Bull. Environ. Contam. Toxicol. (1999) 63:150-156


Bulletin of Environmental Contamination and Toxicology

# Morphological Masculinization in Poeciliid Females from a Paper Mill Effluent Receiving Tributary of the St. Johns River, Florida, USA

S. A. Bortone,[1] R. P. Cody[2]

[1] Florida Center for Environmental Studies, FL, USA
[2] Florida Marine Research Institute, Division of Marine Resources, 100 Eighth Avenue, SE., St. Petersburg, FL 33701-5095, USA

Received: 15 December 1998/Accepted: 4 June 1999

Environmentally induced intersexuality among natural populations of poeciliids was first described by Howell *et al.* (1980). In two paper mill effluent-receiving streams, the Fenholloway River, Taylor Co. and Elevenmile Creek, Escambia Co., Florida, the anal-fins of females of the mosquitofish (*Gambusia holbrooki*) were modified into gonopodium-like structures typical of developing males. Where present, females of two other poeciliids, the least killifish (*Heterandria formosa*) and the sailfin molly (*Poecilia latipinna*) also bore similarly modified anal-fins. Masculinization (arrhenoidy) in response to paper mill effluent was subsequently reproduced in controlled exposures using mosquitofish females (Drysdale and Bortone 1989). Although a number of mechanisms of endocrine-disruption, focusing on the presence in effluent of chemicals with steroidal properties have been proposed (Howell *et al.* 1980; Davis and Bortone 1992) the complex nature of paper mill effluents has made the task of identifying masculinizing chemicals difficult. In controlled exposures, female mosquitofish exposed to the plant sterol, β-Sitosterol, a major byproduct of wood-pulp delignification, in the presence of *Mycobacterium smegmatis* became masculinized (Howell and Denton 1989). McLatchy and Van Der Kraak (1995) provided evidence that exposure to β-Sitosterol reduced gonadal steroid synthesis in goldfish. *Mycobacterium smegmatis* and plant sterols (including β-Sitosterol) are present in waters unimpacted by paper mill effluents, albeit at much lower concentrations.

Although the phenomenon of arrhenoidy has been suspected for a number of other effluent-receiving streams investigations of this kind have not been undertaken. Consequently, the extent of masculinization among females from other natural populations of mosquitofish and other poeciliids exposed to paper mill effluent remains largely unknown.

Elevenmile Creek and Fenholloway River are characterized by relatively small basins, of which, effluent may account for >80% of stream volume in the vicinity of their respective effluent outfall sites. Consequently, effluent concentrations may remain as high as 50% of overall stream volume several km downstream from the source. In larger receiving rivers, effluent concentrations as high as

*Correspondence to:* R. P. Cody

those found in Elevenmile Creek and Fenholloway River are unusual or are restricted to the immediate discharge area. Effluent concentrations of <5% volume are not unusual in larger rivers (e.g., McLeay 1987; Middaugh *et al.* 1997).

Rice Creek is a tributary of the St. Johns River in Florida and is the receiving stream for effluent from a large wood-pulping facility in Putnam County. Similar to Elevenmile Creek and Fenholloway River, Rice Creek represents a relatively small drainage. However, unlike Rice Creek, both Elevenmile Creek and Fenholloway River receive effluent relatively close to their headwaters. In Rice Creek, the effluent discharge site is located just a few km from the mouth of the stream (the confluence of Rice Creek with St. Johns River), thus insuring a more rapid dilution of effluent.

The main objective of this study was to determine whether Rice Creek populations of livebearing poeciliid fishes responded to paper mill effluent exposure in a similar manner to those of Elevenmile Creek and Fenholloway River. The hypothesis tested in this study is that in response to high concentration of paper mill effluent in Rice Creek, inhabitant poeciliid fishes will display evidence of masculinization similar to that of Elevenmile Creek and Fenholloway River populations. Moreover, the extent of masculinization among population samples from Rice Creek may help elucidate the relationship between the expression of arrhenoidy in livebearing poeciliids and exposure to paper mill effluent.

## MATERIALS AND METHODS

A 3 m-seine net (8-mm stretched mesh) was used to collect fish from four sites along Rice Creek (U1, D1-D3) and a single site on Etonia Creek (EC) in March 1997 (Fig. 1). Both Etonia and Rice Creeks are tributaries of the St. Johns River. Etonia Creek flows into Rice Creek approximately 2 km upstream of the confluence of Rice Creek with the St. Johns River. In addition to fresh collections, additional material was available from the University of Florida, Florida Museum of Natural History, Gainesville, Florida (Catalog No. UF28023). This reference collection is referred to herein as D4 Ref1. A collection from the same site (referred to as D4 Ref2) was made by the authors in January 1997. A collection from the Fenholloway River (Fen Ref) provided a reference sample from a population in which arrhenoidy had been documented (e.g., Howell *et al.* 1980; Cody and Bortone 1997). The approximate locations of collection sites relative to the effluent outfall area are as follows: U1, 200 m upstream of the effluent discharge site; D1, 200 m downstream; D2, 400 m downstream; D3, 900 m downstream; and D4 Ref, 3.0-3.2 km downstream. The Etonia Creek reference site (EC) is located 100-200 m upstream of confluence with Rice Creek.

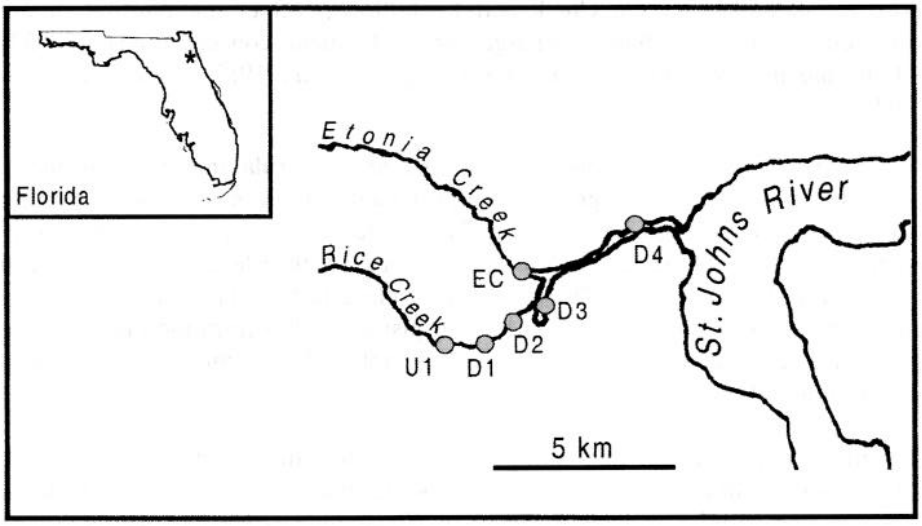


**Figure 1.** Location of collection sites on Rice Creek and Etonia Creek. The effluent discharge site is situated between sites U1 and D1 on Rice Creek. Within the map of Florida, the location of Rice Creek and Etonia Creek sites is denoted by a star.

The reference site chosen from the Fenholloway River was approximately 3.0 km downstream from the effluent discharge site.

All collected fish were fixed in a 10% formalin solution for a minimum of 24 hrs and subsequently preserved in 40% isopropyl alcohol. Mosquitofish females were measured for standard length (SL) and anal fin length (AL). As masculinized females bear conspicuously elongated anal-fins, a simple index of masculinization was calculated as the median AL/SL value for fish measured from a given site. To reduce the influence of size on anal-fin length only fish between 25 and 40 mm SL were measured. The use of larger fish also reduces the possibility that (normally smaller) true males were incorrectly identified as female fish. Females were identified by the presence of a gravid spot on the lower abdomen.

Gross visual evidence of masculinization was verified through inspection of female fish for gonopodial development with the aid of a Wild M5D binocular microscope attached to an Optimas® image analysis system. When present, females of the sailfin molly and least killifish were also examined for similar morphological features of masculinization.

All statistical analyses were performed using Jandel Sigmastat® ver. 2.01 for PC compatible computers. Data were tested for assumptions of normality and equal variance prior to statistical analysis.

## RESULTS AND DISCUSSION

Gonopodial development was evident in females of all three poeciliid species (eastern mosquitofish, least killifish, and sailfin molly) present in Rice Creek. However, only the mosquitofish was abundant and allowed "between-site" comparisons of masculinization effects. Samples of mosquitofish contained between 10 and 90 females (mean = 31.4). Microscopic examination of anal-fins of masculinized females revealed both increased segmentation of anal fin-rays 3-5 and changes in the architecture of terminal segments (Fig. 2).

Morphological evidence of masculinization was found in mosquitofish females from sites U1, D1, D2, and D3 on Rice Creek (Fig. 3). No masculinized fish were found at site D4 on Rice Creek or the Etonia Creek site (EC). The most conspicuously masculinized fish were found at the nearest downstream site D1, located approximately 200 m from the effluent outfall. Although, the number of sites sampled was small, a trend indicative of diminished masculinization associated with increased distance from the discharge site was evident. At each of the sites that contained masculinized fish, females bearing normally developed anal-fins were also found. Consequently, a high degree of variation in relative anal-fin length was characteristic of population samples from sites where masculinization was found (Fig. 3). This was particularly noticeable in the Fenholloway River (Fen) sample. Small sample size probably contributed to the large variance. In contrast, low variation in relative anal fin length characterized D4 and EC sites, where masculinized fish were not found.

The degree of masculinization among female mosquitofish from site D1 of Rice Creek was comparable to that found in fish from the Fenholloway River reference site. Although only two reference collections (1992, 1997) from Rice Creek were available for examination, neither collection contained females with abnormal gonopodial development (Fig. 3). To evaluate statistically the relationship between site and masculinization, median values of AL/SL were examined in a Kruskal-Wallis one-way Analysis of Variance (ANOVA) based on ranks. Median AL/SL values were found to differ significantly between sites ($H = 64.2$, $df = 7$, $p < 0.001$). Pairwise comparisons (Dunn's method) revealed significant differences ($p < 0.05$) between ranks of median AL/SL values for site D1 fish versus those from EC, U1, D3, D4 ref1, and D4 ref2 and for site D2 fish versus fish from EC, D4 ref1 and D4 ref2 sites. Differences between masculinization levels in female fish from the two downstream sites (D1, D2) closest to the

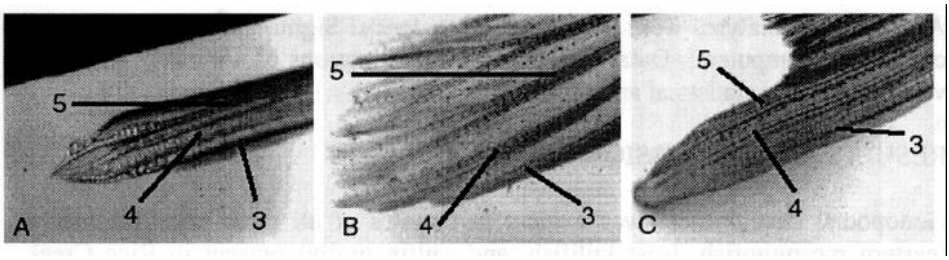


**Figure 2.** Anal-fin morphology of normal and masculinized mosquitofish from Rice Creek. A. Anal-fin morphology of a male. B. Anal-fin structure of an normal (unmasculinized) female. C. Anal-fin structure of a masculinized female. Numbers 3-5 refer to the third-fifth anal fin rays. Note the presence of dorsal projections on the fifth anal-fin ray of the male. Increased segmentation is evident in the third anal-fin ray of the masculinized female.

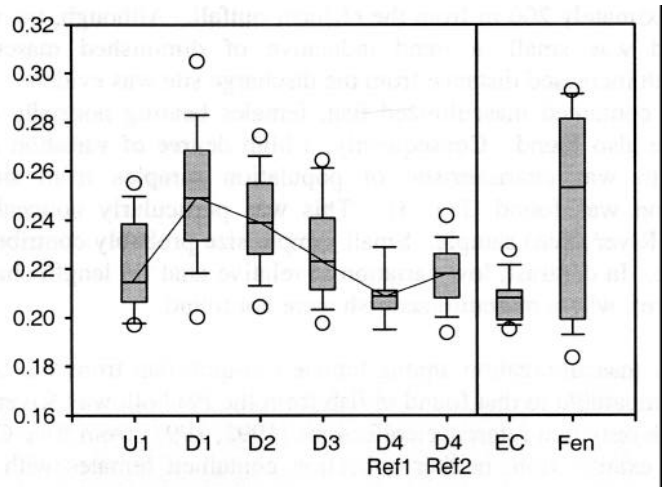


**Figure 3.** Modified box plots of relative anal fin lengths for mosquitofish collected from sites on Rice Creek (U1, D1-D4), Etonia Creek (EC), and the Fenholloway River (Fen). The median = horizontal line through the boxes, boxes are delimited by 50% and 75% quartiles, bars represent 95% confidence intervals, open circles = outliers. Arrow denotes the relative position of the discharge site relative to Rice Creek sites.

effluent outfall and other sites on Rice Creek are consistent with a distance/dose related response to effluent exposure.

Indicative of broad variation in the degree of masculinization among fish from the Fenholloway River site (Fen Ref), no pairwise comparison involving this site was statistically significant. The lack significant differences in quantitative measures of masculinization between Fenholloway River fish and fish from both Etonia and Rice Creeks emphasizes the importance of microscopic examination of anal fin structure as verification of masculinization.

In common with the Fenholloway River and Elevenmile Creek, masculinized fish were encountered at downstream sites with the most masculinized fish occurring at sites closest to the discharge point. Unlike Elevenmile Creek and Fenholloway River, in Rice Creek masculinized fish were found upstream from the effluent outfall. The absence of arrhenoidy among female mosquitofish from upstream sites in both Elevenmile Creek and Fenholloway River was interpreted as evidence that morphological aberrations were directly associated with exposure to paper mill effluent (Howell *et al.* 1980; Rosa-Molinar and Williams 1984). Because of its proximity to tidal St. Johns River, Rice Creek exhibits daily fluctuations in water level of 30-45 cm. Although a net downstream movement of effluent would be expected, tidal ebb and flow results in effluent moving upstream with an incoming tide. As well as affecting the movement of effluent, the tidal cycle (by facilitating movement of fish between sites) may also partially explain the high variance in levels of arrhenoidy exhibited by fish at those sites on Rice Creek. Another difference between the Rice Creek discharge site and that of either Elevenmile Creek or Fenholloway River is the distance to a major diluting water body. Discharge sites for both Elevenmile Creek and Fenholloway River, are located approximately 20 and 25 km upstream from their respective termini. Evidence of arrhenoidy within those populations has been described for fish at downstream sites several km from their respective discharge points (Howell *et al.* 1980; Cody and Bortone 1997). Despite the relatively restricted distribution of arrhenoidy among mosquitofish population samples from Rice Creek, there is a relatively clear association between the degree of masculinization and the level of exposure.

The large number of chemicals present in bleached kraft mill effluent precludes investigation of the masculinization potential of each one. However, as information becomes available on the status of other poeciliid populations exposed to paper mill effluents, comparison of mill characteristics and associated environmental conditions may help in the elucidation of a mechanism of action.

Acknowledgments: We thank Stuart Holm and Myra Carpenter, Georgia Pacific Corp., John Higman, St. Johns Water Management District for assistance in data

collection. Thanks are also due to Florida Museum of Natural History for providing catalogued collections for examination. This study was supported through a grant (R81143-01-0) from the U.S. Environmental Protection Agency to SAB.

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