Bull. Environ. Contam. Toxicol. (1997) 58:429–436

Bulletin of Environmental Contamination and Toxicology

Masculinization of Mosquitofish as an Indicator of Exposure to Kraft Mill Effluent

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Received: 1 June 1996/Accepted: 5 November 1996

Masculinization (i.e., arrhenoidy) of mosquitofish (*Gambusia affinis)* from a small stream receiving kraft mill effluent (KME) in northwest Florida has been previously documented (Howell *et al.* 1980). Responses to their exposure to KME include: precocial maturation of males, incorporation of elements of male reproductive behavior into the female behavioral repertoire, and acquisition of gonopodium-like anal fins in females. Bortone *et al.* (1989) suggested these responses may be used as a potential bioindicator of KME exposure. The latter characteristic (i.e., gonopodium formation as evidenced by the elongation of the anal fin) remains the most conspicuous indication of exposure to KME among mosquitofish. Moreover, a simple estimate of the degree of masculinization may be obtained from a comparison of the anal fin lengths of unexposed and exposed female mosquitofish (Drysdale and Bortone 1989).

The acquisition of male secondary sex characters by KME-exposed female mosquitofish (and other poeciliids) is similar to the response these livebearing fishes make to water-mediated administration of androgens (e.g., Turner 1960). The suspected presence in KME of steroid analogs and precursors capable of mimicking steroidal activity (as part of a general class of compounds known as endocrine disrupters) has been advanced to explain the observed similarity between the responses of poeciliids to KME exposure and exogenous treatment with androgens (Davis and Bortone 1992). However, the isolation of specific chemical agents in KME has proven difficult, largely as a result of inter-mill differences in the degree of effluent treatment which leads to differences in the composition of KME. Additional differences in KME concentration may be due to differences in the amount of discharge and/or its dilution associated with seasonal factors such as increased rainfall and droughts.

Variation in the masculinization response to KME has been noted among poeciliid fishes by Davis and Bortone (1992) who remarked that stream color was darker during periods of drought and that this was accompanied by an increase in the

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level of the masculinization. In the same small stream (i.e., Elevenmile Creek) near Pensacola, Florida where Howell *et al. (1980)* first observed masculinization in female mosquitofish, recent changes in the appearance of the effluent are attributed to process changes at a Pensacola mill.

In addition to process changes, the mill now reclaims approximately 97% of tall oils and terpenes (by-products of the wood pulp delignification process). The phytosterols ß-sitosterol and stigmastanol are major components of tall oils. In experimental exposures these substances induce masculinization in female livebearing fishes (Howell and Denton 1989). Environmental modification of phytosterols to form steroidal compound analogs has been suggested as a possible explanation for the steroidal properties of KME (Howell and Denton 1989; Davis and Bortone 1992). Highly toxic polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) are suspected to originate from the use of molecular chlorine in the bleaching process (Voss *et al.* 1988). A number of physiological and morphological responses have been described for egg-laying fishes exposed to these compounds (Walker and Peterson 1991). However, the role of molecular chlorine in the masculinization of female mosquitofish exposed to paper mill effluent is unknown.

Reduction in the use of molecular chlorine coupled with increased reclamation of tall oils at the Pensacola mill were sufficient criteria to warrant a reexamination of the degree of masculinization among females of the mosquitofish population that inhabits Elevenmile Creek. This reassessment was undertaken partially to test the utility of the mosquitofish as a bioindicator. Questions addressed in this study include: was masculinization evident after process improvements?; If masculinization persisted, was the morphological response different?

MATERIALS AND METHODS

Seine-net collections of the mosquitofish *Gambusia affinis* were made each month during 1995 at three sites in Escambia County Florida (Fig. 1). Two of the sites were located on Elevenmile Creek, which receives kraft mill effluent (KME) from a modern paper mill. The intermittent nature of the headwaters of Elevenmile Creek, and inconsistent abundances of mosquitofish in collections from the upper reaches of the stream precluded the selection of an upstream location as a reference site. The third site, located on Eightmile Creek, which receives no paper mill effluent served as a reference site. Eightmile Creek, a tributary of Elevenmile Creek, converges with Elevenmile Creek approximately 12 km downstream from the effluent outfall. In their initial study, Howell *et al.* (1980) found normal fin morphology in mosquitofish captured above the effluent outfall and from tributaries of Elevenmile Creek. The Eightmile Creek collection site was located approximately 3 km upstream of the confluence of the two streams and was one of the original reference sites examined by Howell *et al. (*1980*).*

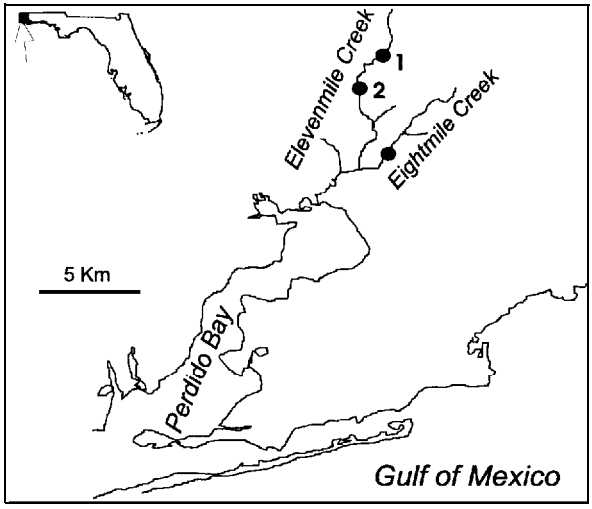

Figure 1. Location of sampling sites on Elevenmile and Eightmile Creeks, Escambia County, Florida. The effluent discharge point is located on Elevenmile Creek, approximately 4 km upstream of site 1.

Fish were absent from the Eightmile Creek site in December 1995. This temporary displacement of individuals from the Eightmile Creek site occurred during record low water temperatures.

Female mosquitofish were measured to the nearest 0.05 mm for standard length (SL) and anal fin length (AL), respectively. Standard length was measured as the distance from the tip of the snout to the insertion of the caudal fin. Anal fin length was measured as the distance from the anterior origin of the anal fin to the distal most point of the longest fin rays. To reduce variance associated with disparate sizes, female mosquitofish less than 25 mm SL and greater than 40 mm SL were excluded from analyses. Prior to analyses data were tested for assumptions of normality and equal variance at $p = 0.05$ using a Kolmogorov-Smirnov test. To simplify interpretation of comparisons between sites, months were grouped into one of two seasonal categories: winter, October through March; and summer, April through September. These categories correspond to periods of minimum and maximum seasonal drought (Palmer hydrological drought indices) in Northwest Florida (National Climatic Data Center 1996).

The main effect of site and the nested effect of season were examined in a multiple analysis of variance in which AL and SL functioned as dependent variables. One degree of freedom, orthogonal comparisons of site and seasonal mean values of SL and AL were made using the least squares method (Dowdy and Wearden 1991).

RESULTS AND DISCUSSION

Visual examination of 736 and 167 female mosquitofish from Elevenmile and Eightmile Creeks, respectively, revealed anal fin elongation (a characteristic of masculinization) among females from Elevenmile Creek sites. Anal fins of female mosquitofish from Eightmile Creek were normal (Fig 2A). Extremely modified anal fins typical of female mosquitofish exposed to concentrated KME (e.g. Fig 2B) were, however, rare among fish from either of the Elevenmile Creek sites. Most individuals were at an intermediate level of masculinization (Fig 2C, 2D).

The effects of site and season (within site), respectively, were significant factors ($F = 50.16$, $p < 0.005$; $F = 31.36$, $p < 0.005$) in determining SL and AL for female mosquitofish. Significant differences between sites for SL ($F = 22.67$, $p < 0.005$) and AL ($F = 62.10$, $p < 0.005$) were detected. Seasonal differences in AL within sites were significant ($F = 28.96$, $p < 0.005$). Seasonal differences were not evident for SL ($F = 0.65$, $p = 0.58$).

Evidence that the degree of masculinization varied seasonally was detected at both KME-receiving sites. At Eightmile Creek, the mean AL for summer months was not significantly different from mean AL recorded for winter months whereas mean AL at both Elevenmile Creek sites was significantly greater for months categorized as summer (Table 1). There were no differences in size (SL) between mosquitofish collected in winter and summer at any of the three sites. The lack of concurrence between results obtained for seasonal comparisons of AL and SL strongly suggests that seasonal differences in the degree of masculinization are not related to fish size. Comparisons of sites revealed that AL was greater at both Elevenmile Creek sites than at the Eightmile Creek site for either winter or summer (Table 1). When SL were compared, no statistical differences were found between fish from Eightmile Creek and Elevenmile Creek (site 1) for months categorized as winter or summer.

The Female *G. affinis* collected from Elevenmile Creek (site 2) were larger than those sampled at either Elevenmile Creek (site 1) or Eightmile Creek. This suggested that the differences in AL of females of *G. affinis* from Eightmile and Elevenmile Creeks were not associated with differences in SL between sites. Eightmile Creek and Elevenmile Creek (site 1) fish were more similar in SL than either site was to Elevenmile Creek (site 2). Consequently, it is reasonable to infer that increased anal fin length of KME-exposed fishes during the summer months was not dependent on fish size (SL).

In summary, masculinization which was evident among KME-exposed female mosquitofish from Elevenmile Creek prior to the initiation of effluent treatment improvements has persisted. Anal fin elongation varied temporally and this variation was unrelated to variation in fish length. The degree of masculinization

Table 1. A summary of seasonal and site comparisons of mean standard lengths (SL) and anal fin lengths (AL) of female mosquitofish. Displayed *p*-values refer to seasonal comparisons within sites, NS = Not statistically significant at $p \leq 0.05$. In comparisons between sites vertical lines connect values that did not differ significantly at $p \leq 0.05$. X = mean, se = standard error.

	SL (mm)					AL (mm)				
	Winter		Summer			Winter		Summer		
Site	$\bar{X}$	se	$\bar{X}$	se	*p*	$\bar{X}$	se	$\bar{X}$	se	*p*
Eightmile Creek	31.15	0.23	30.77	0.26	NS	6.54	0.06	6.43	0.07	NS
Elevenmile Creek 1	31.39	0.22	31.21	0.30	NS	6.95	0.58	7.61	0.10	< 0.005
Elevenmile Creek 2	32.41	0.25	32.83	0.23	NS	7.14	0.06	7.81	0.07	< 0.005

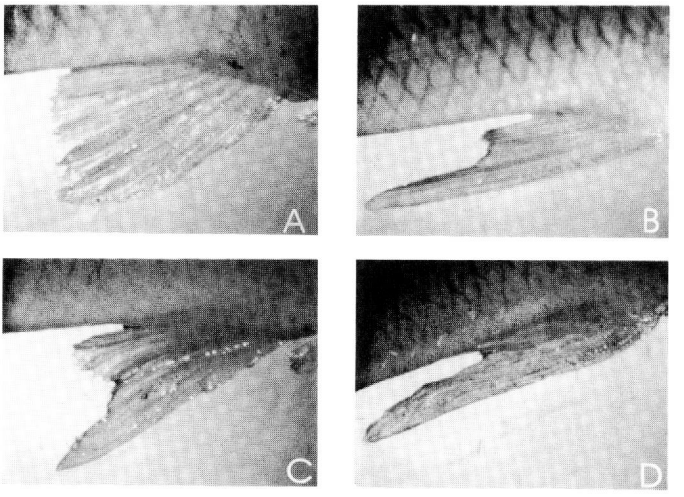

Figure 2. Anal fin morphology of female mosquitofish. (A) Normal fin of a female (SL = 37.0 mm, AL = 7.4 mm) from Eightmile Creek; (B) Extremely elongated fin of a female (SL = 30.9 mm, AL = 9.27 mm) from Fenholloway River (a KME-receiving stream, Taylor Co., Florida) indicative of advanced masculinization; (C), (D) Anal fins of females (SL = 37.0 mm, 35.7 mm, AL = 8.51 mm, 10.0 mm, respectively) from Elevenmile Creek - site 1 and site 2, showing intermediate levels of masculinization. Fish were collected in Nov, Mar, Jan, and Apr, respectively.

among fish collected during summer months (i.e., April through September) was greater than that noted for fish collected during winter (i.e., October through March). Davis and Bortone (1992) remarked that female poeciliid fishes collected from streams receiving KME were smaller, less abundant, and more masculinized during periods of drought. An increase in effluent concentration (inferred from a noted intensification of water staining during periods of drought) was suggested to explain these responses (Davis and Bortone 1992). Months categorized in the present study as summer correspond to a seasonal period of relative drought in northwest Florida. The relative increase in effluent concentration (associated with drought conditions or periods of reduced rainfall) may partially explain temporal differences in the degree of masculinization observed among female mosquitofish in this study.

It is possible that a number of other factors may also influence the degree of masculinization among female mosquitofish. Many poeciliid fishes have increased metabolic activity when water temperature increases (Snelson 1989). Increased metabolism of xenobiotic substances (also associated with an increase in water temperature) may affect the degree of masculinization during summer months. Piferrer *et al.* (1994) showed that genetic female chinook salmon

required only a brief exposure to an aromatase inhibitor during sexual differentiation to induce phenotypic sex change. The timing of initial exposure and duration of exposure to masculinizing agents in the effluent may also influence the degree of morphological change and may make some cohorts of mosquitofish more susceptible to arrhenoidy.

Some notable changes that have occurred at the Pensacola mill include, oxygen delignification in 1986, 50% ClO_2 substitution in 1989, and the elimination of molecular chlorine as a bleaching agent in 1995. In Elevenmile Creek, any differences in morphological response by mosquitofish due to the elimination of molecular chlorine from the bleaching process in 1995 will probably not be evident until cohorts recruited into the population after the process change attain maturity and the individuals that were masculinized prior to changes in the bleaching process are no longer present in the population. Phytosterols present in tall oils cannot be eliminated as a source of masculinization because low levels of tall oils are still present in the effluent. The paucity of highly masculinized females suggests that an overall reduction in the degree of masculinization is associated with tall oil reclamation efforts. Temporal differences in the degree of masculinization may provide a meter for the determination of critical levels, below which there is no morphological response.

Based on previous studies, Davis and Bortone (1992) noted the potential of poeciliid fishes to serve as indicators of paper mill effluent impacted waters. The persistence of masculinization among female mosquitofish exposed to treated KME and temporal variation in the arrhenoid response reaffirms the potential of this assay to assess environmental conditions in effluent receiving waters. Local phenotypic differences may represent adaptive responses to seasons. However, the effectiveness of *G. affinis* and other poeciliid fishes as indicators of environmental conditions within KME-impacted waters will largely depend on regular and consistent monitoring of the status of the morphological response within exposed and unexposed populations.

Acknowledgments. The authors thank J. Brusher, M. Giles, R. Heise, E. Olson, and J. Stolarzyk for field assistance. This study was supported through a grant (R 81143-01-0) from the U.S. Environmental Protection Agency to SAB.

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