Laboratory testing of larvivorous fish Japanese medaka (*Oryzias latipes*) predation ability to copper-treated *Anopheles stephensi* larvae: An alternative method for vector control under low concentration of copper

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**Abstract:** Utilizing larvivorous fish is a promising approach to biological control of malaria. In our laboratory, we have tested fish predation ability to *Anopheles stephensi* larvae under low concentration of copper. We found that at 0.26 ppm larvae were negatively affected, especially in terms of diving ability, which made them easier for the larvivorous fish to catch. This concentration was safe for medaka (*Oryzias latipes*). It needs further study whether it is possible to use this fish together with low concentrations of copper in the limited area where mosquitoes breed.

**Key words:** *Anopheles stephensi*, copper, fish, malaria control, medaka, mosquito larva, *Oryzias latipes*

**INTRODUCTION**

To ensure the successful eradication of malaria via mosquito control, factors such as cost, ease of application in the field, and environmental impact should be considered. The high cost of control through genetic modification, like generating transgenic mosquitoes expressing hemolytic C-type Lectin CELL-III (Yoshida et al., 2007) or expressing anti-parasitic genes in the midgut (Ito et al., 2002) is not practical in the field. Other approaches like generating infertile male mosquitoes have shown promise in field tests (Harris et al., 2011) but still have ethical concerns (Ostera et al., 2011). Efforts using DDT or mass spraying from house to house also have problems in the field.

Adult mosquitoes are difficult to control because they do not stay in one place. Utilizing mosquito nets has been a successful and inexpensive method of controlling adult mosquitoes. In some endemic areas, e.g. India (Bhatt et al., 2012), mosquito nets have proved effective at reducing adult densities. The use of mosquito nets is expanding not only in the form of bed nets but also nets that can cover an entire house like Sumitomo Chemical’s permethrin-impregnated ceiling nets (Kawada et al., 2012). But nets alone are still not enough to control adult mosquitoes. Durability, incorrect use, or poor maintenance reduces its effectiveness over time. The biting behavior of adult mosquitoes also differs with each species or endemic region, and so outdoor contact still poses a threat (Rwegosibora et al., 2002).

These facts encouraged us to find another approach and shift to an easier target, the larvae. Compared to the adult stage, the larval stage is vulnerable. Larvae have to stay in a specific aquatic environment, which can be easier to track or find, compared to adult mosquitoes. One of the most successful malarial eradication efforts in Brazil relied exclusively on larval control (Killeen et al., 2002).

There are many approaches to larval control, for instance using mosquito fish, the removal of breeding sites, or applying oil in breeding areas. However, oil can hinder the growth of vegetation and ruin water quality. Removal of breeding areas is impossible since most countries where malaria is endemic are still developing or undeveloped, which means many natural mosquito-breeding sites exist. Mosquito fish are an option, but our visit to one endemic area in Indonesia (Tarusan, West Sumatra) showed that fish only could not kill all larvae. We found some larvae in ponds filled with local mosquito fish, which showed us that the larvae could survive predation.

Our previous study (Reza et al., 2012) showed that low concentrations of copper could kill larvae or at least jeopardized larval survival in the guppy (*Poecilia reticulata*). We are thinking of the possibility to combine copper treatment with the use of mosquito fish in the field. However, we are aware that fish and the other predator insects like dragonfly (Tollett et al., 2009) are also susceptible to copper. Researchers have listed pos-
sible candidates for fish that can be used to control mosquito larvae (Chandra et al., 2008). *Oryzias latipes* (medaka/Japanese medaka) seems to be a good candidate due to its hardiness and tolerance of brackish water, broad tolerance of temperature (6–40°C Celsius) and availability and acceptance in the field around the world. When compared with other foraging behavior of mosquito fish like gambusia or guppy, medaka is peaceful and rarely attacks the other fish, which makes them possible to be assemblages with other native fish (Chandra et al., 2008). The other reason, medaka is a preeminent fish for investigating effects of carcinogenic and/or toxic waterborne hazards (Hawkins et al., 2003) and easy to maintain and control in the laboratory. In this study, we chose to use this fish in combination with low concentrations of copper (<1 ppm). Our hypothesis was that medaka would kill and eat mosquito larvae more effectively when the larvae were weakened by copper-treatment. Our previous study showed a significant difference in predatory time between low copper-treated larvae vs un-treated larvae. In the current study, we sought the lowest possible concentration of copper, which could be tolerated by medaka and still affect the survival of mosquito larvae. Hopefully this result can lead to another possibility of a simple and inexpensive method to reduce malaria and other vector borne diseases.

**Materials and Methods**

**Mosquitoes, Mice and Fish**

*Anopheles stephensi* Liston, 1901 (strain SDA 500) was reared in our laboratory under room temperature (26°C), 50–70% relative humidity, and a 13 : 11 h of light : dark cycle. From the day of emergence, the mosquitoes were provided with a 5% fructose solution soaked in filter paper. Females five to 20 days after emergence were allowed to feed on anesthetized mice. Three days later, an ovipositing dish was placed in a cage containing gravid females. The eggs were laid on filter paper soaking in the dish. The filter paper with eggs was placed on a 12×20 cm hatching tray containing 500 ml of water. After hatching out, 3–5 mg of carp’s food per tray was sprinkled on the surface of the water twice daily. Twelve to 20 days later, pupae were collected daily and transferred to cages for adult emergence.

Female BALB/c mice were purchased from SLC (Shizuoka, Japan). The mice were fed *ad libitum* and exposed to a 13 : 11 h light : dark cycle. This mosquito-mouse cycle was used to maintain the mosquitoes in our laboratory.

Japanese medaka fish, orange-red strain (*Oryzias latipes* Temminck et Schlegel, 1846) were utilized in this experiment to measure the survival and movement ability of the larvae. The fish were purchased from a local pet shop in Shimotsuke-shi, Tochigi-ken, Japan. They were maintained by twice daily feedings and the water was changed twice a week. One fish ate 50 of 2nd instar or 3rd instar mosquito larvae everyday.

**Preparation for Copper Solutions**

*CuSO₄* solutions were used for the experiments. Copper concentrations were 0.60 ppm, 0.52 ppm, 0.40 ppm, 0.30 ppm, 0.26 ppm, 0.15 ppm, and 0 ppm. Each concentration was allocated to 2 L. *CuSO₄* solutions were prepared by diluting from a standard solution (100 mM) and confirming the ppm level using a Copper Measuring Device (Hanna Instruments, Tokyo, Japan) and Z-5010 Polarized Zeeman Flame Atomic Absorption Spectrophotometer (Hitachi Ltd, Tokyo, Japan).

**Preparation of Mosquito Larvae**

Larvae were fed two times a day and the water was changed every two days to keep it clean. To remove chlorine ions from tap water we used a fine ceramic filter (NGK Insulators, Nagoya, Japan). The 2nd and 3rd instar larvae were used for all experiments.

**Fish Survival Experiment**

For the experiments on exposure to different copper concentrations, ten medaka fish were used for each concentration of copper. Seven containers was prepared and treated with different concentration of copper (0.60 ppm, 0.52 ppm, 0.40 ppm, 0.30 ppm, 0.26 ppm, 0.15 ppm and 0 ppm). Ten fish were put in each container for 14 days and fed with normal daily diet. Fish were checked for mortality and morbidity every day. The number of mortality was recorded and statistically analyzed.

**Fish-Larvae Feeding Time Experiment**

For the experiments on fish-larvae feeding time, the 2nd and 3rd instar larvae were exposed to *CuSO₄* solutions in four different concentrations (0.30 ppm, 0.26 ppm, 0.15 ppm, and 0 ppm) for 48 hours. Fifty larvae were given to five starved medaka fish for each feeding time trial. The total time (seconds) needed by the fish to eat all the larvae in the containers was recorded and statistically analyzed.

**Surface–Bottom (Diving) Comparison**

For the surface-bottom comparison test, about 200 larvae were treated in four different concentrations of *CuSO₄* solutions (0.60 ppm, 0.30 ppm, 0.15 ppm, and 0 ppm). The number of larvae staying on the surface and the number of larvae staying in the bottom were counted at 24 hours and at 48 hours. The numbers of larvae staying at the surface and the bottom were statistically analyzed.

**Results**

Most fish, including medaka, are susceptible to copper. Thus, we tried to find the maximum concentration that can be tolerated by Japanese medaka. Since our
previous study showed that 0.60 ppm of copper was effective against mosquito larvae, we started with this concentration. Figure 1 shows that 0.60 ppm was too toxic, with all the fish dying within 3 days. At 0.52 ppm, 80% mortality occurred after 14 days. The 0.40 ppm concentration resulted in 60% mortality. At 0.30 ppm, 70% of fish survived the experiment. At 0.26 ppm, no fish died until the end of experiment, as same as the control group. This concentration was ideal in this experiment because 0.26 ppm could be tolerated by medaka and also still had negative effect on mosquito larvae survival ability as shown later in the Figure 3. Although 0.15 ppm produced one death, it probably was not related to copper toxicity.

We have also found that fish can acclimate to copper (Erik et al., 2008). We re-exposed the fish of 0.26 ppm group to 0.52 ppm. They survived with no deaths over 2 weeks.

We next tried to find the lowest concentration affecting mosquito larvae (Fig. 2–4). The feeding time for medaka in the 0.30 ppm group after 48 hours’ exposure to copper was significantly faster than the control value ($p<0.05$). This result is consistent with the guppy data in our previous report. We tested 0.26 ppm, the concentration best tolerated by medaka (zero mortality). Figure 3 shows that the larvae exposed to 0.26 ppm copper were eaten faster than those in the control group. Both one and two-tail $t$-tests showed a significant difference ($p<0.05$). Figure 4 shows that the result was not significant at 0.15 ppm. The copper-treated group was eaten in almost the same time as the control.

To confirm that diving ability was truly jeopardized after the copper treatment, we performed a comparison of the larvae among four copper concentrations (0.60 ppm, 0.30 ppm, 0.15 ppm and 0 ppm). Table 1 shows that a large percentage of larvae stayed at the bottom at 24 hours of treatment in the control group (0 ppm). This percentage decreased with the increase
in the concentration of CuSO₄ (0.15 ppm, 0.30 ppm, and 0.60 ppm). The result was statistically significant at 0.30 and 0.60 ppm, but not 0.15 ppm. After 48 hours’ treatment, as shown in Table 2, all CuSO₄ concentrations resulted in a significant difference in comparison to the control. Table 1 and Table 2 confirmed that copper affected the ability of mosquito larvae to dive.

**Discussion**

From the experiment in Figure 1, we concluded that the maximum tolerable copper concentration for Japanese medaka was 0.30 ppm, with minimum mortality (3 out of 10 fish after 14 days). The ideal non-lethal concentration was 0.26 ppm, which was better if we intended to release the fish in copper-treated breeding areas. Since the feeding time experiment in Figure 3 (0.26 ppm vs 0 ppm) showed a significant capture-time, we can utilize medaka at breeding sites with 0.26 to 0.30 ppm of copper.

The lower concentration of 0.15 ppm seemed not to affect the larvae, since the statistical analysis showed no significant difference in capture-time compared to the control group. The highest concentration, 0.60 ppm, from the previous experiment (Reza et al., 2012) was not recommend to be used with Japanese medaka due to the high mortality rates of this fish at this concentration. There is a small tolerance window in the survival rate of the fish treated in 0.26 ppm with the 0.30 ppm. We believe it happened due to the acclimation capability of the fish in toxic environment as reported by some researchers (Erik et al., 2008). Based on their research, it needs some sub-lethal doses of toxin to trigger the repair of damaged cells in the acclimation process in fish. The concentration of 0.30 ppm seemed to be a sub-lethal concentration, since some fish showed morbidity at the beginning (day 1–day 3), then showed recovery after several days. This acclimation is different in each fish individually, that is why not 100% fish survived in this concentration. Therefore, it might be possible to use a larger range of copper concentrations from 0.26 ppm to 1 ppm for easier application due to the fish’s ability to acclimate as reported previously (Sellin et al., 2005). The acclimation is an advantage since mosquito larvae do not have such an ability. The acclimation would start after damage to the gills or respiratory tract, which triggers the renewal of epithelial cells. In mosquito larvae, the damage occurs in the perithropic matrix (Beaty et al., 2002), which is irreversible. There are reports of some copper-tolerant fish (Johnston, 2011). Furthermore, generating a copper-tolerant strain is also possible since copper tolerance in fish is maternally transferred (Peak et al., 2004).

We confirmed the importance of diving ability to the survival of mosquito larvae. Our examination during the feeding-time experiment showed that the fish took longer to capture the larvae in the control group, because the larvae were able to dive to the bottom. The copper-treated larvae lacked this diving ability, which made them easier prey and resulted in a faster capture-time. Table 1 and Table 2 confirmed that copper reduced the ability of the larvae to dive, with longer exposures and higher concentrations having more of an effect.

It needs careful consideration and application of this method in the field, due to the concerns of environmental impact of copper. We are aware that copper also has negative impact to high bioavailability water environment such as river, lake and sea (see the Environmental Health Criteria of WHO for protection in aquatic life in waters with high bioavailability-EHC200, 1998). Therefore, we propose to limit the use of copper in this method to the low bioavailability water environment where mosquitoes breed, such as swamps, stagnant water environment, or paddy fields. The limited application will be easier to control and can avoid environmental damage. Hopefully the combination of biological control using fish and copper can effectively kill or reduce Anopheles larvae in their breeding habitats. There are several Oryzias species distributed in those areas of Southeast Asia where malaria is endemic and we plan to select the best candidate of larvivorous fish. We believe that this combination of larvivorous fish and low concentration of copper, with careful application and strict control can be alternative method to control malaria or other vector borne diseases.

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**CONFLICT OF INTEREST**

The authors have no conflict of interest.

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