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Short-Term Effects of Bolero on the Gill Apparatus of a Small Number of Mosquitofish (*Gambusia affinis*)

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Mosquitofish (*Gambusia affinis*) are widely used to control mosquito populations in California rice fields. The rice herbicide Bolero is used in those same fields (Finlayson and Lew 1985; Ross and Sava 1986). Thiobencarb (S-4-chlorobenzyl diethylthiocarbamate), marketed as Bolero 8EC, is a contact herbicide used for controlling grasses, broadleaf weeds and sedges in rice fields (Cornacchia et al. 1984). Bolero 8EC toxicity in aquatic animals has been reported. The 96-hr lethal concentration (LC50) is 2600 ppb for mosquitofish and 1700 ppb for bluegill (*Lepomis macrochirus*) (Harrington 1990). Field investigations conducted between 1982 and 1990 to determine rice herbicide concentrations in California rice fields and associated agricultural drains found a maximum thiobencarb concentration of 576 ppb (Ross and Sava 1986). In another study, the concentrations of thiobencarb detected in rice field water were approximately 1800, 1200 and 90 ppb on the 2nd, 4th and 10th d after application (California Rice Promotion Board 1992). The maximum recorded concentration of thiobencarb in the Colusa Basin Drain was 19 ppb in 1985 (Finlayson and Lew 1985).

In a degraded aquatic environment, particularly where pollutants occur at chronic and sublethal concentrations, changes in the anatomy and physiology of aquatic animals occur more frequently than mass mortalities. Therefore, one method for assessing the effects of pollutants on freshwater fish is to examine their organs for morphological changes. Gills are a well-known target organ in fish since they are quick to react to unfavorable environmental conditions (Poleksic and Mitrovic-Tutundzic 1994). The large surface area and fine sieve-like structure of gills make them particularly susceptible to continuous exposure to waterborne noxious agents (Lichtenfels et al. 1996). There are numerous reports of fish gill structural changes induced by toxicants and other irritants in the surrounding water (Hinton and Lauren 1990; Narain et al. 1990; Poleksic and Mitrovic-Tutundzic 1994; Heath 1995; Jagoe et al. 1996; Lichtenfels et al. 1996).

MATERIALS AND METHODS

Female mosquitofish (mean fork length 3.2 +/- 0.1 cm) were used to examine the anatomical damage caused by varying levels of Bolero 8EC. The fish were

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subjected to normal cultural practices (ASTM 1980). Mosquitofish were acclimated for 96 hr to laboratory conditions prior to testing, and were housed in glass aquaria with the use of airpumps to maintain an appropriate oxygen supply in the water. Each aquarium was filled with approximately 10 L of deionized water. Fluorescent lighting was turned on between 0600 and 2000 hr. Dissolved oxygen, temperature, and pH were measured in each aquarium daily.

Two trials were performed in which mosquitofish were exposed to varying concentrations of Bolero 8EC. In Trial 1, groups of 3 mosquitofish were exposed to 0, 576, 1200 and 1800 ppb of Bolero 8EC. In Trial 2, groups of 3 mosquitofish were exposed to 0, 19 and 90 ppb of Bolero 8EC. For all groups, the % O_2 saturation remained at approximately 60%. The average temperature and pH in Trial 1 were 68.8 (+/- 1.9) °F and 7.65 (+/- 0.32) respectively. The average temperature and pH in Trial 2 were 70.2 (+/- 1.5) °F and 7.59 (+/- 0.22) respectively.

At the 95th hr of acclimation to laboratory conditions, mosquitofish were transferred from the control and experimental aquaria to separate plastic containers filled with 3 L of deionized water. Aquaria were cleaned with soap and water, and rinsed 3 times. Approximately 10 L of deionized water was added to each aquarium. A micropipet was used to deliver the volume of Bolero 8EC to attain the desired concentrations (18.5 uL, 1800 ppb; 12.2 uL, 1200 ppb; 5.9 uL, 576 ppb; 9.2 uL, 90 ppb; 1.9 uL, 19 ppb). Thirty min were adequate for the dispersion of Bolero in the aquaria with constant stirring. The mosquitofish were placed in the aquaria for 7 d.

The mosquitofish were removed from each aquarium on the 7th d, or before the 7th d if found in a moribund or dying state. Mosquitofish were euthanized individually in a solution of tricaine, and then placed into a labeled vial with 10% formalin. The University Animal Care and Use Committee approved the use of tricaine as an acceptable method of euthanasia.

After preservation, each gill apparatus consisting of four gill arches was removed from the left and right sides of each fish. Microscopic tissue samples were prepared and stained with alcian blue (Johnson 1980). Following the staining procedures, gill tissue was observed under the light microscope at 25, 100, 400 and 1000 power. A total of 168 gill arches and their primary and secondary lamellae were examined.

Cross and micropathological techniques were used in observing and analyzing morphological changes in gills, We compared the amount of damage to the gill apparatus of mosquitofish using the rating system of Poleksic and Mitrovic-Tutundzic (1994). Each fish was evaluated by determining the type and number of gill lesions, and its I value. Histopathological examination also included a search for gill parasites. The types of lesions and their stages are listed in Table 1.

Table 1. Expected gill lesions and their stages (from Poleksic and Mitrovic-Tutundzic 1994).

Gill Lesions	Stage
Hypertrophy of respiratory epithelium	I
Lifting of respiratory epithelial cells	I
Leukocyte infiltration of gill epithelium	I
Thinning of respiratory epithelium	I
Shortening of secondary lamellae	I
Focal hyperplasia of epithelial cells	I
Hyperplasia from the base to approximately half the length of the secondary lamella	I
Irregular hyperplasia of epithelial cells	I
Fusion of the tips of secondary lamellae	I
Hyperplasia of sponge-like eosinophilic cells	I
Fusion of the primary lamellae tips	I
Fusion of several secondary lamellae	I
Hypertrophy and hyperplasia of mucous cells	I
Empty mucous cells or their disappearance	I
Hypertrophy and hyperplasia of chloride cells	I
Chloride cells present in secondary lamellae	I
Lamellar telangiectasia	I
Filament blood vessel enlargement	I
Gill Parasites	I
Hemorrhages with rupture of epithelium	II
Stasis	II
Rupture and peeling of the lamellar epithelium	II
Uncontrolled thickening of proliferated tissue	II
Complete fusion of all the secondary lamellae	II
Fibrosis	III
Necrosis	III

The sum of the number of lesion types within each of the three stages (a= stage I alterations, b= stage II alterations, c= stage III alterations) multiplied by the stage index (10° for Stage I, 10^{1} for Stage II, 10^{2} for Stage III) gives a numerical value for the degree of anatomical damage in a single fish gill. The summary equation is: I= (1)a + (10)b + (100)c. According to Poleksic and Mitrovic-Tutundzic (1994) the scale of the I values and their associated effects is as follows:

I values	Effects
0-10	Undamaged gills
11-20	Slightly to moderately damaged gills
21-50	Moderately to heavily damaged gills
>100	Irreparably damaged gills

This method of calculating a value of I makes it possible to compare the degree of

tissue damage in fish from different situations of pollution, and to correlate the intensity of pollution with the intensity of the changes found (Poleksic and Mitrovic-Tutundzic 1994).

RESULTS AND DISCUSSION

After 7 d, no mosquitofish in control aquaria exhibited moribund or dying states. Gills of all control fish appeared normal and undamaged on gross examination. Examined by light microscopy, sectioned gills of controls had regular primary and secondary lamellae. No fusion between adjacent primary and secondary lamellae was observed (Fig. 1). Mucous cells were located sporadically along the primary lamellae and less frequently along the secondary lamellae. No other gill lesions were observed, and the average I value assigned to all control groups was 0.

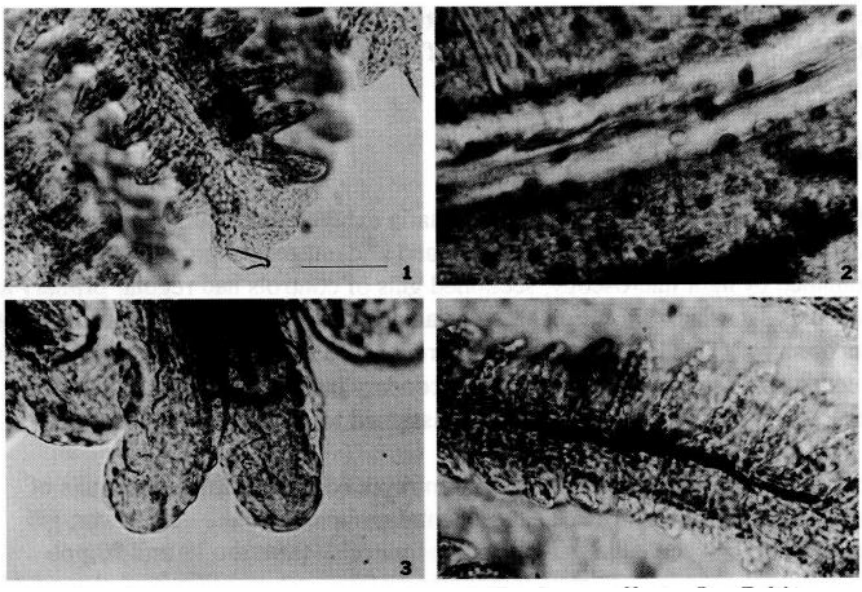
Gross and micropathological examination revealed no parasites in the gills of mosquitofish from control and experimental groups of Trial 1. However, gill parasites were observed in the gills of mosquitofish from the 19 and 90 ppb experimental groups of Trial 2. An average of five gill parasites was observed in all three fish exposed to 90 ppb of Bolero 8EC. Two fish in the 19 ppb experimental group were heavily parasitized (more than 20). Trial 2 controls and one mosquitofish in the 19 ppb group revealed no parasites.

Using light microscopy, a significant inflammatory response was detected in all mosquitofish exposed to Bolero 8EC (Figs. 2-4). The inflammation was characterized by hypertrophy of respiratory epithelium with leukocyte infiltration of gill epithelium, hyperplasia of mucous cells, and empty mucous cells. Gill lesions became more numerous and severe at higher concentrations. Stage II lesions (complete fusion of all secondary lamellae, hemorrhages with rupture of epithelium, stasis) were commonly found in the 1200 and 1800 ppb treatments. We did not observe necrosis in any of the experimentally treated fish. The average I values for the treatment groups are listed in Table 2.

All mosquitofish in the 1200 and 1800 ppb treatments exhibited moribund states before the 7th d. In the 90 and 576 ppb treatments, all mosquitofish appeared healthy after 7 d of exposure. One mosquitofish exposed to 19 ppb was observed in a dying state on the 6th d. A second fish was found in a pre-moribund state at the end of the 7th d. Hemorrhages with rupture of epithelium, stasis, fusion of secondary lamellae, and numerous gill parasites were found in these two fish.

Both of these fish had I values similar to those in the 1200 and 1800 ppb treatments. The third mosquitofish in the 19 ppb treatment did not show any signs of stress or death, and exhibited no gill parasites under light microscopic examination.

Stage I gill lesions occurred in mosquitofish exposed to concentrations of 19, 90



Photomicrographs of cross sections of gills of *Gambusia affinis* after 7 d in control and experimental treatments.
Scale bar, 50 microns.

Figure 1. Control showing the consistent spacing of secondary lamellae.

Figure 2. Showing empty and filled mucous cells of the gill arch.

Figure 3. Showing complete fusion of secondary lamellae at distal end of primary lamellae.

Figure 4. Showing hemorrhages with rupture of endothelium of primary lamellar blood vessel.

and 576 ppb. With increasing Bolero concentrations, stage I and II gill lesions appeared more extensively, thus showing a higher degree of gill damage. Bolero 8EC concentrations of 1200 and 1800 ppb were acutely toxic to non-parasitized mosquito&h and caused moderately to heavily damaged gills. The combination of numerous parasites (more than 20) in mosquito&h gills and 19 ppb of Bolero 8EC caused moderately to heavily damaged gills. Under light microscopy, gill parasites

Table 2. Degree of Gill tissue damage and I values of Parasitized and Non-parasitized mosquitofish from five different concentrations of Bolero 8EC.

Trial 1

Bolero 8EC Concentrations	Group Classifications $(\#)^n$	Average Group I Values	Associated Effects
0 ppb	Controls (3)	0.0	Undamaged gills
576 ppb	Non-parasitized (3)	7.8	Undamaged gills
1200 ppb	Non-parasitized (3)	24.3	Moderately to heavily damaged gills
1800 ppb	Non-parasitized (3)	25.6	Moderately to heavily damaged gills

Trial 2

Bolero 8EC Concentrations	Group Classifications (#)^n	Average Group I Values	Associated Effects
0 ppb	Controls (3)	0.0	Undamaged gills
19 ppb	Non-parasitized (1)	4.4	Undamaged gills
19 ppb	Parasitized (2)	23.9	Moderately to heavily damaged gills
90 ppb	Parasitized (3)	10.5	Slightly to moderately damaged gills

(#)^n: number of fish.

produced stage II lesions (hemorrhages with rupture of epithelium, stasis) at the primary lamellar blood vessels. These observations support a tentative conclusion that moderately to heavily damaged gills (I values of 21-50) producing death of mosquito&h can occur at concentrations of Bolero 8EC of 1200 and 1800 ppb. Additionally, a heavy gill parasite infestation can bring about death at Bolero 8EC concentrations as low as 19 ppb. Due to the small number of fish in the experimental design of this study, one must exercise caution in using these data to predict specific damage.

Harrington (1990) reported the 96-hr LC50 of mosquitofish to Bolero 8EC to be 2600 ppb. Our data indicate that the 96-hr LC50 of mosquitofish to Bolero 8EC is lower. All mosquitofish in the 1800 ppb experimental group exhibited a dying state and were collected before the 3rd d of the experiment. We can roughly estimate that the new 96-hr LC50 lies between 1200 and 1800 ppb. However, a statistically significant LC50 cannot be determined from our data due to the small number of fish in each experimental group.

Mosquitofish are susceptible to a large number of gill parasites. If mosquitofish are heavily parasitized and are weakened by unfavorable environmental conditions, then mortality of mosquitofish can result. *Gambusia affinis* was also chosen for the present study due to its higher resistance to various pollutants compared to many other fishes (Swanson et al. 1996). Gill damage in mosquitofish caused by exposure to Bolero 8EC is an indicator to the damage that can occur in other fishes that are found in Sacramento River valley agricultural drains.

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