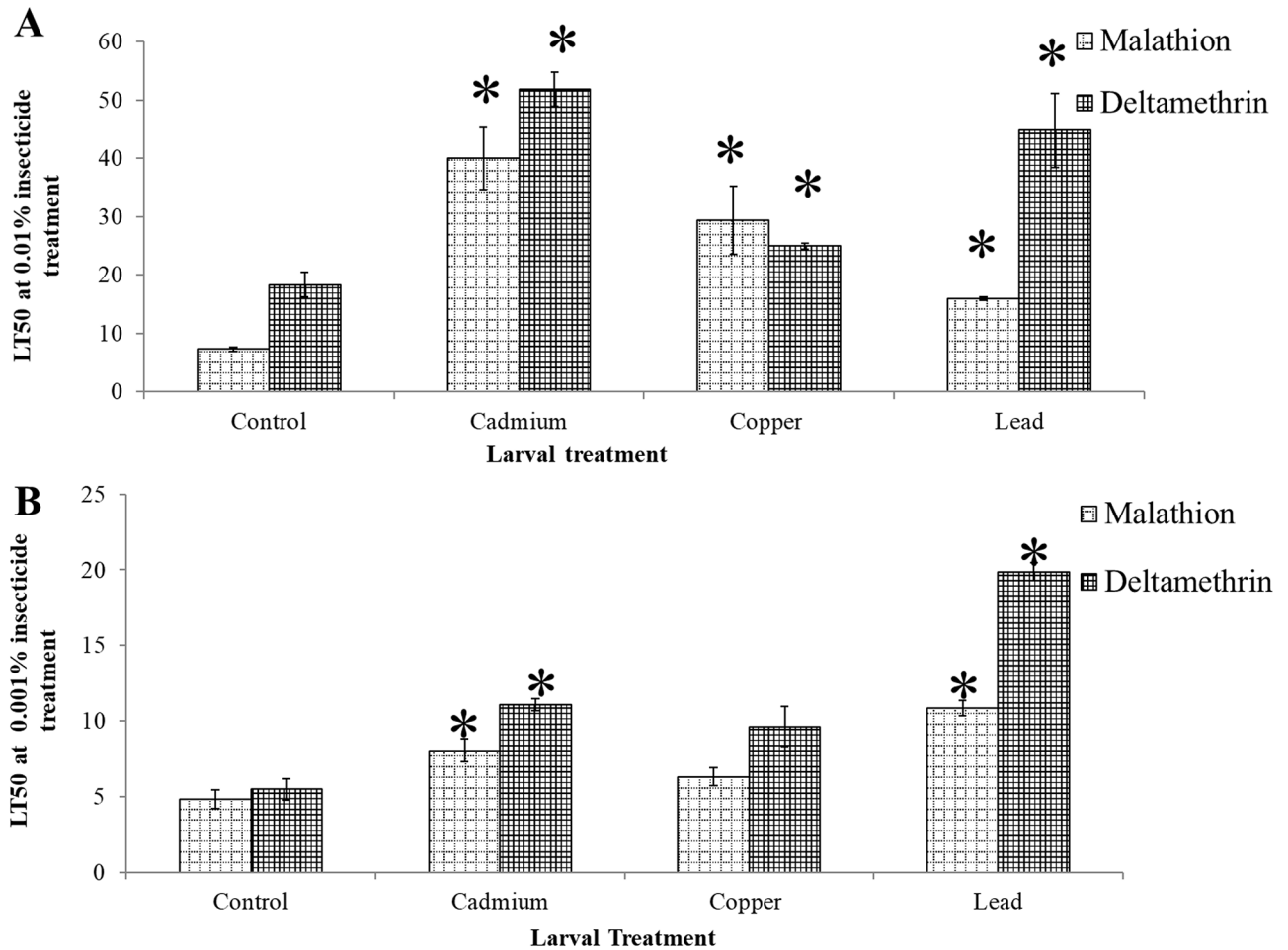
Fig 5. The effect of larval metal exposure on the insecticide tolerance of insecticide susceptible and resistant *Anopheles arabiensis* females. Larval metal exposure resulted in increased tolerance of malathion and deltamethrin in the insecticide resistant SENN DDT strain. All larval metal treatments resulted in a significantly increased lethal time (LT50s) in adult females (A). Larval treatment with cadmium chloride and lead nitrate resulted in a significant increase in malathion and deltamethrin tolerance of adult females of the insecticide susceptible SENN strain. Copper nitrate did not result in a change in tolerance to either insecticide in the SENN strain. Significant changes from the control (p<0.05) are indicated by asterisk (*).

df = 45.8, T = 1.32) or males (2 sample t-test: cadmium- p = 0.82, df = 44.7, T = -0.22; copper- p = 0.66, df = 46.0, T = -0.45; lead- p = 0.90, df = 45.5, T = -0.12). This was also true for SENN DDT males (2 sample t-test: cadmium- p = 0.92, df = 45.3, T = 0.10; copper- p = 0.07, df = 43.3, T = -1.84; lead- p = 0.41, df = 45.9, T = -0.82). There was a significant effect on SENN DDT females where all larval metal treatments decreased GST activity (2 sample t-test: cadmium- p<0.01, df = 46, T = 4.46; copper- p<0.01, df = 46, T = 3.83; lead- p = 0.19, df = 46, T = 7.17) (Fig 6A).

Cytochrome P450 activity was the most affected by exposure of larvae to metals. Although SENN males showed no significant changes (1-way ANOVA: p = 0.45, df = 3, F = 0.90), cadmium treatment did result in a significant increase in P450 activity in SENN females (2 sample t-test: cadmium- p = 0.01, df = 45.3, T = -3.03; copper- p = 0.56, df = 41.6, T = -0.59; lead- p = 0.08, df = 45.8, T = 1.18). For SENN DDT, lead treatment did not induce significant changes, while cadmium and copper treatment resulted in significant increases in enzyme activity in females (2 sample t-test: cadmium- p = 0.03, df = 43.9, T = -2.19; copper- p<0.01,
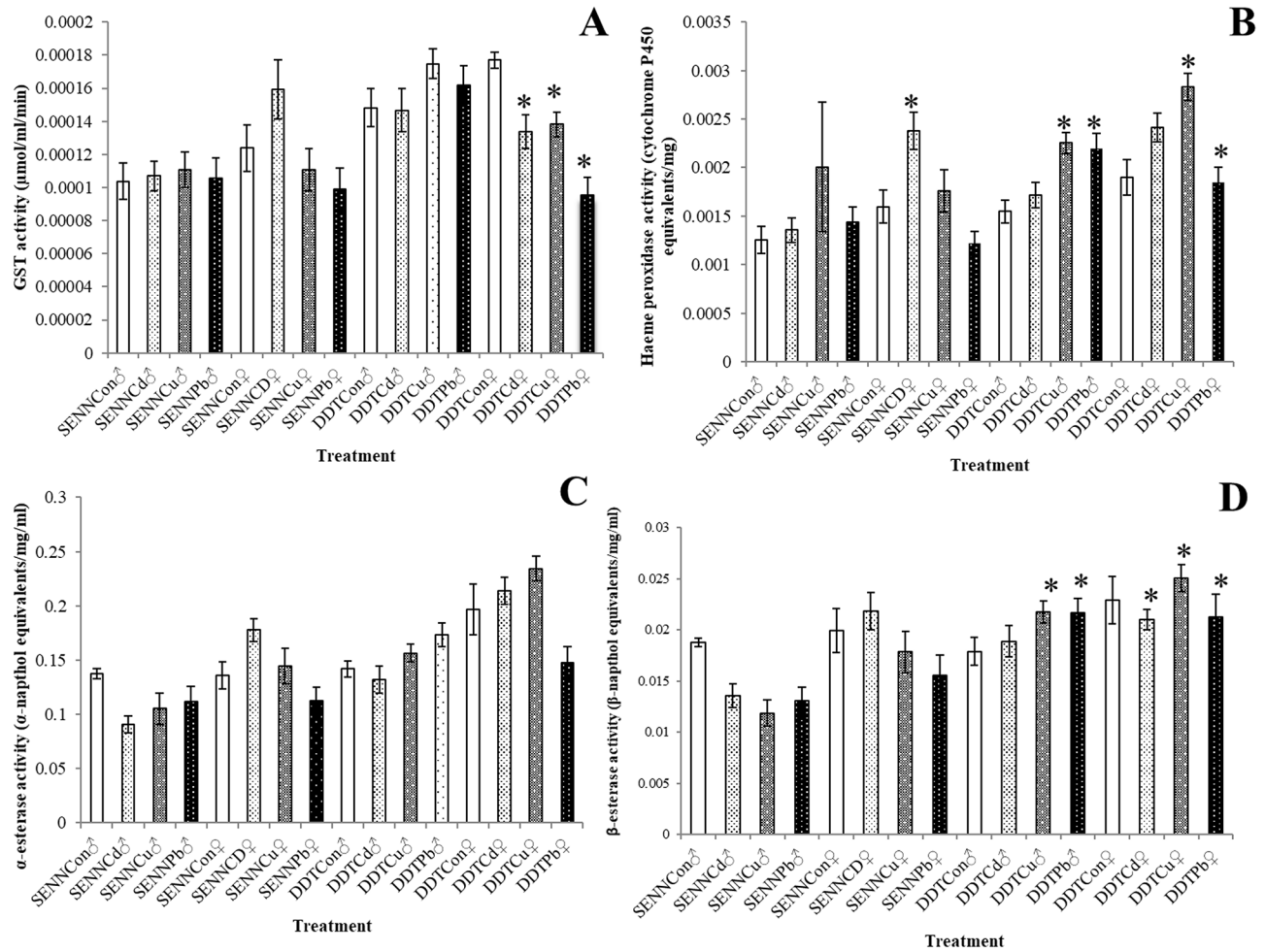
Fig 6. The effect of larval metal exposure on the detoxification enzyme activity of insecticide susceptible and resistant *Anopheles arabiensis* adults. Glutathione S-transferase (GST) activity was significantly decreased in SENN DDT females after all metal treatments, but no other treatments resulted in changes in GST activity (A). Cytochrome P450 activity, however, was significantly affected by certain treatments. SENN females showed a significant increase in activity after larval cadmium exposure. SENN DDT adult males showed significantly increased P450 activity after larval copper nitrate and lead nitrate exposure, while SENN DDT female enzyme activity was significantly increased after cadmium chloride and lead nitrate exposure (B). Neither α-esterase activity (C) nor β-esterase activity (D) was significantly altered in adults after larval metal exposure. Significant changes from control treatment ($p < 0.05$) are indicated by asterisk (*).

df = 43.1 T = -4.04; lead- p = 0.82, df = 44.6, T = 0.22). Although cadmium treatment did not increase P450 activity in females, copper and lead treatment did result in an increase in enzyme activity (2 sample t-test: cadmium- p = 0.35 df = 45.54, T = -0.94; copper- p<0.01, df = 45.9, T = -4.33; lead- p<0.01, df = 42.9, T = -3.32) (Fig 6B).

Alpha esterase activity was not affected by larval treatment with metals in either of the strains (1-way ANOVA: SENN males—p = 0.20, F = 1.56, df = 3; SENN females—p = 0.12, F = 1.99, df = 3; SENN DDT males—p = 0.14, F = 1.89, df = 3; SENN DDT females—p = 0.20, F = 1.56, df = 3). Beta esterase activity was not increased in SENN males (1-way ANOVA: p = 0.14, F = 1.82, df = 3) or SENN DDT females (1-way ANOVA: p = 0.09, F = 2.27, df = 3). In contrast, larval metal treatment did increase beta esterase activity in SENN females (1-way ANOVA: p = 0.02, F = 3.85, df = 3) and SENN DDT males (1-way ANOVA: p = 0.01, F = 4.24, df = 3).

The effect of larval metal treatment on adult oxidative stress enzyme activity

Larval metal exposure did not have a significant effect on the catalase activity of SENN females (1-way ANOVA: p = 0.13, df = 3, F = 1.95) or males (1-way ANOVA: p = 0.18, df = 3, F = 1.65). Similarly, SENN DDT males did not show increased catalase activity (1-way ANOVA: p = 0.31, df = 3, F = 1.21). In contrast, SENN DDT female catalase activity was strongly affected by larval cadmium and copper exposure (1-way ANOVA: p<0.01, df = 3, F = 138), but not lead exposure (1-way ANOVA: p = 0.24, df = 1, F = 1.42) (Fig 7A).

Larval metal exposure variably affected Glutathione peroxidase activity. For the SENN strain peroxidase activity was decreased in males (1-way ANOVA: p<0.01, df = 3, F = 8.61) and females treated with copper and cadmium (1-way ANOVA: p<0.01, df = 2, F = 10.0), but not lead (1-way ANOVA: p = 0.19, df = 1, F = 1.77). In contrast, peroxidase activity was varied in SENN DDT. For SENN DDT males, lead and cadmium treatment did not have a significant effect on peroxidase activity (1-way ANOVA: p = 0.78, df = 2, F = 0.25), but larval copper treatment resulted in a significant decrease (1-way ANOVA: p = 0.05, df = 1, F = 3.84). For SENN DDT females, cadmium treatment did not have a significant effect on peroxidase activity (2-sample t-test: p = 0.39, df = 46, T = 0.86), but copper treatment did result in a significant increase in activity (2-sample t-test: p = 0.02, df = 46, T = -2.41) as did lead treatment (2-sample t-test: p<0.01, df = 46, T = -6.07) (Fig 7B).

Superoxide dismutase activity was most affected by larval metal treatment. For SENN females, cadmium treatment did not have a significant effect (2-sample t-test: p = 0.39, df = 46, T = -0.86), while copper and lead treatment resulted in a significant increase in peroxidase activity (1-way ANOVA: p<0.01, df = 2, F = 35.9). For males, all three lead treatments resulted in a significant increase in enzyme activity (1-way ANOVA: p = 0.02, df = 3, F = 3.41). Similarly, all three metal treatments significantly increased peroxidase activity in both SENN DDT males (1-way ANOVA: p<0.01, df = 3, F = 12.6) as well as females (1-way ANOVA: p<0.01, df = 3, F = 6.28) (Fig 7C).

Discussion

Metal pollution is one of the most important anthropogenic pollutants *Anopheles* mosquitoes are exposed to, both in urban and rural areas. Members of the *Anopheles gambiae* complex are known to breed in unpolluted, temporary bodies of water [7]. These include important malaria vector species that have adapted to these polluted environments [8, 11, 12] and these adaptations are likely to affect life history traits of epidemiological importance [2, 13].

The concentrations of the metals chosen in this study represent amounts that are legally tolerable tolerated in water bodies, showing that even these amounts exert a significantly measurable biological effect on vector mosquitoes i.e.in water bodies. data from this study suggest that increased pollution augments the expression of insecticide resistance in resistant populations. Although this study did not show an overall effect on adult longevity which is therefore unlikely to affect transmission dynamics [38, 39], the data do indicate that insecticide resistant individuals are advantaged under polluted conditions owing to their enhanced detoxification capabilities. This is because insecticide resistant mosquitoes have a higher capacity to cope with pollutants as demonstrated by significantly higher lethal doses of all three metals. This confirms the findings of Poupardin *et al*. [13]. Importantly, besides being able to survive higher doses of larval metal exposure, insecticide resistant mosquitoes also do not show increased costs to fitness (such as reduced adult longevity) following exposure to metal pollutants as compared to insecticide susceptible mosquitoes [40]. This dynamic suggests that metal-polluted environments may inadvertently select for or enhance resistance to insecticides in vector
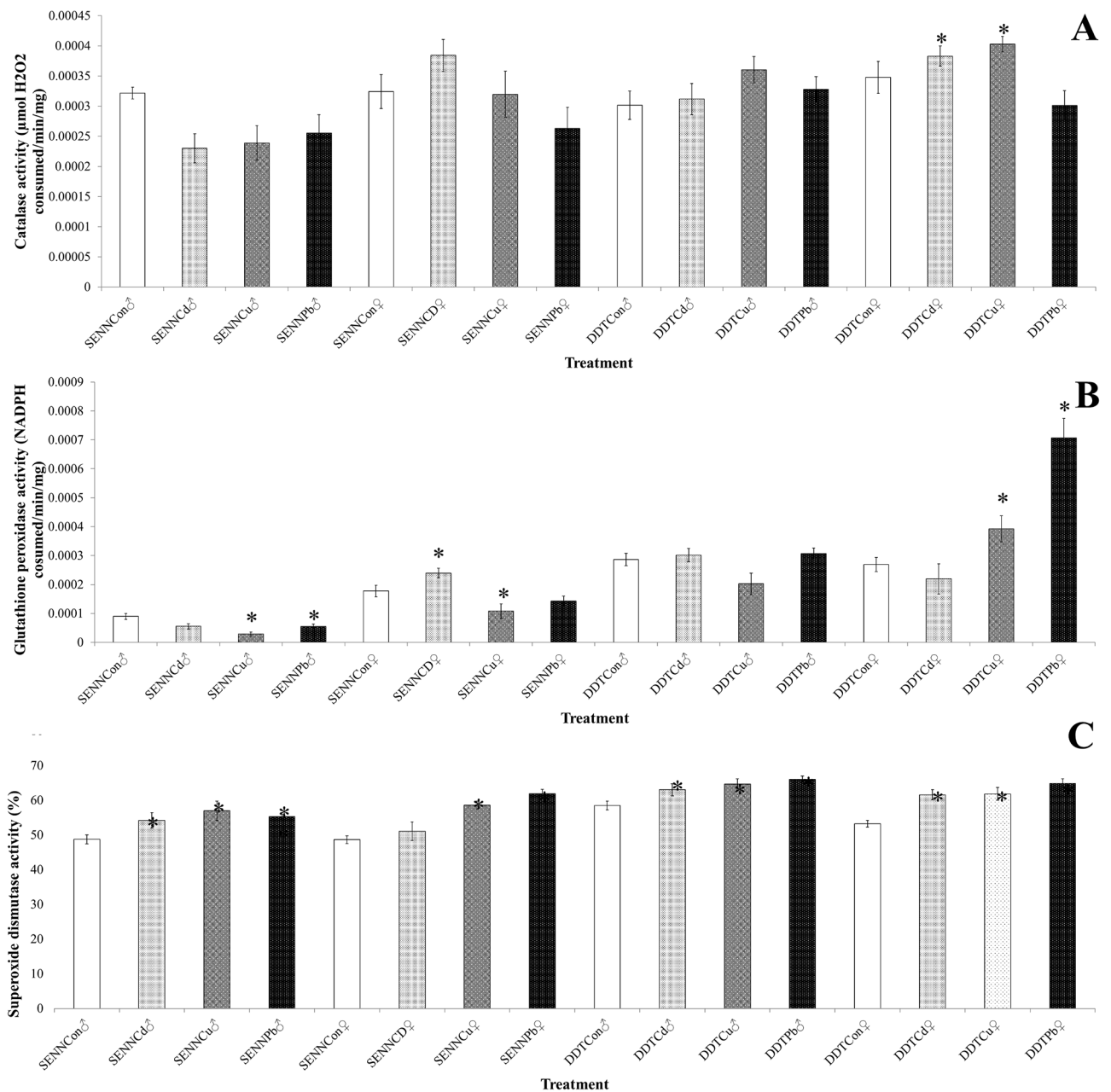
Fig 7. The effect of larval metal exposure on the oxidative stress enzyme activity of insecticide susceptible and resistant *Anopheles arabiensis* adults. Larval metal exposure had a highly variable effect on the three classes of oxidative stress defence enzymes. Catalase was least affected by larval metal exposure, with only SENN DDT females showing increased activity after larval copper nitrate and cadmium chloride exposure (A). Glutathione peroxidase activity showed the greatest variability after larval metal treatment. In the insecticide susceptible SENN strain, all three larval metal treatments significantly decreased peroxidase activity in adult males. In SENN females, although copper nitrate treatment significantly decreased peroxidase activity, cadmium treatment significantly increased adult peroxidase activity. In contrast, larval metal treatment had no effect on adult peroxidase activity in males of the resistant SENN DDT strain, while lead nitrate treatment resulted in a significant increase in activity in females (B). Superoxide dismutase activity was the most uniformly affected, with only lead treatment failing to elicit a significant increase in superoxide dismutase activity (C). Significant changes from the control (p<0.05) are indicated by an asterisk (*).

populations, which could affect subsequent malaria transmission intensity in insecticide-based control settings depending on the strength of the resistance phenotypes concerned.

In terms of life history traits there appears to be a size trade-off in adult insecticide resistant individuals which do not appear to be associated with decreased larval development time i.e.

insecticide resistant mosquitoes that eclose from pollutant exposed larvae are generally smaller. The direct effect of this size differential on malaria transmission is unclear because there are hypotheses that suggest that smaller adults blood feed more frequently (so as to augment their energy reserves and complete their gonotrophic cycles) causing a higher intensity of transmission, as opposed to larger adults that may feed less frequently but are also longer-lived, thereby increasing their transmission potential. [36, 41–44]-44]

It is worth noting that the increased tolerance to insecticide following metal pollutant exposure in both insecticide resistant and susceptible strains mirrors findings from Kenya where the presence of lead correlated positively with the presence of *An. gambiae* and *Ae. aegypti* larvae [10]. The high lethal doses tolerated by *An. arabiensis*, as well as the fact that lead does not affect larval development while augmenting insecticide tolerance, is an important consideration because lead exposure generally affects crucial life history traits in exposed mosquitoes.

When examining the biochemical basis of these responses, it appears that the key detoxification enzymes are the cytochrome P450s and β-esterases. No significant changes were noted in the α-esterases following exposure to metal pollutants and the only significant change in GST activity was decreased activity in insecticide resistant females. Beta esterases were not affected in the insecticide susceptible strain, but increases were noted in males and females of the resistant strain. For the cytochrome P450s, cadmium exposure induced an increase in the activity of insecticide susceptible females. For insecticide resistant mosquitoes, two of three treatments increased cytochrome P450 activity in both males and females. Cytochrome P450 activity is crucial in metal tolerance. Metal tolerant *An. gambiae* females generally showed increased CYP6 expression, while males typically showed decreased CYP6M2, CYP6P3 and CYP6Z1 expression [23]. It is important to note that the data from this study was obtained from metal naïve populations as opposed to metal tolerant strains, in contrast to the Musasia *et al*. [23] study. This study suggests that an interplay between the Phase I enzymes the β-esterases and the cytochrome P450 proteins may be the key mediators in increased insecticide tolerance in adults eclosed from larvae exposed to metal pollutants.

Oxidative stress defence plays an important role in insecticide resistance [30, 45] with increased oxidative stress defence playing a key role in insecticide tolerance [33]. Understanding the effect that larval metal pollution has on adult oxidative stress response is therefore important, not only because of the potential effect on the expression of insecticide resistance in adult mosquitoes, but also because metal pollution is also a major source of oxidative stress in numerous organisms [46]. Glutathione peroxidase activity displayed a sex-specific induction in adults after larval metal exposure. While pollutant exposure reduced peroxidase activity in susceptible males, it increased the activity in females. Similarly, in resistant females, activity was significantly increased, while no changes were noted in males. Superoxide dismutase activity was increased in all treatments except cadmium treatment in susceptible females. Increased defence against oxidative stress following exposure to metal pollutants likely advantages adult mosquitoes in an insecticide polluted environment because defence against oxidative damage also inadvertently leads to enhanced expression of certain insecticide resistance phenotypes via increased expression of GSTs [33].

It is concluded from this study that there is an enzyme-mediated positive link between tolerance to metal pollutants and insecticide resistance in adult *An. arabiensis* mosquitoes. Furthermore, exposure of larvae to metal pollutants may have operational consequences under an insecticide-based vector control scenario by increasing the expression of insecticide resistance in adults, and by affecting certain life history traits. Of the three metals examined, copper nitrate was the most toxic, while neither insecticide resistant nor susceptible larvae were unduly affected by exposure to lead.

Supporting information

S1 Table. Concentrations used to determine lethal concentrations of metals salts. The exact concentration points used to determine the LD50 values for each strain are detailed in this table.
(XLSX)

S2 Table. Supplementary developmental parameters. The days of first and final pupation, as well as final pupation percentages for both SENN and SENN after metal treatment are detailed in this table.
(XLSX)

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Author Contributions

Conceptualization: Shüné V. Oliver.

Data curation: Shüné V. Oliver.

Formal analysis: Shüné V. Oliver.

Funding acquisition: Shüné V. Oliver.

Investigation: Shüné V. Oliver.

Methodology: Basil D. Brooke.

Project administration: Shüné V. Oliver.

Writing – original draft: Shüné V. Oliver.

Writing – review & editing: Basil D. Brooke.

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