Sri Lanka J. Aquat. Sci. **20 (1)** *(2015): 1-10*

Effect of glyphosate-based herbicide, Roundup™ on territory deference of male *Oreochromis mossambicus* (Osteichthyes, Cichlidae) associated with mating behaviour

K.V. Sandun N. Bandara and Upali S. Amarasinghe*

Department of Zoology and Environmental Management, University of Kelaniya, Kelaniya GQ11600, Sri Lanka

*Corresponding author (zoousa@kln.ac.lk)

Abstract

African cichlids are important constituent species in tropical inland fisheries and *Oreochromis mossambicus* supports profitable fisheries in many countries of the world. This species exhibits elaborate courtship behaviour. Building of spawning nests by males, aggressive behaviour and breeding colouration are important behavioural patterns of this species associated with reproduction. As *O. mossambicus* is found in reservoirs of Sri Lanka which are situated in agricultural areas of the country, reservoir populations of this species are vulnerable to exposure to agrochemicals at least at sub lethal levels. As 'Roundup™' is one of the widely used herbicides in the agricultural areas of the country, the present study was undertaken to investigate its impacts on territorial behaviour of *O. mossambicus*.

Adult *O. mossambicus* collected from the wild was acclimatized to laboratory condition and exposed to sub-lethal glyphosate concentrations (0 ppm, 5 ppm, 8 ppm and 10 ppm). The territorial behaviour of males measured as intensity of colour change, chasing distance, chasing occurrence, area of territory maintained by nest builders, dorsal fin erection and status of the bites has altered significantly in the aquaria with all three concentration of herbicide.

Keywords: cichlids, glyphosate, herbicide, *Oreochromis mossambicus,* reproductive behaviour

Introduction

Exotic cichlids especially tilapias were introduced to the Asia in the first half of twentieth century (Ling 1977) with a view to developing aquaculture and capture fisheries. Thereafter tilapia became a constituent species in the capture fisheries in tropical Asia including Sri Lanka (De Silva *et al*. 2004). In Sri Lanka exotic cichlids, especially *Oreochromis mossambicus* and *O. niloticus* are the mainstay in the inland fishery (Amarasinghe and Weerakoon 2009). As the inland fisheries production is a cheap source of animal protein for the rural communities, its contribution to the food security is an indispensable aspect to be considered in the national development priorities in Sri Lanka.

In many cichlid fisheries, recruitment is essentially governed by their reproductive behaviour and mouth breeding habits that ensure larval survival (Fryer and Iles 1972; Turner and Robinson 2000). Disruption of reproductive behaviour of cichlids may therefore bring about low recruitment resulting in long-term effects of reduced fish yields. In *O. mossambicus*, males are more aggressive than females and build spawning nests. They also develop secondary sexual characters, such as long extensions to the soft dorsal and anal fins, exaggerated jaws and elaborate genital papillae (Turner 1986; Turner and Robinson 2000). Male *O. mossambicus* is aggressive and exhibits territorial behaviour to keep away other male counterparts in the community (Turner and Robinson 2000).

As the inland fishery in Sri Lanka is mainly dependent on the magnitude of irrigation reservoirs of the country, contamination of agro-chemicals in the reservoir ecosystems is quite possible, which might pose adverse impacts on inland fisheries. It is therefore important to investigate whether there would be any impact of agrochemical contamination on the territorial behaviour associated with reproduction of *O. mossambicus,* one of the dominant species in the inland fishery of Sri Lanka.

Glyphosate (Roundup™; *The trade name is used for product identification purposes only and does not imply endorsement*) is a major herbicide applied in Sri Lankan agriculture. Langiano and Martinez (2008) have shown that short-term exposure to Roundup™ at sub-letal concentrations induced biochemical, physiological and histological alterations in the neotropical fish, *Prochilodus lineatus*. In Nigeria, laboratory studies indicated that glyphosate herbicide is toxic to Nile tilapia juveniles and caused histopathological changes in different organs such as gills, liver, kidney and brain (Ayoola 2008). Adverse histopathological impacts of glyphosate on guppy, *Poecilia reticulata* are reposted by Chandrasekara and Weeratunge (2011). It is however, reasonable to expect that extremely high levels of agro-chemicals are rarely added to reservoirs due to dilution effect, but low levels which are sub-lethal. These sub-lethal levels are may have some effect on the normal behavioural patterns of fish. As such, the present study was carried out to investigate whether sub-lethal levels of glyphosate would have some effects on territorial behaviour of male *O. mossambicus*.

Materials and Methods

Studies were conducted from December 2010 to September 2011. Adult breeding sized Mozambique tilapia (*O. mossambicus*) males (100-120 g in body weight and 18.0 - 21.1 cm in total length) and females (60- 80 g in body weight and 14.0-17.0 cm in total length) were collected from Palliyawatta area of the Negombo lagoon, Sri Lanka as they are believed to be non-hybrids because salinity barrier prevents *O. niloticus* entering into the estuary. Fish were bought to the laboratory in the oxygenated polythene bags. They were acclimatized to laboratory conditions until they were adapted to formulated food in a cement aquarium (2.8 x 2.8 x 0.8 m) filled with aerated aged tap water under natural photo-period for nearly three weeks prior to the experimentation. During acclimatization period, fish were daily provided with commercially available food pellets around 08:00 h at a daily ration of 5% of the body weight.

Range finding test for glyphosate was not possible for adult fish of *O. mossambicus* under the existing laboratory facilities. Also due to lack of data for glyposate LC_{50} values for adult *O. mossambicus*, the value reported for *O. niloticus*, which was 16.7 mg l^{-1} (http://www.pesticideinfo.org/List_AquireAll.jsp?Species=485&Effect=&offset=1100) was used as the approximate value of lethal concentration in the present experiment. Four fibre-glass aquaria of 1.1 m diameter were prepared for the experiment. To prevent contamination of fibre-glass with the herbicide, the inner surfaces of tanks were covered with thick black polythene. The bottom was filled with properly washed coarse sand with 10 cm thickness to provide substrate to *O. mossambicus* for nest building. The four tanks were filled with aged tap water with known volume maintaining the water depth of 50 cm. All tanks were properly aerated. One tank was assigned as the control. The other three tanks were assigned sub-lethal concentrations of 5 ppm, 8 ppm and 10 ppm of glyphosate. For each tank, 9 male and 3 female *O. mossambicus* of more or less similar size were introduced. After male *O. mossambicus* started building nests at the bottom, nesting behaviour of fish was observed daily during mid-day between 11:30 hrs to 14:00 hrs. The observations made were, intensity of colour change in males; aggressive behaviour of nest building males to keep away other males; and approximate area of home range that each nest builder maintains. These behavioural patterns were thought to be sufficient to investigate the degree of territorial behaviour of male *O. mossambicus* to keep away other males as part of reproductive behaviour.

Each observation which lasted for 10 minutes was carried out for every nest builder separately. The level of colour change was assigned a score depending on the colour intensity (i.e. very dark: 0.8; dark: 0.6; light: 0.4; very light: 0.2).

A wire grid of 5 cm x 5 cm was placed on each fibreglass tank to facilitate measuring the chasing distance of territorial males. During the 10 minutes observation period, instances of chasing occurrence of fish were also counted. Occurrence of dorsal fin erection during chasing was assigned as 1 and non-occurrence as 0. Biting of males during chasing was assigned as 1 and non-biting as 0.

The "territory" of nest building male was defined as the area where fish swims around based on the chasing distance of each male, and approximate area of "territory" was measured by counting 5 cm x 5 cm squares in the grid. Chasing direction of each territorial male was determined from the middle of the nest.

The 10-minute observations were repeated 10 times for each male, which gave 30 observations for all three trials. After each trial, all fish were removed and the tanks were emptied. The adult males and females were replaced with new fish. This experiment was repeated three times.

One-way analysis of variance (ANOVA) was used to compare the mean values of chasing distance, chasing occurrence, approximate area of territory that each nest builder maintains and colour changes of males in the four treatments. When the results indicated significant differences ($p<0.05$), Scheffe's test was used for pair-wise comparisons. Chi-square (χ^2) test was used for analyzing frequencies of dorsal fin erection and biting of males. All statistical analyses were performed using SPSS statistical software (version 15). The overall behavioural patterns of *O. mossambicus* of four different treatments (with three replications each) were analyzed using non-metric multidimensional scaling (nMDS) (Clarke and Gorley 2001) to investigate the underlying pattern of multivariate data sets in relation to glyphosate concentration. Here, in order to reduce the effect of the greater values on the final analysis, the data were subjected to square root transformation before analysis. This analysis was performed using PRIMER 5 software (Clarke and Gorley 2001).

Results

Colour pattern of males

The mean colour pattern of territorial males of *O. mossambicus* in the four treatments are significantly different ($F = 37.30$; $p< 0.001$). According to Scheffe's test, mean colour pattern of control tank was higher than those of the other treatments. Also mean colour pattern in 5 ppm treatment was significantly higher than that of 10 ppm treatment (Figure 1A).

Chasing distance of males

The highest chasing distance of territorial males was reported in the control tanks and with increasing concentration of glyphosate, chasing distances drastically reduced with significant differences in mean values ($F = 270.58$; $p<0.01$). Pairwise comparisons indicated that the mean value of chasing distance at 8 ppm treatment was not significantly different from that of 10 ppm treatment (Figure 1B).

Chasing occurrence of males

Mean values of number of chasing occurrences per 10 minutes were significantly different in the four treatments ($F = 109.82$; $p<0.001$) and pair wise comparison indicated that the value in control tank was significantly higher than those in other tanks ($p<0.05$). However, chasing occurrences were not significantly different in the tanks with three glyphosate concentrations (Figure 1C).

Size of territory

Mean territory sizes of territorial males ranged from 0 cm^2 to 4800 cm^2 in the four treatments. The ln (x+1) transformed territory size to minimize the non-normality indicated significant differences ($F = 28.60$; $p<0.001$). Pair-wise comparison showed that the value in control tank was significantly higher than in other treatments ($p<0.05$), but was not significantly different between 8 ppm and 10 ppm treatments ($p>0.05$) (Figure 1D).

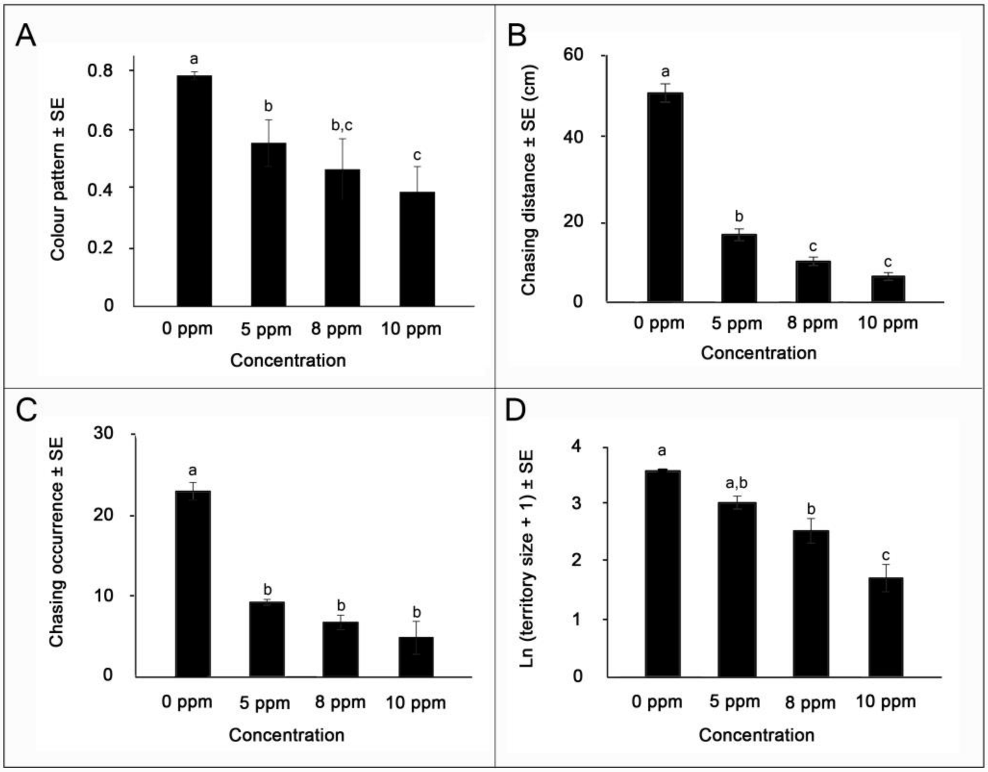

Figure 1. Territorial behavioural patterns of male *O. mossambicus* in four treatments (A) Colour patterns; (B) Chasing distance; (C) Chasing occurrence; (D) Ln (Territory size + 1). Vertical error bars are ± SE. The bars with similar letters are not significantly different at 5% probability level (Scheffe's test: $P>0.05$).

Dorsal fin erection of males

Number of occasions of dorsal fin erections against non erections of dorsal fin is shown in Table 1. Contingency test (R*C χ^2 test) indicated that observed differences were significant at 5% level ($\chi^2 = 32.2$; $p<0.05$).

Table 1. Positive and negative frequencies of dorsal fin erection in territorial males.

	Control	5 ppm	8 ppm	10 ppm
Positive frequencies	581	173	71	72
Negative frequencies	536	211	130	120

Bites

Numbers of bites observed in territorial males of *O.mossambicus* in four treatments are given in Table 2. These observed frequencies are significant at 5% level ($\chi^2 = 80.6$; $p<0.05$).

Table 2. Positive and negative frequencies of bites observed in territorial males.

	Control	5 ppm	8 ppm	10 ppm
Positive frequencies	486	110	45	34
Negative frequencies	631	274	156	158

Non- metric multidimensional scaling

The non- metric multidimensional scaling (nMDS) plot (Figure 2) had the stress value of 0.01 so that ordination of observations in a two-dimensional plane is adequate to describe the similarities of the observed values (Clarke and Gorley 2001). The results indicate that the overall territorial behaviour of *O. mossambicus* in control tanks is different from those in the tanks with three concentrations of glyphosate.

Figure 2. Two-dimensional Non-metric Multi-Dimensional Scaling plot of overall behavioural patterns of male *O. mossambicus* in tanks of four treatments. A: Control; B: 5 ppm; C: 8 ppm: D: 10 ppm.

Chasing direction of male O. mossambicus

Chasing direction and frequency of occurrences of territorial males of *O. mossambicus* indicate that roundup had distinguishable effect on territorial behaviour of male *O. mossambicus* (Figure 3).

Discussion

Glyphosate has effective controlling ability of competing vegetation, rapid interaction in soil and low mammalian toxicity (Franz *et al*. 1997). Therefore it is widely used throughout the world. Nevertheless, glyphosate toxicity has been reported in varying degrees. For example, in a study carried out in pine plantations in northern California indicated that repeated field application of glyphosate had minimal effects on soil microbial communities (Busse *et al*. 2001).

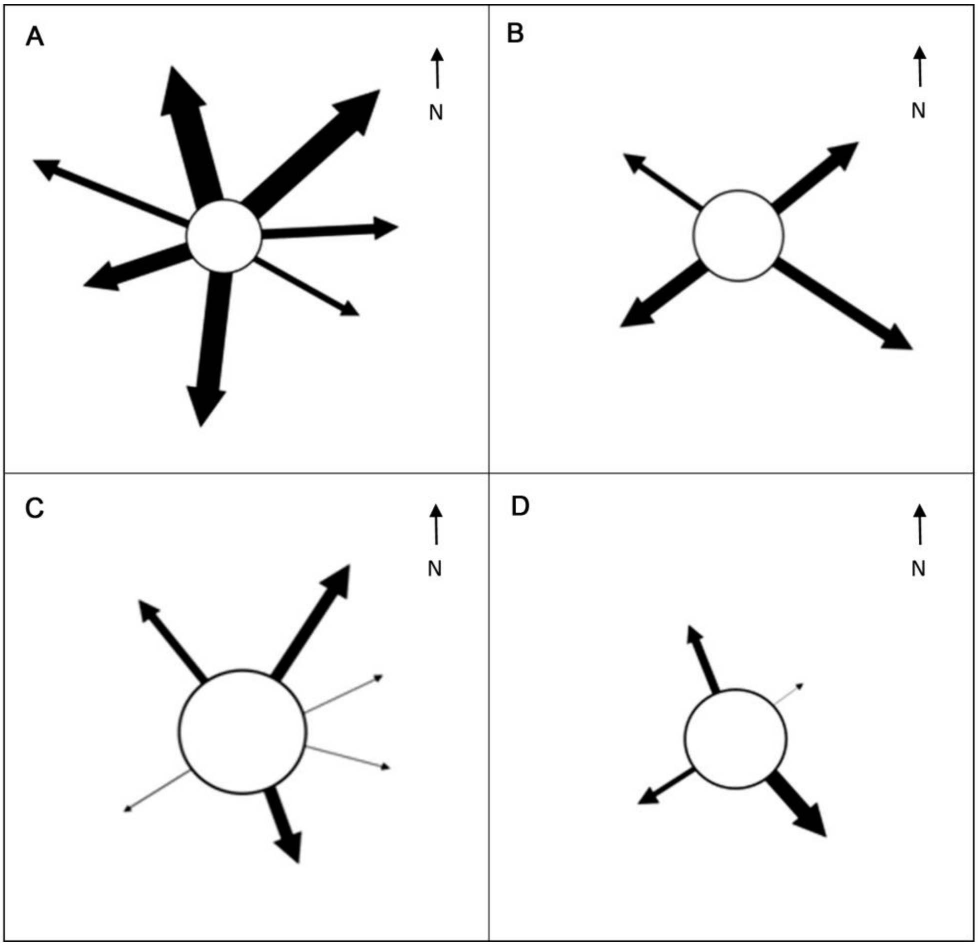

Figure 3. Chasing directions of male *O. mossambicus* in tanks with different herbicide concentrations. A: Control; B: 5 ppm; C: 8 ppm; D: 10 ppm. The lengths of arrows indicate relative chasing distance. The thicknesses of the arrows indicate relative occurrence of chasing.

However, present study indicated impairment of territorial behaviour of *O. mossambicus* even at sub-lethal levels of glyphosate, which might in turn affect reproductive performance. Although this herbicide is generally considered as somewhat environmentally friendly, the toxicity of the surfactant, polyoxyethyleneamine (POEA), is reported to be greater than the toxicity of glyphosate alone (Bradberry et al. 2004). In the commercial formulation, isopropylamine (IPA) salt of glyphosate and 15% POEA are found. The current chronic kidney disease of unknown etiology in most of the rice farming areas of Sri Lanka is hypothesized to be due to the unique metal chelating properties of glyphosate (Jayasumana *et al*. 2014).

Short-term exposure to glyphosate at sub-lethal concentrations is reported to induce biochemical, physiological and histological alterations in fish (Langiano and Martinez 2008). Histopathological changes are reported to be caused by this herbicide in different organs such as gills, liver, kidney and brain (Ayoola 2008; Chandrasekara and Weeratunge 2011).

In *O. mossambicus*, behavioural changes due to glyphosate, as shown in the present study might be through its impact on physiology of fish. It could be possible that alteration of aggressive behaviour associated with nest building of male *O. mossambicus* by glyphosate may be due to disruption of reproductive hormone even at sub-lethal levels. The typical breeding colour (uniformly black, apart from red edges to the unpaired fins and a white chin and operculum) of male *O. mossambicus* (Lanzing and Bower 1973), which was frequently visible in the control tank, was not observed in the treatment tanks with three concentration of glyphosate. Various colour patterns are produced in *O. mossambicus* with the development and localization of melanophores (Lanzing and Bower 1973), and change in colour pattern in fish exposed to sub-lethal concentrations of glyphosate is possibly due to impairment of this process.

Aggressive encounters (e.g., chasing distance, bites and erected dorsal fin) of male with adjacent territories were initially vigorous in control tank, but the severity of the encounters soon declined with the increase of herbicide concentration. Sub-lethal concentrations of agrochemicals are associated with subtle changes in behaviour and physiology that impair reproduction (Khan and Law 2005). Laboratory experiments have shown that sub lethal concentrations of agrochemicals can affect many aspects of fish biology, including behavioural patterns such as incessant jumping and gulping of air, restlessness, surface to bottom movement, sudden quick movement and resting at the bottom (Ayoola 2008). Present study also indicated that in experimental tanks with herbicide, behavioural patterns were disrupted.

Reproductive behaviour of animals is important for maintaining viable population sizes. Any disruption of reproductive behaviour would affect their population growth. In *O. mossambicus*, reproductive performance is known to be essentially governed by nesting behaviour (Oliveira and Almada 1996). As sub-lethal concentrations of roundup alter the nesting behaviour and aggressive behaviour of male *O. mossambicus*, their reproductive performance could be adversely affected by this herbicide.

Such sub-lethal concentration of glyphosate may occur in irrigation reservoirs from the surface run off from agricultural lands. Hence, through the alteration of the reproductive behaviour, there may be long term adverse impacts on the *O. mossambicus* populations in reservoirs. Poor reproductive performance of *O. mossambicus* would result in recruitment failures in reservoir fisheries. As *O. mossambicus* is the mainstay in the inland fishery of Sri Lanka as a source of cheap animal protein for rural communities (Amarasinghe and Weerakoon 2009), any adverse impact on reservoir populations of *O. mossambicus* may cause significant damages to the rural economy of the country. As such, proper management of the application of this herbicide in agricultural lands should be seriously considered to ensure sustainability of inland fishery of Sri Lanka.

Acknowledgements

The authors wish to thank Mr. R.P.K.C. Rajapakse of the Department of Zoology and Environmental Management, University of Kelaniya for his support to prepare figures.

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