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SHORT COMMUNICATION

Increased tolerance of *Anopheles gambiae* s.s. to chemical insecticides after exposure to agrochemical mixture

THERESIA E. NKYA[1,2,3*], FRANKLIN W. MOSHA[3], STEPHEN M. MAGESA[4] and WILLIAM N. KISINZA[1]
[1]*National Institute of Medical Research, Amani Research Centre, P.O. Box 81, Muheza, Tanzania*
[2]*Laboratoire d'Ecologie Alpine, UMR CNRS-Université de Grenoble 5553, Grenoble Alpes, France*
[3]*Kilimanjaro Christian Medical University College, Tumaini University Makumira, Moshi, Tanzania*
[4] *RTI International-Tanzania, Dar es Salaam, Tanzania*

Abstract: Resistance of mosquitoes to insecticides is mainly attributed to their adaptation to insecticide-based vector control interventions. Although pesticides used in agriculture have been frequently mentioned as an additional force driving the selection of resistance, only a few studies were dedicated to validate this hypothesis. The objective of this study was to investigate the effect of exposure of the malaria mosquito, *Anopheles gambiae s.s.* larvae for 72h to sub-lethal concentrations of the agrochemical mixture (pesticides, herbicides and fungicides). Their subsequent tolerances were measured to deltamethrin (pyrethroid), DDT (organochlorine) and bendiocarb (carbamate) currently used for vector control. The mean LC_{50} was determined and tolerance ratios for larvae exposed to agrochemical comparatively with unexposed larvae were calculated and expressed as fold increased tolerance. Bioassays revealed a significant increase in larval tolerance to detamethrin (1.83-2.86 fold), DDT (1.31-1.53 fold) and bendiocarb (1.14-1.19 fold) following exposure to 0.1 µM and 1µM agrochemical mixture. The observed increased tolerance in this study is likely to be based on metabolic resistance mechanisms. Overall, this study reveals the potential of agrochemicals to increase the tolerance of mosquito larvae to chemical insecticides.

Keywords: agrochemicals, agriculture, pesticide, *Anopheles gambiae,* malaria, insecticide resistance

Vector control programmes have shown success in the last decade through the use of chemical insecticides. However the success of these control programmes is now threatened by the increase in resistance to these insecticides in mosquito populations (Hemingway *et al*., 2004). Insecticide resistance is believed to be mainly caused by high insecticide treated mosquito nets (ITNs) and indoor residual spraying (IRS) coverage, or recurrent space spraying interventions (N'Guessan *et al*., 2007; Protopopoff *et al*., 2008). However, studies have pointed out the possible role of agricultural pesticides in selection of inherited resistance mechanisms or in the higher tolerance of mosquitoes to insecticides (Diabate *et al.*, 2002; Nkya *et al.*, 2013). While insecticide resistance is rising dramatically in Africa, deciphering how agriculture affects resistance is crucial for improving resistance management strategies. Pesticides used in agriculture have been frequently mentioned as an additional force driving the selection of resistance. However, only a few studies were dedicated to validate this hypothesis and characterize the underlying mechanisms.

In West Africa, the increased rate of pyrethroid resistance was often attributed to the massive use of DDT and pyrethroids in cotton growing areas (Yadouleton *et al*., 2011). Northern Tanzania has an historical recording of intensive agriculture with massive use of insecticides of various classes, but a moderate insecticide pressure from vector control activities (T.E. Nkya, unpubl). In this area, high resistance of *Anopheles arabiensis* to pyrethroids has been reported (Matowo et al., 2010) which could be partly attributed to the use of agrochemicals (pesticides, herbicides and fungicides). It is at larval stage that these mosquitoes come in contact with these

* Correspondence: Theresia E. Nkya; E-mail: theresia_nkya@hotmail.com

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agrochemicals that could lead to development of resistance in adult mosquitoes to insecticides. However, in Tanzania no study has explored the direct link of these agrochemicals and insecticide resistance in malaria vectors.

Most susceptibility tests are aimed at adult mosquitoes, looking at their tolerance based on knockdown/mortality effects and metabolic resistance. As most resistance alleles can be inherited through generations with no phenotypic resistance observed, the spread of resistance will continue to be a problem if we continue to ignore the importance of larvae in early detection of resistance. It can be hypothesized that in areas of intensive agriculture, agrochemicals found in mosquito breeding sites may induce particular enzymes involved in the degradation of chemical insecticides, leading to an increased tolerance of mosquitoes to insecticides.

In the present laboratory experiment, we investigate the capacity of pesticide, herbicides and fungicides to modify the tolerance of *An. gambiae s.s.* (from here on referred to as *An. gambiae*) larvae to three different chemical insecticides used worldwide for mosquito control: deltamethrin, dichlorodiphenyltrichloroethane (DDT) and bendiocarb. We exposed laboratory population of *An. gambiae* larvae not previously exposed to agrochemicals and fully susceptible to all insecticides for 72 hours to sub-lethal concentrations of agrochemical mixture before comparing their larval tolerance to insecticides.

This laboratory experiment reports the influence of agrochemicals in selecting for insecticide resistance in larvae. A laboratory strain of *An. gambiae* (Kisumu strain, susceptible to all insecticides) was reared in standard insectary conditions (26 °C, 8 h/12 h light/dark period, tap water) and used for all experiments. Larvae were reared in the insectary, fed on fish food (Tetramin®) for 3 days before exposure for 72h to a mixture of chemicals likely to be found in mosquito larvae habitats in agricultural areas across Tanzania. The agrochemicals included herbicide, paraquat dichloride (Gramoxone^{R}), Gama cyhalothrin (Healarat®), cypemethrin 10% and chlropyrifos 35% (Dudu® All 450EC), Chlorpyrifos (Dursban®4E), endosulfan (Thionex® 35EC), Mukpar-Dimethoate (Dimethoate® 40%EC) and chlorothalonil (Banico® 500 SC).

Pre-exposures to agrochemical mixture were performed in triplicate with 100 homogenous second stage *An. gambiae* larvae in 200 mL of tap water containing 50 mg of larval food (Tetramin®). Concentrations of agrochemicals used for larval pre-exposure were chosen according to the concentrations likely to be found in mosquito breeding sites in agricultural areas (T.E. Nkya, unpubl). Prior to bioassays with insecticides, larvae were exposed for 72h to 0.1 or 1 µM agrochemical mixture. After 72h, fourth stage *An. gambiae* larvae were collected, rinsed twice in tap water and immediately used for bioassays. Larval bioassays were conducted on larvae exposed to agrochemical mixture and unexposed larvae (controls) with the 3 chemical insecticides deltamethrin, DDT and bendiocarb. Bioassays were performed in triplicate with 25 larvae in 50 mL insecticide solution and repeated 3 times. Four different insecticide concentrations leading to larval mortality ranging from 5% to 95% were used. Deltamethrin and bendiocarb were used at 5µg/L-120µg/L, while DDT was used at 90µg/L-1440µg/L.

Larval mortality was recorded after 24h contact with insecticide and further analyzed using the Probit analysis (PoLo Plus 1.0, LeOra software). For each insecticide, the mean LC_{50} was determined and tolerance ratios for larvae exposed to agrochemical compared to those unexposed were calculated and expressed as fold increased tolerance. For each insecticide, tolerance ratios consisted of LC_{50} obtained for the exposed strain divided by LC_{50} obtained with the unexposed (control) Kisumu strain. Because comparison of LC_{50} values may not well represent differential tolerance across all concentrations of insecticides used for bioassays, differential insecticide tolerance between larvae exposed to each agrochemical concentration and controls was further analyzed as described in (Poupardin *et al*., 2008) by generating a Generalized Linear Model (GLM) from mortality data followed by a likelihood ratio test using R software (R Core Team, 2007).

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Exposing *An. gambiae* larvae to sub-lethal concentrations of agrochemical mixture (herbicides, pesticides and fungicides) for 72 h affected their subsequent tolerance to insecticides. Overall, exposing larvae to these agrochemicals increased larval tolerance to insecticides with a more pronounced effect observed with higher concentrations of the mixture (Table 1). Larval tolerance in $LC_{50}$to the pyrethroid insecticide deltamethrin increased significantly after exposure to 1 µM and 0.1µMof the agrochemical mixture (2.86-fold, p<0.001 and 1.83-fold, p<0.01, respectively). Larval tolerance to the organochloride insecticide DDT increased after exposure to both 1 µM (1.53-fold, p<0.01) and 0.1µM (1.31-fold, p<0.01) agrochemical mixture. Larval tolerance to bendiocarb (carbamate) was only slightly enhanced after exposure to the highest concentration of agrochemical mixture (1.19-fold, p<0.05, respectively) but no significance fold change was observed to the lowest concentration of the mixture.

During the recent decades, the amount of agrochemicals (pesticides, herbicides and fungicides) used in both intense agriculture and small scale urban farming, has dramatically increased. Although the effect of these chemicals on human health is intensively studied, their impact on insecticide resistance remains poorly understood. The classes of insecticide used for public health and agricultural share the same mode of action. This laboratory experiment clearly shows the impact of these chemicals on the tolerance of mosquito larvae to chemical insecticides. We showed that the presence of these agrochemicals in water where mosquito larvae develop can significantly increase their tolerance to insecticides, particularly deltamethrin and DDT. One could argue that residual insecticides from agriculture found in mosquito breeding sites may not reach sufficient levels, as to be lethal to mosquitoes. However, continuous exposure of sub-lethal doses of insecticides may select for resistance as shown in this experiment. This experiment revealed that cocktails of distinct pesticides from different chemical classes, currently used in agriculture throughout Tanzania, showed a synergistic effect on mosquito leading to tolerance to insecticides.

Although the increase in insecticide tolerance reported in this study is low, it still shows that the presence of agrochemicals may contribute to insecticide tolerance in mosquito larvae. This suggests that resistance mechanisms expressed at the adult stage can be rapidly selected even when selection targets the larval stage as suggested. This could lead to multi-resistant phenotype which can be the consequence of the selection of different mechanisms with limited trade-off between them or the selection of particular genes conferring cross-resistance to different insecticide classes. This laboratory experiment did not explore the resistance mechanisms involved. However, previous field studies have associated metabolic resistance with intensive agriculture in Tanzania. In Moshi, Tanzania, the resistance of *An. arabiensis* to pyrethroids through metabolic resistance was associated with pesticide usage in an intensive agriculture area (Matowo *et al*., 2010). More recently, another Tanzanian field study comparing urban, agricultural and low pollution areas pinpointed the elevated resistance level of *An. gambiae* found in proximity of intensive agriculture and identified candidate genes associated with the use of pesticides in agriculture and insecticide resistance (Nkya et al., 2014).

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Table 1: Differential tolerance of *An. gambiae s.s.* larvae to deltamethrin, DDT and bendiocarb after exposure for 72 h to agrochemical mixture

Treatment	Deltamethrin			DDT			Bendiocarb		
	LC_{50} µ/L (CI 95%)	FC increase tolerance	Likelihood ratio test *P*-value	LC_{50} µ/L (CI 95%)	FC increase tolerance	Likelihood ratio test *P*-value	LC_{50} µ/L (CI 95%)	FC increase tolerances	Likelihood ratio test *P*-value
Control	6.79 (2.62-10.56)	--	--	265.41 (198.63-356.20)	--	--	24.34 (6.10-39.82)	--	--
0.1µM agrochemical mixture	12.43 (9.26-14.19)	1.83	**	347.69 (316.01-386.32)	1.31	**	24.74 (20.39-29.01)	1.14	ns
1µM agrochemical mixture	19.43 (16.43-22.34)	2.86	***	407.56 (284.65-786.84)	1.53	**	29.15 (20.47-41.58)	1.19	*

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Overall, our experiment demonstrated that the agrochemical mixture likely to be found in mosquito breeding sites in agricultural areas increase tolerance of mosquito larvae to different classes of insecticides. Considering the persistent contamination of mosquito breeding sites by agrochemicals and the potential effect of phenotypic plasticity, the question of long-term impact of agrochemicals on development of insecticide resistance presents an important future research direction. This study has shown that agrochemicals do affect tolerance of *An. gambiae* to insecticides. It is important that studies are undertaken to investigate the long term impact of this agrochemical mixture on *An. gambiae* larvae, in selection for resistance and the resistance mechanisms involved, to confirm the actual involvement of agriculture in the selection of resistance in mosquitoes. Any strategy of resistance management needs to consider the different selection pressures and allow accurate resistance monitoring. Lessons from this study could be essential to draw guidelines for the Tanzania nationwide insecticide resistance surveillance to ensure efficiency and sustainability in national control strategies.

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