Bull. Environ. Contam. Toxicol. (1989) 43:370–377

Morphological and Behavioral Characters in Mosquitofish as Potential Bioindication of Exposure to Kraft Mill Effluent

Stephen A. Bortone,[1] William P. Davis,[2] and Charles M. Bundrick[3]

[1]Biology Department, University of West Florida, Pensacola, Florida 32514, USA; [2]U.S. Environmental Protection Agency, Gulf Breeze, Florida 32561, USA and [3]Institute for Statistical and Mathematical Modeling, University of West Florida, Pensacola, Florida 32514, USA

Intersexuality in the form of arrhenoidy, induced by environmental factors, is documented for the mosquitofish, Gambusia affinis (Howell et al. 1980). The masculinized condition of the secondary sex characters (chiefly the anal fin) among female mosquitofish occurs in stream sections receiving kraft mill effluent (KME), but is entirely absent both upstream of the effluent discharge and in tributaries to the main stream. In addition, this phenomenon has been observed at another stream site receiving KME in two additional poeciliid fish species (Howell et al. 1980; Bortone & Drysdale 1981). Concomitant with the morphological changes induced in mosquitofish exposed to KME, changes in behavior (Howell et al. 1980) as well as reproductive potential (Rosa-Molinar & Williams 1984) have been reported. Laboratory studies indicate that arrhenoidy can be induced in young female mosquitofish exposed in to KME (Drysdale 1984). The induced changes closely resemble responses that mosquitofish display after having been exposed to various androgens (Turner 1960).

Although the specific chemicals or factors actually responsible for induction of arrhenoidy among these fish have not yet been identified, it is known that a wide variety of potential compounds occur as by-products from the processing of wood pulp (Keith 1976). The purpose of this study was to investigate the morphological and behavioral responses of mosquitofish environmentally exposed to KME and to evaluate the potential of these responses as bioassay endpoints. A method to quantify the morphological or behavioral responses of mosquitofish should provide an in situ bioindicator to assess the impact of KME discharge has on receiving water biota.

MATERIALS AND METHODS

Morphological comparisons using discriminant function analysis consisted of fish from four treatment groups: (1) males and (2) females from a stream that received KME (Elevenmile Creek in the Perdido Bay Drainage approximately 1 - 5 km downstream of the effluent discharge; see Howell et al. [1980] for a description of the study site), and (3) males and (4) females from water that did

Send reprint requests to: SA Bortone

not receive KME (a 1 hectare pond in Escambia Co., Florida, approximately 15 km East of Elevenmile Creek and part of the Escambia River Drainage). Fish were preserved in 10% formalin for 1 wk and later transferred to 40% isopropyl alcohol. All morphometric characters were recorded from fish according to the methods of Hubbs & Lagler (1964) with the following emendations: preanal length, shortest distance between the anus and the anterior tip of the lower jaw; postanal length, shortest distance between the anus and the center of the hypural base; and anus to gonopore distance (anus - gonopore dist.), shortest distance between these structures. Measurements were recorded to the nearest 0.01 mm using an ocular micrometer except standard length, total length, preanal length, and postanal length which were to the nearest 0.1 mm, using a dial caliper.

The SAS statistical package (SAS 1979) was used to conduct discriminant function analyses on the morphometric data (excluding the standard length measurement) by converting all morphometric measurements to hundredths of standard length to reduce the impact of size on the analysis.

Specimens for the behavioral comparison and evaluation also consisted of four treatment groups: males and females from water that had received KME (the same general locations in Elevenmile Creek as above); and males and females from a drainage pond receiving no KME in Cantonment, Florida. Fish were encircled by a fine (3.2 mm) mesh minnow seine. Within 1 hr of capture, fish were transferred to a 150-L outdoor fiberglass holding tank containing noncontaminated freshwater with aquatic vegetation. After 2-4 wk, fish were transferred to a similar holding tank in the laboratory and acclimated for 1-2 mo at 20-22°C and illuminated for a photoperiod of 12L-12D using daylight, fluorescent lamps.

Behavioral observations were made on pairs of fish placed in a 3.8-L glass jar containing 1.9 L of pond water at a temperature of 20-21°C. During the afternoon fish were observed after a 1 hr acclimation to the observation jars. Initially, fish placed in the observation jars exhibited erratic behavior; however, after about 30 min they appeared unstressed.

Twenty replicate pairs of fish from the four treatment groups were observed for 10 min periods during which they were scored for the frequency of occurrence of five behavioral characters. We chose the following characters and their definitions for recording fish interactions: Approach - fish, slowly but deliberately, moved toward the other; Chase - fish abruptly and quickly moved toward the other; Display - fish's body remained rigid, quivering slightly with fins held erect; Thrust - fish's erect anal fin was moved toward the gonopore of the other fish (usually following an approach or chase); Penetrate - contact with the gonopore was made with the erect or extended anal fin (usually following a thrust).

Fish from the four treatment groups were observed (i.e., F = females not previously exposed to KME, G = females captured from stream water known to receive KME and displaying arrhenoidy, M = males not exposed to KME, and B = males captured from water known to receive KME) in the following combinations: B x G, F x M, F x G, F x F, and G x M. Treatment groups from twenty replicates of each combination were statistically compared, using the Student-Newman-Keuls test, for the average frequency of occurrence of each behavior as observed during the 10 min observation period. No fish was observed for more than one period and assignment to pairwise combinations was done without regard to body size.

RESULTS AND DISCUSSION

Morphometric data comparing the treatment groups of mosquitofish are presented in Table 1. Each morphometric variable was divided by the standard length of that individual to help standardize the data and to reduce the impact of size on the analyses. Discriminant function analyses were conducted to determine the efficacy of using morphometric data to correctly assign individuals to their respective treatment group. Standard length was excluded from the analyses. Using 14 morphometric variables, the discriminant function analysis indicated extremely good treatment group assignment (99.2% correct assignment). Only one fish was incorrectly assigned; in that instance a non-KME exposed male was assigned into the KME-exposed male treatment group.

Table 1. Summary of morphometric data used to compare male and female mosquitofish exposed or unexposed to KME. Numbers indicate the mean character value in mm followed by the standard deviation.

Character	Male Control n=22	Female Control n=38	Male Exposed n=31	Female Exposed n=39
Standard Length	20.55±1.52	27.59±3.64	17.67±3.05	27.25±4.86
Total Length	25.75±1.87	34.37±4.18	21.96±3.86	34.21±6.14
Preanal Length	10.02±0.82	15.95±2.43	8.63±1.29	15.45±2.98
Postanal Length	11.88±1.09	12.78±1.34	9.95±2.28	12.84±1.93
Predorsal Length	12.76±0.95	18.22±2.77	10.99±1.72	17.52±3.42
Body Depth	4.52±0.34	7.16±1.63	3.87±0.75	7.12±1.68
Caudal Ped. Depth	2.97±0.25	3.89±0.65	2.64±0.51	4.18±0.78
Head Length	4.98±0.37	6.74±0.66	4.38±0.66	6.48±1.16
Dorsal Fin Height	4.69±0.57	5.84±0.62	4.09±0.86	6.47±1.34
Anal Fin Height	7.05±0.63	6.00±0.44	5.81±1.05	6.71±1.11
Pectoral Fin Length	4.05±0.47	5.11±0.48	3.50±0.73	5.33±1.11
Pelvic Fin Length	1.91±0.30	2.96±0.37	1.53±0.34	2.67±0.73
Orbit Length	1.77±0.16	2.19±0.13	1.53±0.22	2.10±0.32
Anus - Gonopore Dist	0.74±0.19	1.01±0.16	0.54±0.17	1.01±0.26
Interorbital Width	2.36±0.19	3.59±0.50	1.98±0.35	3.28±0.63

One of the goals of this study was to develop bioassay endpoint criteria for rapid identification of fish responses to KME exposure. Therefore, additional discriminant function analyses were performed using still fewer morphometric characters. To reduce the number of variables per analysis, an ANOVA procedure was conducted on the morphometric data. The variables total length, head length and pectoral fin length were eliminated as a result of this procedure because each variable had an F-value less than 3.88 (df = 3, 126), and the amount of variation each variable contributed to their respective model was always less than 8.5%. Using this data set of eleven variables, reduced by F-value criteria, correct treatment group assignment was still high (95.3%). Misclassifications occurred with regard to KME exposure within sex. No misclassification occurred between sexes. The number of variables used to correctly identify treatment groups was further reduced by examination of the correlation coefficients between all morphometric variables. Variables displaying a significant ($p < 0.05$) corrélation with at least 10 or more variables were considered redundant, contributing little to assignment of individuals to treatment groups. The variables postanal length and orbit length were therefore eliminated (leaving a total of nine variables). The discriminant function procedure was still able to establish correct treatment group assignment at a high level (95.3%). Generally, misassignment remained as previously described with the exception of an individual KME-exposed male being assigned to the KME-exposed female treatment group.

A summary of the behavioral responses of pairwise interactions for the four treatment groups is presented in Table 2. The number of interactions observed during a 10-min observation period was generally low with the most frequent behavior being Approach and Penetrate the least. There was a statistically significant tendency for females to have a much lower number of behavioral responses than males during the observation period. There was no significant tendency for KME-exposed females to have a significantly higher number of behavioral responses than non-KME exposed females. There was, however, an indication that KME-exposed females were statistically similar to males for the behavioral character Display.

Males from both treatment groups showed their behavioral characters more frequently during the observation period than females of either treatment group. There, however, was a slight tendency for non-KME males to show the behavioral characters more frequently than the KME exposed males. Notable was the tendency for non-KME exposed males to show the highest frequency of behavioral characters of all pairwise combinations when interacting with non-KME exposed females (i.e., the "normal" interaction).

The discriminant function analyses indicated that correct treatment group assignment occurs at a high level among mosquitofish males and females, whether or not they have been exposed to KME. Morphological

Table 2. Summary of behavioral responses and results of Student-Newman-Kuels test comparing the means of the behavioral traits (per 10 min observation period) to characterize the four reproductive groups of mosquitofish.

Behavioral Character									
Approach		Chase		Display		Thrust		Penetrate	
Type	Mean	Type	Mean	Type	Mean	Type	Mean	Type	Mean
F x F	3.1	F x G	0.5	G x M	0.3	F x M	0.0	F X M	0.0
F x G	6.0	G x M	0.7	F x F	0.4	F x G	0.0	F X G	0.0
G x M	6.1	F x F	0.9	F X G	0.8	F x F	0.0	F x F	0.0
G x B	6.8	G x B	1.4	F x M	0.9	G x M	0.0	G x F	0.0
G x F	7.4	G x F	1.7	G x B	1.4	G x B	0.1	G x B	0.0
F x M	8.8	F x M	2.0	G x F	2.9	G x F	0.2	G x M	0.0
B x G	14.2	M x G	5.0	M x G	4.3	B x G	3.1	M x F	0.1
M x G	15.6	B x G	6.7	B x G	5.5	M x G	3.3	M x G	0.3
M x F	16.9	M x F	8.6	M x F	6.3	M x F	3.4	B x G	0.4

F = control females, G = KME exposed females, M = control males, B = KME exposed males (the first letter in the pairing indicates the reproductive group being compared as a result of the pairwise observations). Treatment groups with statistically similar means (at the 0.05 level) are indicated by a continuous vertical line.

character analysis shows potential use as a bioindicator of exposure to KME. There were few incorrect classifications to treatment groups and these occurred mostly for KME-exposed males classified as non-KME exposed males. On a morphometric basis, males are less affected by the masculinization factor than females and it would seem reasonable to expect misclassifications to occur among them. The discriminant function analysis has potential as a useful and effective technique for identifying masculinized females resulting from exposure to KME.

The behavioral characters were ineffective as a measure or bioassay endpoint to discriminate between treatment groups. Correct sex assignment is likely with this method but correct treatment group recognition is unlikely. Lack of behavioral distinctions among treatment groups may be due to several reasons: The behavioral characters selected may be somewhat arbitrary, insensitive and/or ambiguous. Although these characters were comparable to those recognized by Itzkowitz (1971) and Martin (1975), the assumption of significance of these characters to the reproductive success of the species may be invalid. Perhaps, if another suite of characters had been chosen, a more discriminating behavioral measurement might have resulted. The test conditions in the present study may not have been appropriate for this species. Modifications of the scoring protocol or, perhaps, testing during or immediately after exposure to KME might improve the reliability of a behavioral test. Seasonal maturation patterns or reproductive condition may have contributed to variation in behavior and, therefore, the unreliability of these characters to permit discrimination among treatment groups. It was noticed in an earlier trial study conducted during November-December that few or no behavioral interactions occurred between the individuals in the treatment group pairs. Although characters related to reproductive condition were observed during the March-April testing period reported here, it is possible that significantly more behavioral interactions might occur during other parts of the reproductive season. Additionally, if more behavioral characters had been scored and more frequent interactions had been observed, then perhaps a discriminant function analysis would have permitted a high degree of treatment group assignment.

Howell et al. (1980) indicated that male and female mosquitofish exposed to KME were more aggressive in courtship than unexposed mosquitofish. Schröder & Peters (1988) were able to demonstrate that the reproductive behavior in male guppies was useful as a bioindicator of low concentrations of aquatic pollutants. Our results indicate a lower level of aggressive reproductive behavior in male and female fish exposed to KME in comparison to non-KME exposed fish of the same sex.

Identification of onset and quantification of the degree of the masculinization response among poeciliid fishes exposed to KME continues to be a problem. Denton et al. (1985) and Howell & Denton (1989) indicated that plant sterols such as stigmastanol and ß-sitosterol can induce masculinization in female mosquitofish after these sterols interacted with the bacterium, Mycobacterium smegmatis. This masculinization phenomenon, besides being a continuum of intersexual states, additionally may be a response to a secondary biodegradation of KME constituents rather than a reaction to a specific pollutant compound. However, the total impact on the aquatic community must be considered in the design of waste treatment processes and the discharge of treated effluents. The morphometric responses presented in this study offer a useful bioindicator and environmental signal to recognize individual mosquitofish responding to environmentally induced intersexuality. Further investigations are needed to resolve additional effects that this induction may represent both to this species' reproductive success, environmental fitness as well as life history attributes of other species in these impacted habitats. Schooling behavioral responses to KME have been noted for fish (Myllyvirta & Vuorinen 1989) and may also serve as a useful indicator of KME. It should be noted, however, that it is important to determine if these alterations in behavior are indeed toxic responses (Ruben & Wagner 1989).

Acknowledgments. We thank Charles Ashton for his perspectives on the behavior of mosquitofish. In addition we thank Rudolf Reinboth for providing laboratory facilities, and Jerry Oglesby for help with the statistical analyses and R. Alani Davis for assistance in the field. WM Howell constructively reviewed an earlier draft of the manuscript. Contribution No. 659, Environmental Research Laboratory, US EPA, Gulf Breeze, FL 32561.

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Received November 4, 1988; accepted March 6, 1989.