Abnormal Expression of Secondary Sex Characters in a Population of Mosquitofish, Gambusia affinis holbrooki: Evidence for Environmentally-Induced Masculinization
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Paper mill effluents are discharged into Elevenmile Creek at Cantonment, Escambia County, Florida. The total population of mosquitofish, *Gambusia affinis holbrooki*, inhabiting the stream below the effluent discharge exhibits abnormal sexuality. All females are strongly masculinized, displaying both physical secondary sex characters and reproductive behavior of males. Males exhibit precocious development of physical secondary sex characters and reproductive behavior. Elevenmile Cr. above the paper-mill effluent, as well as tributaries to this creek, all contain *Gambusia* with normal secondary sex characters. This evidence strongly suggests that some yet unidentified chemical or combination of chemicals associated with the paper-mill effluent exerts a strong androgenic effect upon this population. This constitutes the first report of possible environmentally-induced masculinization involving a total natural population of vertebrates.

**THE mosquitofish, *Gambusia affinis*, is a live-bearing, freshwater member of the Poeciliidae. It is a diminutive species rarely exceeding 46 mm standard length. Sexual dimorphism is pronounced in that mature males are distinctly smaller than mature females and exhibit an anal fin which is modified into a complex intromittent device, the gonopodium, which is used to transfer sperm packets into the female genitalium (Figs. 1A, 2A). The gonopodium is an elongation and modification of anal-fin rays 3, 4 and 5. The gonopodial rays have evolved a functional independence from the more posterior rays and can rotate forward and laterally to facilitate copulation. The gonopodial tip is equipped with barbs and spines which serve as holdfast devices during gonopodial insertion into the female genitalium (Rosen and Gordon, 1953). Female anal-fin rays are unmodified (Figs. IB, 2B). Several days after internal fertilization, a dark gravid spot appears in the pregnant females on each side of the abdomen (Fig. 1B). This spot enlarges as pregnancy proceeds and is prominent near the time of parturition (Breder and Rosen, 1966).

Expression of the dimorphic secondary sex characters in *Gambusia* seems stable. During the past 100 years, ichthyologists have collected and studied thousands of specimens of *Gambusia*, and no report has appeared of masculinization, hermaphroditism, or sex reversal within natural populations of this fish. Herein, we report the discovery of masculinization involving a total natural population of *Gambusia a. holbrooki* inhabiting a stream receiving paper-mill effluents. All females within this stream are strongly masculinized, possessing a male-like gonopodium (Figs. 3, 4C–F) and displaying male reproductive behavior. All males exhibit precocious secondary sex characters and reproductive behavior.

**MATERIALS AND METHODS**

**Materials.**—Masculinized specimens of *Gambusia a. holbrooki* were collected in Elevenmile Creek during 1978 at the following sites in Escambia Co., FL: U.S. Rt. 90-A bridge, 8 km S Cantonment (sec. 3, T1S, R31W), 24 June, 15 and 19 July and 26 Aug.; Co. Rt. 297-A bridge, 6.5 km S Cantonment (sec. 2, T1S, R31W), 15 July; 3.2 km W of Co. Rt. 297, 4 km S of U.S. Rt. 90 (sec. 33, T1S, R31W), 5 and 26 Aug.; U.S. Rt. 90 bridge, 5.5 km S of I-10 (sec. 22, T1S, R31W), 5 Aug.; Co. Rt. 86 just S of paper-mill property, 3.7 km S Cantonment (sec. 14, T1N, R31W), 19 July.

Normal specimens of *G. a. holbrooki* were collected during 1978 at the following sites in Escambia Co., FL: Headwaters of Elevenmile Cr., above paper-mill effluent, along Rt. 29 at Cantonment (sec. 11, T1N, R31W), 15 July and 26 Aug.; trib. to Elevenmile Cr., on Co. Rt. 297-A, 1.6 km N jct with Co. Rt. 97, 5 km S Cantonment (sec. 22, T1N, R31W), 19 July; trib. to Elevenmile Cr. at jct Klondike and Deerfield Rds., 1 km S of I-10 (sec. 10, T1S, R31W), 5 Aug.; Eightmile Cr., 1.6 km SW U.S. Rt. 90, 1 km W Co. Rt. 297 (sec. 26, T1S, R31W), 5 Aug.;
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Fig. 1. Normal specimens of Gambusia affinis holbrooki collected above paper-mill effluent. Note sexual dimorphism. A) male, 25 mm SL, showing gonopodial differentiation of anal fin. B) Pregnant female, 31 mm SL, showing typical unmodified anal fin and dark gravid spot on abdomen above anal fin origin.

Drainage pond trib. to Elevenmile Cr., along E bank, 3.7 km W Co. Rt. 297, 4 km S U.S. Rt. 90, 5 Aug.; land excavation pond trib. to Elevenmile Cr., on Saufley Field Rd., 0.8 km W Co. Rt. 297 (sec. 33, T1S, R31W), 5 Aug.; farm pond behind houses on E bank of Elevenmile Cr., 3.2 km E Co. Rt. 297, 5.5 km E on Saufley Pine Rd. from Rt. 297 (sec. 33, T1S, R31W), 5 Aug.

For behavioral studies, 50 masculinized Gambusia (35 ♀♂ and 15 ♂♂) were collected in Elevenmile Cr. at Co. Rt. 297-A bridge, 6.5 km S Cantonment, Escambia Co., FL. Additionally, 50 normal Gambusia (32 ♀♀ and 18 ♂♂) were collected from the headwaters of Elevenmile Cr. above the paper-mill effluent, along Rt. 29 at Cantonment. The specimens were placed in well-aerated, styrofoam containers and brought back immediately to the laboratory where they were transferred to 38-L aquaria for holding and five days acclimation prior to observations of their behavior.

Fig. 2. Anal fins of normal specimens of Gambusia affinis holbrooki from above paper-mill effluent. These are fins of specimens shown in Fig. 1. A) Gonopodium of mature male showing normal differentiation and elongation of fin rays 3, 4 and 5. Note presence of hooks and spines. B) Anal fin of mature female showing unmodified rays.

Gross determination of sex.—500 specimens were examined for development of gonopodium and gravid spots. Of these, 127 specimens from the portion of Elevenmile Cr. below the paper-mill effluent discharge, and 59 specimens from above the discharge, were dissected and their gonads were examined with a dissecting microscope.

Histological determination of sex.—Permanent microscope slides were made according to the following technique: whole fish were fixed for 24 hr in Davidson's fluid and then gradually dehydrated with ethyl alcohol. Clearing was done by gradual dealcoholization with 3:1 toluene: Terpineol solution. Infiltration was accomplished by gradual replacement of toluene-Terpineol with paraffin. Embedding was done in paraffin. The embedded tissue was then sectioned at 7–12 microns, mounted onto slides and stained with hematoxylin and eosin according to Galigher and Kozloff (1964). Longitu-
Fig. 4. Gonopodial structure in masculinized Gambusia affinis holbrooki collected below paper-mill effluent. The photomicrographs were taken from the sequence of specimens as shown in Fig. 3. A) Immature male gonopodium. B) Mature male gonopodium. C) Mature gonopodium of female. D) Slightly regressed gonopodium of female. Note reduction of spines on third anal ray and hooks at gonopodial tip. E) Regressed gonopodium of female showing no spines on third anal ray and no gonopodial hooks. F) Mature gonopodium of female. The gonopodia shown in B and C represent the typical structure of those of normal males above paper-mill effluent and in tributaries.

Fig. 5. Map of Elevenmile Creek, Escambia County, Florida. Dark circles indicate collecting sites where masculinized Gambusia affinis holbrooki were taken. Dark squares are sites where normal G. a. holbrooki were collected. The normal Gambusia were collected only above the paper-mill effluent and in tributaries to Elevenmile Cr. The two collections west of Saufley Field were made in permanent freshwater ponds adjacent to Elevenmile Cr. Masculinized Gambusia were limited to the paper-mill effluent portion of Elevenmile Cr. This distribution strongly indicates a direct relationship between the paper-mill effluent and masculinization in Gambusia.

Observations of reproductive behavior.—The following combinations were introduced into 38-1
aquaria and their reproductive behavior was observed: 1) 3 pregnant, masculinized females and 3 normal, control females; 2) 2 pregnant, masculinized females; 3) a precociously masculinized male and a normal female; 4) a normal male, a precociously masculinized male and 3 normal females; 5) a normal male and female, used as a control.

RESULTS

Paper-mill effluents are discharged into Elevenmile Cr. at Cantonment, Escambia Co., FL, 20 km NW of Pensacola. From this point, the creek flows approximately 20 km before entering Perdido Bay (Fig. 5). All 350 females, 13-46 mm SL, collected within this stream below the paper-mill effluent exhibited abnormal sexual expression by possessing varying degrees of gonopodial development (Figs. 3, 4C–F). Histological sections and gross inspection of the gonads of females showed ovarian tissue with either developing ova, mature eggs or embryos. No evidence of testicular tissue was found among the female gonads, suggesting that hermaphroditism does not exist within this population. Thus, the females are apparently only masculinized. Males, too, are abnormally masculinized in that they develop physical secondary sex characters earlier than expected. Immature males only 12–13 mm SL (Fig. 3A) displayed precocious elongation of anal rays 3, 4 and 5 (Fig. 4A). In males from 13–18 mm SL these elongated rays were differentiated into mature gonopodia (Figs. 3B, 4B). In the normal population of Gambusia above the paper-mill effluent, the gonopodium does not differentiate in males until they attain 18–22 mm SL. There is apparently some factor exerting a strong androgenic effect on the males within Elevenmile Cr. causing their precocious sexual development. Histological and gross inspection of male gonads showed only testicular tissue.

Masculinization within this population of Gambusia is limited to the paper-mill effluent portion of Elevenmile Cr. (Fig. 5). This distribution is strong evidence for some relationship between the masculinization and some component of the effluent-discharge. All Gambusia collected in Elevenmile Cr. above the paper-mill effluent, as well as in tributaries to this creek, exhibited normal sexual expression (Fig. 5). An additional 3,000 specimens collected in adjacent stream systems from Mobile, AL eastward to Panama City, FL were all normal with regard to secondary sex characters.

Since the normal reproductive behavior of Gambusia has been well documented (Krumholz, 1948; Rosen and Gordon, 1953; Rosen and Tucker, 1961; Peden, 1970), we felt it pertinent to observe the reproductive behavior of the masculinized Gambusia and compare it with that of normal Gambusia from above the paper-mill effluent. When three pregnant, masculinized females were placed into an aquarium with three normal, control females, the masculinized females exhibited only behavioral patterns of typical males, i.e., chasing the control females with gonopodial swinging and thrusting, in vain attempts at fertilization. Although the three masculinized females seemed predominantly interested in the normal females, they occasionally chased each other with gonopodial swinging and thrusting at each other’s genital region. When two masculinized females were placed into an aquarium with each other, both exhibited only typical male reproductive behavior. When a precociously masculinized male and a normal female were placed into an aquarium, the male displayed typical, but more aggressive courtship than normal, control males. When a normal male, a precociously masculinized male, and three normal females were placed together, the masculinized male displayed dominance over the normal male, chasing him into corners. The masculinized male was then free to court the normal females without competition from the normal male. When a normal male and a normal female were placed together, they exhibited only normal behavior as has been described.

Chromosome studies on both the masculinized population and normal Gambusia from above the paper-mill effluent showed a karyotype typical of G. a. holbrooki, i.e., 46 acrocentric and 2 small submetacentric chromosomes (Black and Howell, 1979). No heteromorphic sex chromosomes were distinguishable such as those found in G. a. affinis (Black and Howell, 1979).

DISCUSSION

Laboratory experiments using androgenic hormone treatments have produced precocial appearance of secondary sex characters in males and masculinization in females of Gambusia and seem to parallel the situation found in Elevenmile Cr. Turner (1941, 1942a,b) induced precocious gonopodial development in immature males of G. affinis with ethynyl tes-
testosterone. This hormone also led to gonopodial development in young female G. affinis. Similar experiments have been performed on two other live-bearing species: the guppy, Poecilia (= Lebistes) reticulata and the swordtail, Xiphophorus helleri. Régnier (1938) induced some of the gonophonus infection in the Elevenmile Cr. population of both of these species by testosterone propionate injections. Eversole (1941) found that the hormone, pregnenolinone (=ethynyl testosterone), fed to guppies from birth, caused precocious male secondary sex characters and prevented the development of female characters. These experiments with three live-bearing fishes show that the genetic factors for gonopodial development are present in the female but remain latent in the absence of an androgenic hormone.

Besides androgens, a number of other conditions or agents have been shown to induce masculinization in female swordtails: treatment with pregnant mare serum and chorionic gonadotropin (Régnier, 1938; Baldwin and Li, 1942), X-rays (Vivien, 1950), incomplete hypophysectomy (Vivien, 1952), old age (Esenberg, 1926) and parasites (Wurmbach, 1951; Forsellius, 1957). Atz (1964) suggested that these conditions or agents have an adverse effect either directly on the ovary or indirectly by reducing the ability of the pituitary gland to produce sufficient gonadotropin. Because of these adverse conditions, the ovary assumes an altered metabolism and begins to secrete androgen that is responsible for the development of male secondary sex characters. The mycete parasite, Ichthyophonus hoferi, commonly infects fishes and undoubtedly has caused masculinization in various live-bearing aquarium fishes (Atz, 1964). It was suggested that Ichthyophonus itself might produce an androgenic substance; however, Atz felt that the fungus infection might induce the female fish to produce the androgen. We found no evidence of Ichthyophonus infection in the Elevenmile Cr. population of Gambusia.

What chemical or combination of chemicals present in Elevenmile Cr. causes masculinization in Gambusia? The State of Florida, Department of Environmental Regulation, Pensacola, FL, has periodically monitored Elevenmile Cr. since 1970. Their reports show that the stream exhibits strong turbidity with a large quantity of suspended solids. Dissolved oxygen ranges from 1.6 to 8.0 ppm. There is a strong BOD. Chlorides, phosphates, nitrates, ammonia, grease, phenol and coliform bacteria were present in significant amounts. Water temperatures and pH were within normal limits as compared with nearby streams. While the highly guarded techniques of processing paper vary among competitive paper companies, Environmental Protection Agency data (U.S. Environmental Protection Agency, 1976) show that most paper-mill effluents contain dissolved organics, methanol, turpenes, acetone, fatty acids, cellulose decomposition products and non-degradable organic fractions such as lignins and tannins. Substances harmful to aquatic life are sulphides, mercaptans, resin-acids, fatty acids, soaps, turpenes, chloride and caustic soda. These latter pollutants have not been monitored by the Florida Department of Environmental Regulation in Elevenmile Creek.

The idea that paper-mill effluents might have an ontogenetic effect on fish populations was confirmed recently when McLean and Brown (1974) exposed juvenile coho salmon for 200 days to neutralized, filtered bleached kraft mill effluent. The exposed fish had a mean weight twice that of the controls. The precocious growth rate of the fish living in the effluent was considered to be due to induced behavioral changes, increased nutrient supply, effluent salinity, hormetic effects and hormonal changes. The period of exposure was too short to ascertain whether or not secondary sex changes would take place.

The most reasonable hypothesis is that some chemical or combination of chemicals in the paper-mill effluent exerts a strong androgenic effect on the Elevenmile Cr. population of Gambusia. An alternate hypothesis is that organic materials in the effluent stimulate the growth and maintenance of a parasite which infects and masculinizes Gambusia. However, no such organism was found. Still another hypothesis is that although masculinization in Gambusia is atypical, it does occur in natural populations, and that it is selectively advantageous for survival in stressful environments. This latter hypothesis does not seem likely since many populations of Gambusia have been observed in stressful environments and no evidence for masculinization has ever been presented for these populations. Future studies utilizing component chemicals from the paper-mill effluent may shed light on the factor(s) that is exerting the androgenic effect. Discovery of this unique population of Gambusia should provide endocrinologists, biochemists, geneticists,
behaviorists, environmental biologists, evolutionary biologists and others, with a natural laboratory within which to study many phenomena associated with the determination and expression of sex in vertebrates.

On 25 July 1980 additional evidence for environmentally induced masculinization of G. affinis was recorded. Of a total of 50 fish there were: 25 females with gonopodia; 6 females with apparently normal anal fins; 15 males; and 4 immature individuals. These fish were collected from the Fenholloway River, Taylor County, Florida, 5 km downstream from the location of a paper mill.

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Literature Cited


