

The effect of pollution on the competitive dynamics of *Anopheles arabiensis* Patton, 1905 and *Culex quinquefasciatus* Say, 1823 (Diptera: Culicidae)

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Culex quinquefasciatus Say, 1823 and *Anopheles arabiensis* Patton, 1905 (Diptera: Culicidae) are often found breeding in the same water sources and engage in interspecific competition. Although *Cx. quinquefasciatus* is known to proliferate in a range of polluted environments, the ability of *An. arabiensis* to proliferate in polluted water has only been reported relatively recently. The effects of pollution and insecticide resistance on this competitive interaction are unknown. This study examined the effect of pollution on the dynamics of the interspecific competition. Three laboratory strains were used in this study: an insecticide susceptible and an insecticide resistant *An. arabiensis*, and an insecticide resistant *Cx. quinquefasciatus*. Larval pollutant tolerances of each strain were assessed and compared by determining the lethal concentration at 50% mortality (LC_{50}). The larvae from each strain were exposed to either inorganic fertiliser or copper nitrate, following which eclosion success was assessed. The results showed that the insecticide resistant strains had higher emergence rates when reared in polluted conditions without competition, with the *Cx. quinquefasciatus* strain showing the highest rate of eclosion. This species also had a higher tolerance for metal pollution than the *An. arabiensis* strains. The effects of pollutants on oviposition choice were examined. Pollution altered adult oviposition choice. The effect of larval metal exposure had variable effects on the activity of metabolic detoxification enzymes. An insecticide resistant phenotype had greater tolerance to pollutants and possibly developmental advantages based on a variable detoxification response to the pollutant. Pollution can therefore alter interspecific competition dynamics between the malaria vector *An. arabiensis* and *Cx. quinquefasciatus*.

INTRODUCTION

The *Anopheles gambiae* Patton, 1905 complex (Diptera: Culicidae) consists of nine morphologically identical species of which six are either primary or secondary vectors of malaria. Primary vector species in this complex include *An. gambiae* sensu stricto, *An. arabiensis* and *An. coluzzii* (Sinka et al. 2010). One of the defining characteristics of the *An. gambiae* complex is that member species prefer to proliferate in shallow, sunlit, temporary bodies of relatively clean water (Gillies & Meillon 1968; Sinka et al. 2010). This is in contrast to *Culex* mosquito species, which have been found to proliferate in heavily polluted water, such as sewage and chemically treated water of septic tanks (Calhoun et al. 2007; Charlwood 1994; Correia et al. 2012).

The level of anthropogenic pollution is increasing (Donohue & Garcia Molinos 2009; Matson et al. 2002), with *Anopheles* mosquitoes evidently adapting to breeding in polluted waters, most notably *An. gambiae* (Antonio-Nkondjio et al. 2011; Awolola et al. 2007) and *An. arabiensis* (Jones et al. 2012). As a result, the diversity of breeding habitats utilised by the *An. gambiae* complex is expanding, placing these species in increased competition with other mosquitoes, such as those of the *Culex pipiens* Say, 1823 complex (Diptera: Culicidae) of which *Cx. quinquefasciatus* is a member.

Anopheles arabiensis is a major malaria vector in southern Africa (Sinka et al. 2010). This adaptable vector poses a particular challenge to those vector control methods that are based on the indoor application of insecticides [i.e. indoor residual spraying (IRS) and insecticide-treated bed nets] (Kitau et al. 2012), enabling this species to sustain low-level outdoor transmission, known as residual malaria (Killeen 2014). *Anopheles arabiensis* can flourish in agricultural and urban settings, making the relationship between this species and land use important for the control of malaria transmission (Githeko et al. 1996; Ijumba & Lindsay 2001; Mwangangi et al. 2006).

Anopheles arabiensis breeds in the same habitats as the common house mosquito, *Cx. quinquefasciatus*. As such, these two species are subject to interspecific competition. Interspecific competition between mosquito larvae has previously been examined in laboratory and field settings (Kirby & Lindsay 2009; Reiskind & Lounibos 2009; Kweka et al. 2012). The effect of environmental pollution on this dynamic, particularly between *An. arabiensis* and *Cx. quinquefasciatus*, has not been examined. Furthermore, the role of existing insecticide resistant phenotypes in larval interspecific competition in polluted conditions has not been evaluated.

Pollutant exposure during the immature aquatic stages may have consequences for subsequent adult mosquito life history traits (Oliver & Brooke 2018a; Oliver & Brooke 2018b). Examination of the effect of agricultural and urban pollutants on both malaria vectors and non-vectors is therefore important. Due to their preferred breeding sites, members of the *An. gambiae* complex

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and the *Cx. pipiens* complex would therefore be subject to the same environmental stressors. This has the potential to alter disease transmission dynamics. *Anopheles* mosquitoes transmit malaria, while *Culex* mosquitoes transmit arboviruses and filarial nematodes (Bartlow et al. 2019). The effects of competition on their relative abundances could translate into changes in the rates of disease transmission.

The aim of this study was to examine interspecific larval competition between *An. arabiensis* and *Cx. quinquefasciatus* in relation to common pollutants and insecticide resistance. The effects of pollution on competitive success were examined, as were the effects of levels of pollution on detoxification enzyme activity in larvae.

MATERIALS and METHODS

Laboratory-reared *Cx. quinquefasciatus* and *An. arabiensis* strains were utilised in this study. The two strains of *An. arabiensis* used were SENN and SENN-DDT. The SENN-DDT strain was selected for DDT resistance specifically, but also displayed resistance to deltamethrin, permethrin, λ -cyhalothrin (pyrethroids) and malathion (organophosphate) (Oliver & Brooke 2013). An insecticide resistant strain of *Cx. quinquefasciatus*, QUINQS, was included in the study for comparative purposes. This strain was resistant to DDT and pyrethroids.

Original sources of founding populations for mosquito colonies

Anopheles arabiensis (SENN strain): The insecticide susceptible SENN strain was originally from Sennar, Sudan, and has been maintained as a laboratory colony at the Botha de Meillon insectary, Johannesburg, South Africa, since 1980.

Anopheles arabiensis (SENN-DDT strain): The insecticide resistant SENN-DDT strain was selected from the Sudanese SENN strain by continuous selection with 4% DDT, and has been maintained as a laboratory strain at the Botha de Meillon insectary, Johannesburg, South Africa, since 1995.

Culex quinquefasciatus (QUINQS): The insecticide resistant QUINQS strain was originally from Johannesburg, South Africa, and has been maintained as a laboratory strain at the Botha de Meillon insectary, Johannesburg, South Africa, since 2010.

Rearing conditions

All larvae were fed a mixture of Tetrafin™ Plus (480 mg in 15 mL dH₂O) and/or a standard diet consisting of powdered dog biscuits and yeast in a 5:1 ratio (Hunt et al. 2005).

Determination of relative pollutant toxicity in *Culex quinquefasciatus* and *Anopheles arabiensis*

Dose response experiments were used to determine the lethal metal concentrations that would induce a larval mortality of 50% (LC₅₀). The source of the metal pollutants for cadmium, copper and lead were cadmium chloride (CdCl₂), copper nitrate (Cu(NO₃)₂) and lead nitrate (Pb(NO₃)₂). Wondersol™ (Wondersol™ All Purpose Fertiliser) was the commercial fertiliser used for testing purposes. The concentrations of nitrogen (N), phosphorus (P) and potassium (K) in Wondersol™ were 103.5 g/kg (N), 23.0 g/kg (P) and 59.5 g/kg (K) respectively. In order to test the effects of N, P and K on larvae separately, rather than in combination (viz. Wondersol™), urea was used as a source of N, superphosphate as a source of P and potassium chloride as a source of K. Twenty-five fourth instar larvae were exposed to the selected concentrations for each pollutant treatment and the test replicated four times. Fourth instar larvae, the final larval stage before pupation, were selected due to their relatively large size. The relatively large size of the larvae made it easier to select equivalently sized individuals for a homogenous test population. All experiments were

performed in 100 mL of distilled water (dH₂O) in paper cups. All experiments were performed at 25 °C (± 2 °C) and 80% humidity (± 5%). All experiments were set up during the light period of the insectary. During the 24-hour experimental period, the larvae were exposed to a 12:12 hour light/dark cycle with a 30-minute duration for dawn/dusk. Treatment concentrations follow WHO guidance on larviciding (WHO 2005) and are detailed in Table 1a–c. Each exposure lasted 24 hours at which point mortality was recorded. This provided an indication of the ability of each strain to cope with pollution stress. Lethal concentrations (LC₅₀s) for each strain were determined using a probit method of analysis (Finney 1952). If the control mortality exceeded 5% Abbott's formula was applied as a correction (Abbott 1925).

Table 1a. Metal treatment concentrations (i.e. exposures) used for fourth instar SENN, SENN-DDT, and QUINQS mosquito larvae

Mosquito strain	Metal	Concentrations (mg/L)				
SENN	Cd	0.0625	0.0312	0.0156	0.0078	0.0039
	Cu	0.0625	0.0312	0.0156	0.0078	0.0039
	Pb	8000	4000	2000	1000	N/A
SENN-DDT	Cd	0.25	0.13	0.0625	0.0312	0.0156
	Cu	4	2	1	0.5	N/A
	Pb	8000	4000	2000	1000	N/A
QUINQS	Cd	2	1	0.5	0.25	0.125
	Cu	10	8	4	2	1
	Pb	8000	4000	2000	1000	N/A

Table 1b. Inorganic fertiliser component treatment concentrations (i.e. levels of exposure) used for fourth instar SENN, SENN-DDT, and QUINQS mosquito larvae

Mosquito strain	Inorganic fertiliser component	Concentrations (mg/L)				
SENN	Urea	160	140	120	100	80
	Superphosphate	80	40	20	10	5
	Potassium chloride	40	35	30	25	20
SENN-DDT	Urea	160	140	120	100	80
	Superphosphate	80	40	20	10	5
	Potassium chloride	40	35	30	25	20
QUINQS	Urea	100	80	60	40	20
	Superphosphate	80	40	20	10	5
	Potassium chloride	50	45	40	35	30

Table 1c. Wondersol™ NPK fertiliser treatment concentrations (i.e. exposures) used for fourth instar SENN, SENN-DDT, and QUINQS mosquito larvae

Mosquito strain	Inorganic fertiliser	Concentrations (mL fertiliser/100 mL dH ₂ O)				
SENN	Wondersol™	8	6	4	2	1
SENN-DDT	Wondersol™	8	6	4	2	1
QUINQS	Wondersol™	8	7	6	5	4

The effect of pollution on interspecific competition between *Culex quinquefasciatus* and *Anopheles arabiensis* larvae

Culex quinquefasciatus and *An. arabiensis* (SENN and/or SENN-DDT strains) first instar larvae were placed together in containers (415 × 295 × 40 mm) filled with 1 L dH₂O. Each tray contained two hundred larvae (one hundred from each species/strain) in combinations of either QUINQS and SENN or QUINQS and SENN-DDT. The experimental set up was replicated in triplicate with larvae emerging from different egg batches. The separation of SENN and SENN-DDT was performed to allow clear phenotypic distinction among adults, which would not be possible with the morphologically indistinguishable *An. arabiensis* strains and allowed enumeration of emergent adults. Controls were left untreated, while exposure trays were treated with either 0.2% Wondersol™ (V:V), or 1.86 µg/L copper nitrate. The Wondersol™ concentrations were based on a value equivalent to a LC₂₅ value for all three strains. The concentration chosen for copper nitrate was the maximum acceptable toxicity concentration (MATC) for this metal. Copper nitrate was chosen as cadmium chloride proved highly toxic to the *An. arabiensis* strains, while lead nitrate was highly tolerated (as summarised in the LC₅₀ data obtained from the relative pollutant toxicity study described above). Larvae were allowed to pupate and the numbers of adults emerging were counted. To control for the effect of pollution without competition, each of the strains was reared separately in polluted environments at the same concentrations used for the competition study. One hundred QUINQS, SENN and SENN-DDT first instar larvae were each placed separately into trays containing 1 L of dH₂O, supplemented with either fertiliser or copper nitrate. Larvae reared in clean dH₂O were used as a control. The experiment was replicated three times from three different cohorts arising from three separate egg batches.

Oviposition site choice

SENN, SENN-DDT and QUINQS adults emerging from larvae reared under standard insectary conditions were collected. Adults were collected before the age of 24 hours and segregated by sex until the experiment was performed. Samples from each strain were kept separately in a male:female ratio of 2:1 ($n = 50$ males, $n = 25$ females). They were allowed to feed to repletion on a human volunteer (co-author SVO) at three and seven days. At the age of 11 days they were allowed to oviposit, during which they were offered a choice of an egg plate containing either clean water, water polluted with 0.7% NPK fertiliser, or water polluted with 0.7 mg/L copper nitrate. Blood feeding was performed as per ethics waiver S Oliver 03-01-2018 from the University of the Witwatersrand. Both the polluted water and clean water egg plates were available in the same cage. The experiment was replicated three times, with adults arising from three independent egg batches. Each of the biological replicates consisted of three technical replicates ($n = 225$ females, $n = 450$ males).

Assessment of *Anopheles arabiensis* SENN and SENN-DDT larval detoxification enzymes

The effect of metal pollutants on larval detoxification enzymes was assessed to determine the species-specific mechanisms for coping with elevated metal concentrations. Previous studies on the effect of metal concentrations on larval detoxification enzyme levels in SENN and SENN-DDT adults indicated that cytochrome P450, glutathione S-transferase (GST), general esterases and glutathione peroxidases were all affected (Oliver & Brooke 2018b). The effects of metal concentrations on these larval enzymes were therefore examined to assess if similar effects would be found in larvae rather than adults. Metals were selected, as there were large differences and variations in tolerance

in this class of xenobiotic, based on the results obtained from the pollutant tolerance study. Furthermore, the role of enzymes in the detoxification of metals, in the adults of the strains selected for the study, have been described in earlier studies (Oliver & Brooke 2018b). Larvae were reared from hatchlings to the fourth instar, under untreated (control) and treated (metal) conditions. Metal treatments were conducted at the MATCs (0.36 µg/L CdCl₂, 1.86 µg/L Cu(NO₃)₂, and 4.39 µg/L Pb(NO₃)₂) (Mireji et al. 2008; Mireji et al. 2010). Forty-eight samples were taken from each treatment, repeated for both species/strains. Larvae were rinsed in dH₂O to avoid metal contamination in the homogenate. Two larvae were used per reaction. Samples were homogenised using a Qiagen TissueLyser II. Samples were stored at -70 °C until required for further use. All biochemical assays were conducted as per Oliver & Brooke (2017). In brief, samples were assessed for metabolic detoxification enzyme activities (cytochrome P450, glutathione S-transferase and general esterase activity) and oxidative stress enzyme activity (glutathione peroxidase). Cytochrome P450 activity was measured as haeme peroxidase activity, determined by the peroxidation of 3,3',5,5'-tetramethyl benzidine, measured as an end product at 650 nm. Glutathione S-transferase activity was measured as the conjugation of chlorodinitrobenzene, measured kinetically at 340 nm. General esterase activity was measured as the conversion product of 1-Naphthol or 2-Naphthol acetate to naphthol, measured at 570 nm. All the quantified products were standardised by dividing each reaction by total amount of protein per sample. Endpoint assays were determined by calibration to a standard curve (Cytochrome C: Sigma-Aldrich Product Number C2867 at concentrations of 0, 0.25, 0.5, 1 and 2 M; 1-Naphthol: Sigma-Aldrich Product Number N1000; 2-Naphthol: Sigma-Aldrich Product Number 185507 at concentrations of 0, 25, 50, 100, 200 and 400 M). All calorimetric readings were performed using a Multiskan Ascent 96 well microplate reader (AEC Amersham: Thermo Scientific).

Statistical analysis

All statistical analyses were performed at a 95% confidence interval. Samples were tested for normality using the Shapiro-Wilks test (Shapiro & Wilk 1965). Lethal concentrations were determined using probit analysis (Finney 1952). Differences in mean enzyme activity and the ovipositional activity index (OAI) (Kramer & Mulla 1979) were determined either with a two-sample *t*-test, or a one-way ANOVA, with a Tukey HSD as a post hoc test (Tukey 1949). Bartlett's test for equality of variance was performed for each ANOVA (Bartlett 1937). The multiple comparisons between means in the enzyme assays were corrected by the Benjamini-Hochberg procedure. All statistical analyses were performed using either IBM SPSS version 22 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp), or Statistix 8 (Analytical Software, Tallahassee, FL).

RESULTS

Relative toxicity and larval tolerance to pollutants

The capacity of fourth instar larvae to tolerate metal salts and fertiliser were used as a proxy for the capacity to cope with pollutants in nature. The LC₅₀ values for metal salt exposures show that both SENN-DDT and QUINQS larvae coped with Cu(NO₃)₂ and CdCl₂ stress significantly better than SENN (one-way ANOVA: cadmium: $p < 0.01$, $F_{(2,8)} = 17.5$; copper: $p < 0.01$, $F_{(2,8)} = 18.8$) (Table 2).

An LC₅₀ estimate for Pb(NO₃)₂ was not possible for the QUINQS strain, as they were extremely tolerant to this pollutant, necessitating an LC₃₀ estimate instead. Based on the estimates for LC₃₀, QUINQS and SENN-DDT showed a significantly higher tolerance to Pb(NO₃)₂ than SENN (one-way ANOVA: $p =$

0.04; $F_{(2,8)} = 5.21$). When comparing LC_{30} values for QUINQS and SENN-DDT, the QUINQS strain showed a significantly higher LC_{30} value (two-sample t -test: $p = 0.01$; $t = 4.85$) (Table 2).

SENN-DDT showed the highest tolerance to urea (one-way ANOVA: $p = 0.04$, $F_{(2,8)} = 5.98$). Tolerance to superphosphate was low across all strains, with no significant difference between them (one-way ANOVA: $p = 0.13$, $F_{(2,8)} = 2.84$). The QUINQS strain showed the highest tolerance to potassium chloride (KCl) (one-way ANOVA: $p = 0.01$, $F_{(2,8)} = 9.43$, Tukey's critical $Q = 4.34$) (Figure 1C). The tolerance of the larvae to fertilisers in combination showed no significant differences between strains (one-way ANOVA: $p = 0.1$, $F_{(2,8)} = 3.49$) (Table 2).

Table 2. Lethal concentrations (LC_{50}) of pollutants. Concentrations are given in mg/L and confidence intervals are given in parentheses. Lead nitrate values represent LC_{30} values, as well as extrapolated LC_{50} values

LD ₅₀ mg/L	Strain		
	SENN	SENN-DDT	QUINQS
Cadmium chloride	0.03 (0.01–0.6)	0.12 (0.03–0.52)	1.04 (0.49–1.44)
Copper nitrate	0.04 (0.02–0.08)	1.01 (0.26–2.38)	7.79 (6.34–10.31)
Lead nitrate (LD_{30})	42.6 (41.5–120.6)	53.3 (34.6–120.6)	60.8 (41.5–120.6)
Lead nitrate (LD_{50})	60.8 (41.5–122.9)	93.2 (66.6–223.9)	93.3 (63.4–224.1)
Urea (N)	9.79 (8.99–10.48)	10.75 (9.97–11.46)	9.20 (8.40–10.49)
Superphosphate (P)	1.83 (0.93–3.25)	1.70 (1.02–2.61)	2.63 (2.08–3.38)
Potassium chloride (K)	2.94 (2.81–3.19)	3.04 (2.86–3.23)	3.79 (3.59–3.97)
NPK combination fertiliser	5.49 (4.38–7.78)	4.98 (3.47–11.23)	5.75 (5.43–6.12)

The effects of fertiliser and exposure to metal pollutants on adult emergence under competitive conditions

As adult emergence is a crucial measure of life history success, it can be used to assess the effect of pollutant concentrations on larvae and hence give insight into competitive success. Adult emergence was used rather than development rate for this purpose. This was because development rate is normally measured as time to pupation, and the pupae of the different species could not be accurately identified. Under control conditions, QUINQS had a greater number of adults emerging compared to both SENN and SENN-DDT (one-way ANOVA: $p = 0.04$, $F_{(2,6)} = 6.12$, Benjamini-Hochberg correction: SENN vs QUINQS: $p = 0.04$, $q = 0.04$; SENN-DDT vs QUINQS: $p = 0.02$, $q = 0.03$). When all three strains were reared as single strains in water polluted with fertiliser, SENN had a significantly lower rate of emergence than QUINQS (one-way ANOVA: $p = 0.03$, $F_{(2,6)} = 6.43$; Benjamini-Hochberg correction: SENN vs QUINQS: $p = 0.01$, $q = 0.02$; SENN-DDT vs QUINQS: $p = 0.08$, $q = 0.08$; SENN vs SENN-DDT: $p = 0.2$, $q = 0.14$). When exposed to elevated metal concentrations, both SENN and SENN-DDT had a lower rate of emergence than QUINQS, although SENN-DDT had an increased rate of emergence compared to the SENN strain (one-way ANOVA: $p < 0.01$, $F_{(2,6)} = 29.01$, Benjamini-Hochberg correction: SENN vs QUINQS: $p < 0.01$, $q < 0.01$; SENN-DDT vs QUINQS: $p = 0.02$, $q = 0.02$; SENN vs SENN-DDT: $p < 0.01$, $q < 0.01$) (Figure 1A).

In competitive conditions, both *An. arabiensis* strains, SENN and SENN-DDT, were at a competitive disadvantage when exposed to elevated levels of fertiliser in water, with lower rates of adult emergences than QUINQS (one-way ANOVA: $p < 0.01$, $F_{(3,8)} = 12.5$; Benjamini-Hochberg correction: SENN vs QUINQS: $p < 0.01$, $q < 0.01$; SENN-DDT vs QUINQS: $p < 0.01$, $q < 0.01$) (Figure 1B). No significant differences were observed in adult emergence when strains were cohabiting in water with elevated metal concentrations (Figure 1C).

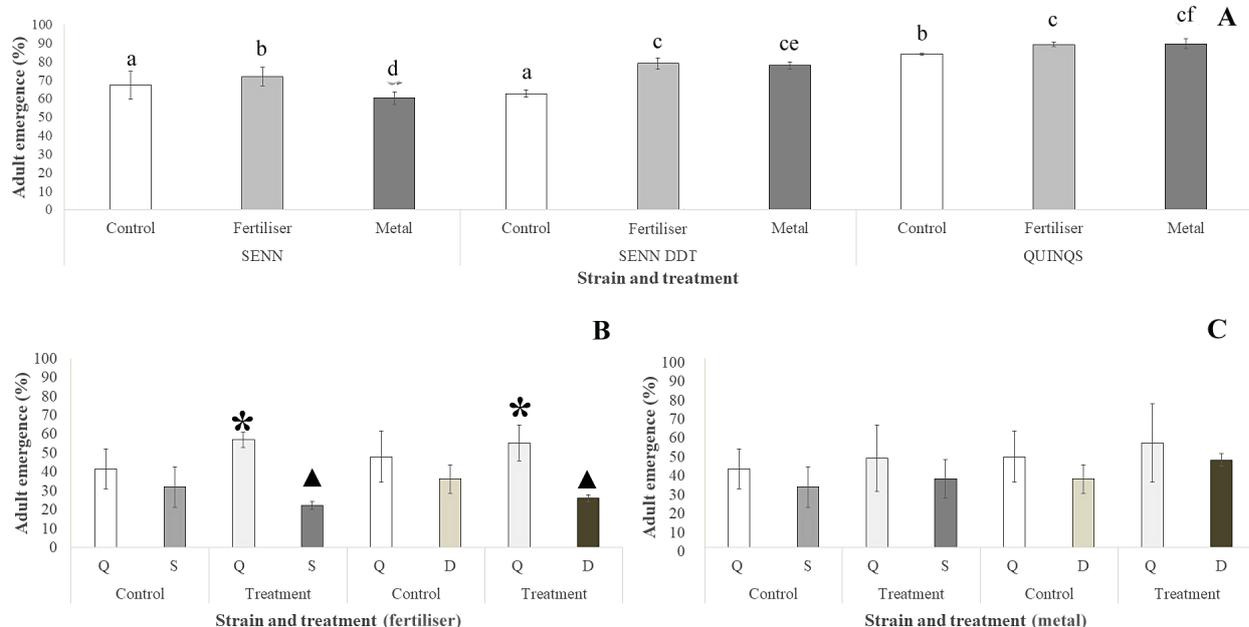


Figure 1. The effect of larval pollutant exposure on the emergence of *Anopheles arabiensis* and *Culex quinquefasciatus* adults.

A: Emergence of SENN (*An. arabiensis*, insecticide susceptible), SENN-DDT (*An. arabiensis*, insecticide resistant) and QUINQS (*Cx. quinquefasciatus*, insecticide resistant) under polluted conditions, without competition. Letters indicate significant differences, B: Emergence of SENN (S), SENN-DDT (D) and QUINQS (Q) under competitive conditions with NPK fertiliser pollution. Significantly more QUINQS adults emerged under competition conditions, while adult emergence was significantly reduced in SENN and SENN-DDT. C: Emergence of SENN (S), SENN-DDT (D) and QUINQS (Q) under competitive conditions with metal pollution. No significant changes were observed. In all cases, asterisks (*) indicate a significant change from the control, while triangles (▲) indicate a significant difference between strains

Oviposition site choice

Under control conditions, the SENN strain laid an average of 22.56 eggs per female, while the SENN DDT strain laid 14.94 eggs per female. The SENN strain laid an average of 9.95 eggs in metal-treated water and 27.19 eggs in fertiliser treated water. The SENN-DDT strain laid 13.08 eggs in metal-treated water and 19.31 eggs in fertiliser treated water. The OAI was calculated for each strain and treatment (Table 3). Metal treatment was found to be an attractant for the QUINQS strain, but a repellent for the SENN strain. Although metal concentration resulted in a negative OAI, it did not exceed the threshold for classification as ovipositioning repellent. Fertiliser, by contrast, was an attractant for SENN-DDT and QUINQS. Although the SENN strain had positive OAI values for the fertiliser treatment, the values were not large enough for it to be considered an attractant. Both treatments demonstrated that elevated concentrations of metals and fertiliser could be considered attractants, although there was no significant difference in the OAI of the treatments for the QUINQS strain (one-way ANOVA: $p = 0.99$, $F_{(1,8)} = 0.00$). There was a significant difference between the OAI for concentrations of metal and fertiliser for the SENN (one-way ANOVA: $p = 0.01$, $F_{(1,8)} = 20.1$) and SENN-DDT strains (one-way ANOVA: $p = 0.04$, $F_{(1,8)} = 9.15$).

Assessment of the *Anopheles arabiensis* SENN and SENN-DDT enzyme detoxification systems

Cytochrome P450 activity in the *An. arabiensis* strains was increased by exposure to copper (two-sample t -test: SENN: $p = 0.02$, $t = 2.36$; DDT: $p = 0.01$, $t = -2.66$). In contrast, while lead

Table 3. Oviposition activity index (OAI) for SENN, SENN-DDT and QUINQS adults when ovipositing in metal or fertiliser polluted water. The mean OAI and standard deviation (in parentheses) are given

Treatment	Mosquito strain		
	SENN	SENN-DDT	QUINQS
Metal	-0.45 (± 0.15)	-0.25 (± 0.19)	0.36 (± 0.09)
Fertiliser	0.14 (± 0.01)	0.35 (± 0.05)	0.36 (± 0.09)

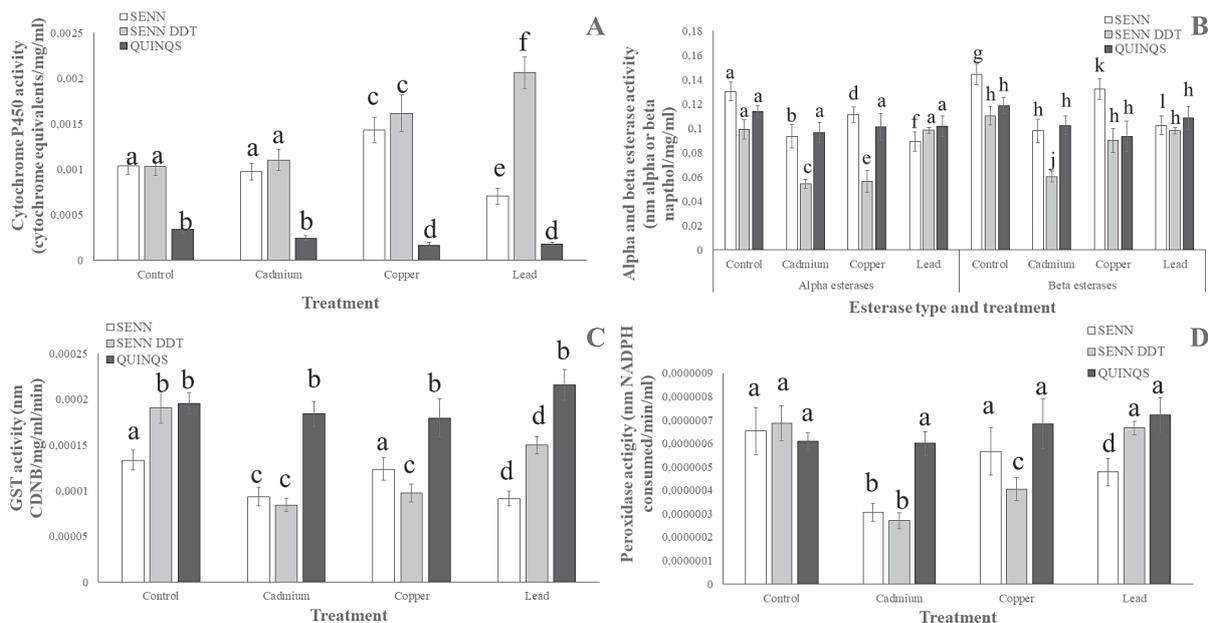


Figure 2. The effect of metal pollutant exposure on the detoxification enzyme activity of *Anopheles arabiensis* and *Culex quinquefasciatus* fourth instar larvae. A: Cytochrome P450 activity in fourth instar larvae measured as haeme peroxidase activity. B: General esterase (α -esterase and β -esterase) activity in fourth instar larvae. C: Glutathione S-transferase activity in fourth instar larvae. D: Glutathione peroxidase activity in fourth instar larvae. Differences in activity are indicated by different lowercase letters

exposure increased activity in SENN-DDT (two-sample t -test: $p < 0.01$, $t = -5.18$), it decreased activity in SENN (two-sample t -test: $p = 0.01$, $t = 2.64$). Cytochrome P450 activity in QUINQS was always lower than for the *An. arabiensis* strains (one-way ANOVA: $p < 0.01$, $F_{(11;288)} = 36.5$), with copper and lead exposure associated with significantly lower levels of activity than the control (one-way ANOVA: $p = 0.03$, $F_{(2;54)} = 3.46$) (Figure 2A).

Alpha esterase activity in SENN decreased after all metal treatments (one-way ANOVA: $p < 0.01$, $F_{(3;95)} = 5.49$). Only cadmium and copper treatment significantly decreased α -esterase activity in SENN-DDT (one-way ANOVA: $p < 0.01$, $F_{(3;95)} = 15.4$). Activity in the QUINQS strain remain unchanged regardless of metal treatment (one-way ANOVA: $p = 0.51$, $F_{(3;95)} = 3.46$). Similarly, with β -esterases all metal treatments significantly reduced activity in the SENN strain (one-way ANOVA: $p < 0.01$, $F_{(3;95)} = 7.05$). For SENN-DDT, only the cadmium treatment significantly reduced β -esterase activity (one-way ANOVA: $p < 0.01$, $F_{(3;95)} = 10.4$). Beta esterase activity in QUINQS remained unchanged regardless of treatment (one-way ANOVA: $p = 0.28$, $F_{(3;95)} = 1.30$) (Figure 2B).

The GST activity in SENN-DDT decreased significantly after all treatments (one-way ANOVA: $p < 0.01$, $df_{(3;95)} = 18.4$), but only for cadmium and lead treatments in SENN (two-sample t -test: cadmium: $p = 0.01$, $t = 2.67$; lead $p < 0.01$, $t = 3.11$). GST activity in the QUINQS strain was unaffected by metal treatment (one-way ANOVA: $p = 0.39$, $F_{(3;95)} = 1.01$) (Figure 2C).

Glutathione peroxidase activity was similar to GST activity in that cadmium and lead activity decreased in SENN (one-way ANOVA: $p = 0.02$, $F_{(3;95)} = 3.26$), while copper and cadmium activity reduced in SENN-DDT (one-way ANOVA: $p = 0.02$, $F_{(3;95)} = 5.92$). As with most enzymes, the peroxidase activity in QUINQS remained unchanged (one-way ANOVA: $p = 0.58$, $F_{(3;95)} = 0.65$) (Figure 2D).

DISCUSSION

Interspecific competition between disease vector species has the capacity to alter the dynamics of disease transmission (Impoinvil et al. 2008). To date, interspecific competition studies have tended to focus primarily on competition between mosquitoes that breed in containers (e.g. Alto et al. 2013; Camara et al. 2016;

Farjana et al. 2012; Lounibos et al. 2016; Noden et al. 2016; Yee et al. 2014), or inter-anopheline competition (e.g. Davies et al. 2016; Gimonneau et al. 2014; Koenraadt & Takken 2003; Schneider et al. 2000), while studies on *Culex/Anopheles* interspecific competition have often focussed on succession in agricultural settings (Muturi et al. 2008; Muturi et al. 2007; Mwangangi et al. 2006).

The data presented here showed a complex interaction between the aquatic stages of *Culex* and *Anopheles* mosquitoes, mediated by exposure of larvae to pollutants and the presence/absence of insecticide resistant phenotypes. In the absence of competition, insecticide resistance confers an advantage in polluted aquatic environments. The insecticide resistant SENN-DDT (*An. arabiensis*) and QUINQS (*Cx. quinquefasciatus*) strains were shown to be at an advantage, due to greater adult emergence after exposure to polluted water.

An examination of larval eclosion in polluted conditions (without competition), showed that rates of emergence for SENN-DDT in these conditions were better for both metal and fertiliser treatments than the control. For QUINQS only the fertiliser treatment resulted in an increased rate of emergence compared to the control in conditions without competition. While no effects on adult emergence (in competitive conditions) were observed for metal treatments, there was a marked difference when subjected to the fertiliser treatment. The QUINQS strain performed significantly better in terms of adult emergence following fertiliser treatment under non-competitive conditions. This carried through to the competition study. SENN, which showed no advantage under fertiliser polluted conditions, and SENN-DDT, which showed increased adult survival under polluted conditions, were subsequently disadvantaged when competing with QUINQS.

These data indicated that *Cx. quinquefasciatus* should predominate when competing with *Anopheles*, but this has not always been observed in the field. A study of competition between *An. gambiae* s.s. and *Cx. quinquefasciatus* found little difference in life history traits between the two species, except for a decrease in adult size in *An. gambiae* (Kweka et al. 2012). Succession studies in a Kenyan rice field demonstrated that *An. arabiensis* and *Cx. quinquefasciatus* were the dominant species with abundances of 45% and 35.8% respectively (Muturi et al. 2007). This indicated that neither species had a marked advantage over the other in these conditions. There have been reports of different dominant species in different environments. For example, *Cx. quinquefasciatus* and *An. arabiensis* have been found to be dominant species in Kenyan rice paddies (Muturi et al. 2008; Muturi et al. 2007). This could be related to various physico-chemical parameters favouring a particular species (Jacob et al. 2005). Interspecific differences in the level of predation have also been reported for *Cx. quinquefasciatus* and *An. Gambiae* (Muturi et al. 2010), so this may also be factor in *An. arabiensis*.

When examining the lethal doses of the pollutants used here, QUINQS showed the highest tolerance to metal concentrations, although this did not translate into an advantage in terms of emergent numbers. Field studies have demonstrated varying tolerance to toxins, in which *An. arabiensis* coped better with the larvicide Agnique® (Bashire et al. 2008), while *Cx. quinquefasciatus* coped better with a crude extract from castor oil plant (*Ricinus communis* L.) (Malpighiales: Euphorbiaceae) leaves (Elimam et al. 2009a). Furthermore, leaf extracts of *Calotropis procera* (Aiton) Aiton fil. (Gentianales: Apocynaceae) proved more toxic to *Cx. quinquefasciatus* larvae than *An. arabiensis* and resulted in fewer eggs being laid by *Cx. quinquefasciatus* females, in water polluted with the extract (Elimam et al. 2009b). None of these studies took into account the insecticide resistance status of the larvae. The results of our study indicated that resistance to

insecticides could provide a significant competitive advantage for resistant mosquitoes under polluted conditions.

A direct comparison of the findings in this study to other studies on interspecific competition is challenging. Examinations of *An. arabiensis* and *Cx. quinquefasciatus* interspecific competition has not been reported as often as that for intra-*Aedes* competition. This is notable, as *An. arabiensis* and *Cx. quinquefasciatus* have been found in the same containers more than would be expected by chance alone (Muturi et al. 2008). The effect of fertiliser and metal concentrations have also not been reported. In terms of environmental conditions, *Culex* larval habitats have previously been positively associated with the level of dissolved oxygen (DO) and concentration of total dissolved solids (TDS) and generally occurred in water with a higher electrical conductivity (Muturi et al. 2008). It would be useful to conduct the present study in field or semi-field conditions, as the larval habitat itself, over and above the physico-chemical parameters of the breeding site, could affect the outcome of competition (Mwangangi et al. 2008). A study of this type would be able to test the postulated competitive advantage insecticide resistant individuals have over others in polluted conditions. It is also possible that the pollutants may have a hormetic effect, as the insecticide malathion can improve life history traits in *Cx. pipiens* by reducing density effects (Muturi et al. 2010).

The choice of site for oviposition represents a significant ecological factor as well and may have a direct influence on how species coexist in sympatry. There was a difference in ovipositional activity between the species and two *An. arabiensis* strains. Both the fertiliser and metal acted as an attractant for the QUINQS strain. Metal was a repellent for the SENN strain, while fertiliser was an attractant for the SENN strain. The differences in the effect of fertiliser on oviposition site choice have been observed in the two *An. arabiensis* strains before (Samuel et al. 2020; Oliver 2021). This indicates that species-specific oviposition choice of site may be directly affected by the presence or absence of resistant phenotypes, and that these choices are likely to affect subsequent life histories, especially survival to adulthood. A previous study demonstrated that oviposition by *An. arabiensis* and *Cx. quinquefasciatus* in water with elevated concentrations of fertiliser was greater than in water polluted with a pesticide. Pollution also had differential effects on oviposition site choice in these species, with *An. arabiensis* laying more in an ammonium sulfate treatment, but *Cx. quinquefasciatus* laying more in a diammonium phosphate (DAP) treatment (Kibuthu et al. 2016). Although this study demonstrated interspecies differences, the resistance phenotype was not taken into account in this study. The positive effects of fertiliser application have been demonstrated in various examples, with the application of ammonium sulphate fertiliser increasing the amount of anopheline and culicine immatures in the field (Mutero et al. 2004).

Examination of the response of larval detoxification enzymes to metal exposure gives some insight into how the chemoprotective system may operate. As cytochrome P450s are metal-inducible proteins (Musasia et al. 2013), it is noteworthy that the highly metal tolerant QUINQS strain showed very low basal activity for this group of enzymes. Copper and lead exposure reduces this already low level of P450 activity. At the larval stage, cytochrome P450 appears to play a crucial protective role in *An. arabiensis*, as most other enzyme systems are reduced in response to larval exposure to metal.

Detoxification enzyme activity in the QUINQS strain is remarkably resilient to change, with no metal treatments reducing or increasing activity, except for the reduction in cytochrome P450 activity following copper and lead exposure. Relative to the *An. arabiensis* strains, it appears that GST is the most important larval chemoprotective enzyme in this strain.

After all metal exposures, this was the only enzyme class that was significantly higher than that reported for the *An. arabiensis* strains.

SENN and SENN-DDT demonstrated markedly different tolerances to insecticide in both the adult and larval stages. It is therefore interesting to note that, with the exception of glutathione S-transferases (GSTs), the basal detoxification enzyme activity levels in both strains were the same. It is also noteworthy that the pattern of activity in the *An. arabiensis* glutathione peroxidases were similar to the activity patterns of GSTs, indicating that the changes observed in peroxidase activity were specifically changes in glutathione peroxidase activity, which some GSTs display (Sheehan et al. 2001). Neither of these classes (i.e. GST, peroxidases) increased in *An. arabiensis* larvae in response to metal exposure. Cytochrome P450 activities increased in two of three treatments in SENN-DDT. This indicates that the cytochrome P450s may play a role in copper and lead tolerance in this strain.

A previous study examined the enzyme activity in SENN and SENN-DDT adults after larval exposure to the same metals at the same concentration (Oliver & Brooke 2018b). There were marked similarities in the enzyme activities of the adults of the previous study and the larvae of this study. This included a general decrease in GST activity and an increase in cytochrome P450 activity in adults. The significant reductions in activity observed in α -esterases were not observed in adults of either strain. By contrast, β -esterases in SENN-DDT adult males increased rather than decreased, as observed in the larvae. The results for the adult SENN-DDT females were all significant, but variable, with copper causing a significant increase in β -esterase activity. The generally conserved metabolic detoxification enzyme profile suggests that an increase in metal tolerance in adults is a direct consequence of increased enzyme activity induced at the larval stage in response to pollutant exposure.

The limitations to this study must be noted. The difficulty in establishing a LC_{50} value for lead nitrate in the *Cx. quinquefasciatus* strain was a notable limitation, as it was not possible to make the LC_{30} estimates with the same level of confidence as for an LC_{50} estimate. Furthermore, the analysis of all the stressors in a symmetric 1:1 manner was limited in scope. This was due to the analysis of factors as single stressors, in order to prevent confounding effects. Furthermore, the study was also limited by the lack of an insecticide susceptible *Cx. quinquefasciatus* strain for a comparative assessment and absence of wild counterparts for evaluation.

We concluded that pre-existing insecticide resistant phenotypes may not only have an adaptive advantage in water with elevated concentrations of fertiliser and metals, but may also respond to concentrations of pollutants at a molecular and enzymatic level. Insecticide resistant phenotypes also appear to have affected the interspecific competition dynamic, with increased insecticide resistance reducing relative competitive success in unpolluted conditions, but generally increasing success in polluted conditions. These interactions are potentially influenced by numerous ecological factors, in addition to the aforementioned molecular and enzyme-mediated responses in the larvae.

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COMPETITION STATEMENT

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

ACSN performed the experiments, collected and analysed the data and contributed to the initial draft. BDL assisted with the optimisation of the experiments and contributed to the initial drafts. JM and BDB assisted with data analysis and contributed to the initial drafts. SVO conceived the project, obtained funding and produced the final draft of the manuscript.

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