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Masculinization of Female Mosquitofish By Exposure to Plant Sterols and *Mycobacterium smegmatis*

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A study by Howell et al. (1980) revealed a population of the sexually dimorphic poeciliid mosquitofish, Gambusia affinis holbrooki, in a stream receiving papermill effluents. In this population, the females were strongly masculinized showing both physical secondary sex characteristics and reproductive behavior of males. The most conspicuous feature of the females was the presence of a fully developed gonopodium, an intromittent organ, charactistic of male livebearer fishes. It was concluded that some chemical or combination of chemicals associated with papermill effluent was exerting a strong androgenic effect upon this unique population of fish. From this study, plant sterols have come to be suspected as the chief source of androstane steroids which can alter sex characteristics (Rosa-Molinar and Williams 1984). It is well documented that plant sterols are widespread within the plant kingdom, especially among pine trees used in the pulping industry, and that sitosterols are the most abundant of these sterols (Rydholm 1965, Pollak and Kritchevsky 1981). Furthermore, it is known that these plant sterols may be broken down by microorganisms to produce androgenic steroids (Marsheck et al. 1972, Charney et al. 1967). This current report communicates that certain plant sterol degradation products are sufficient to masculinize Gambusia affinis under laboratory conditions. The mechanism whereby this occurs may possibly explain the presence of certain populations of masculinized Gambusia in nature.

MATERIALS AND METHODS

Gambusia affinis affinis are sexually dimorphic species of fish widely distributed in nature. Males have anal fins well structured into elongate gonopodia while females are larger in size and lack gonopodia (Fig. 1, A-D). Specimens of Gambusia were collected locally and the females were kept in holding tanks until time of experimental use.

The plant sterols used in this study were predominantly β-sitosterol (24α-ethyl-5-cholesten-3β-ol), and stigmastanol (24α-ethyl-5α-cholestan-3β-ol). Because large quantities of pure sterols were unattainable, anhydrous commercial preparations from soybean extracts were used which contained a predominance of one or the other sterol types. One preparation contained 60% β-sitosterol and 35% campesterol (SIGMA), and a second contained 65% stigmastanol and 30% β-sitosterol (SIGMA).

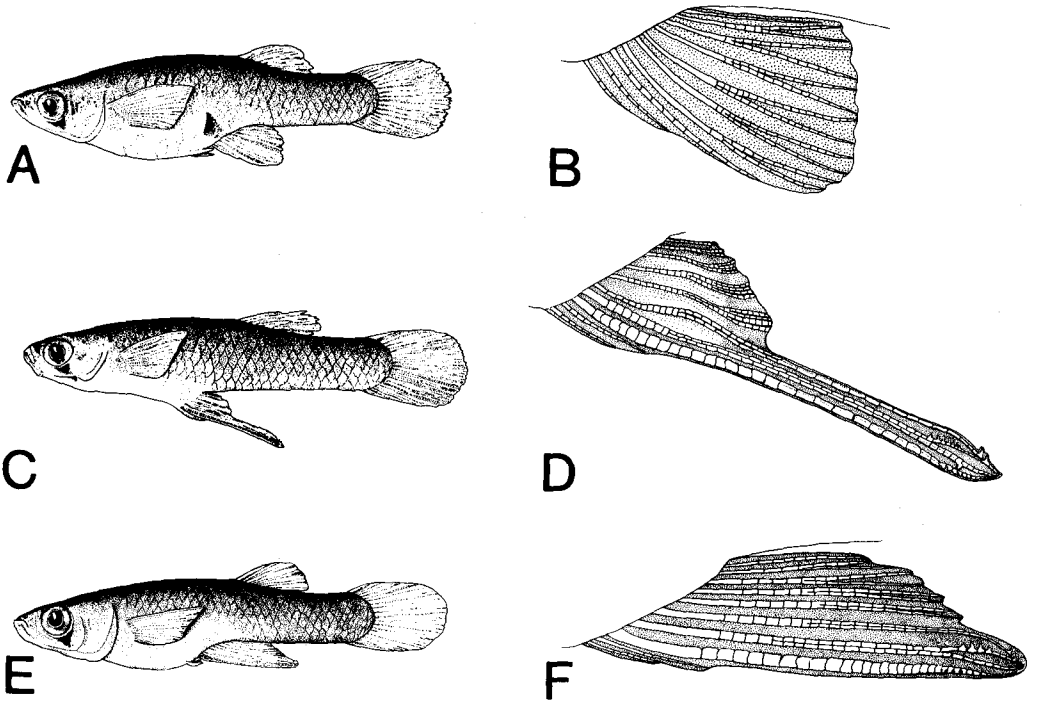

FIGURE 1.

A. Normal, control female Gambusia a. affinis, 35.5 mm in standard length (SL)
B. Anal fin of normal female above, showing unmodified anal fin
C. Normal, control male Gambusia a. affinis, 22.2 mm SL
D. Anal fin of normal male above, showing rays which have been modified into an intromittent device, the gonopodium. Rays 3, 4, and 5 are elongated and terminate in a hooked, holdfast copulatory structure.
E. Sterol-treated female Gambusia a. affinis, 23.2 mm SL, showing a slightly elongated anal fin
F. Anal fin of sterol-treated female above, showing elongation and modification of rays 3, 4, and 5 into a male-like gonopodium.

Mycobacterium smegmatis in nutrient broth was used as the organism to degrade the plant sterols used in this experiment. The method for exposing Gambusia to phytosterols and Mycobacterium was of special design. A total of 60 mature females 24mm to 36mm standard length was placed into 2 liter beakers with each beaker housing 3 specimens. A cellophane bag, approximately 20 ml in volume, was added to each beaker. Each bag contained 1 gram of either 60% β-sitosterol or 65% stigmastanol; 0.5 ml of a thriving culture of Mycobacterium smegmatis, and 10 ml of distilled water. Before immersing the bags in beakers containing Gambusia, they were perforated several times with a fine point dissecting needle to allow possible degradation products to diffuse out of the bag into the liter of dechlorinated water surrounding the specimens. The insoluble fraction of phytosterol mostly remained inside the bag so that the greater portion of it did not come into contact with the Gambusia.

Control systems were established similarly to those described in the system above, but without added plant sterol and Mycobacterium. Neither the experimental nor the control systems were aerated, and the temperature was kept between 28°C and 32°C. All systems were monitored daily until microscopic examination showed that a definite change had occurred in the anal fin rays of the Gambusia.

RESULTS AND DISCUSSION

All Gambusia exposed to plant sterols and Mycobacterium developed male-like gonopodia (Fig. 1, E-F). The thirty specimens exposed to degraded stigmastanol developed gonopodial characteristics within six days whereas those exposed to degraded β-sitosterol showed anal fin ray changes a few days later. More specifically, periodic examinations showed that the third ray began to thicken with elongated growth of the third, fourth, and fifth rays after six or more days. As growth became less marked, differentiation of the various specialized segments of a bony gonopod appeared. This was highlighted by the presence of proximal teeth, distal teeth, and a blade after two or more weeks (Fig. 1F). These characteristics did not regress when the transformed fish were removed to their natural environment which was free of plant sterol products. Examinations of autopsied female gonads revealed no sign of testicular tissue, suggesting that sterol exposure did not cause any noticeable degree of hermaphroditism. None of the female Gambusia exposed to plant sterol alone, or to Mycobacterium alone, showed a change in anal fin ray structure. It is our considered opinion that both β-sitosterol and stigmastanol are capable of being degraded by Mycobacterium into substances capable of modifying anal fin rays of Gambusia. Both of these substances are present in different amounts in the two soybean extracts used in this study. Campesterol is not a component in one sterol extract but comprises 35% of the other extract. It is probable that all three compounds are degraded into androstane-like compounds. Because stigmastanol was the most abundant substance in the second extract used, and since it was this extract that produced anal fin

ray changes before the β-sitosterol-campesterol extract, it is concluded that stigmastanol produces more substances of an androstane nature than either β-sitosterol or campesterol. However, it is difficult to be definite about an activity series without first purifying the components in question. This currently is being done.

An important point in this study is whether or not the C_{29} phytosterols used in these experiments can be degraded into androgenic C_{19} steroids. Marsheck et al. (1972) have demonstrated that certain Mycobacterium species can degrade sitosterols and other soya sterol residues into 30% yields of 1,4-androstadiene-3,17-dione and 13% yields of 4-androstene-3,17-dione in approximately five days. They further report that the 4-androstene-3,17-dione nucleus is further degraded to produce a 2.5% yield of testosterone. At one time, the bioconversion of soybean sterols to β-sitosterol, campesterol, and stigmasterol was considered an alternative source of intermediates for steroid manufacture (Rydholm 1965).

A second point of relevance is the capacity of C_{19} steroids to modify the anal fins of female Gambusia and other live-bearing fishes to produce male-like gonopodia. Turner (1941) demonstrated that methyltestosterone in amounts as low as 0.05 µg per liter of water can accomplish this transformation. From visual inspection, it was degraded slowly over periods of weeks. Thus, it is reasonable to assume that enough androgenic material may be produced in continuous fashion to induce morphological change in anal fins. Eversole (1941) found that the masculinizing effect of pregneninolone and testosterone on the anal fin of Lebistes reticulatus is the most sensitive and quickest androgenic response observed for that species. Hamon (1945), found similar occurrences in Gambusia holbrooki and so did Regnier (1938) for Xiphophorus helleri and Grobstein (1940) for Platypoecilus maculatus when these organisms were exposed to testosterone propionate.

A final consideration is whether or not the laboratory findings of masculinized Gambusia are consonant with masculinized forms occurring in nature. Howell et al. (1980) found masculinized Gambusia affinis holbrooki as far downstream as 4 miles from the location where pulp chemicals were being discharged. Masculinized forms were not found above the point where papermill effluents were entering the stream. These observations were confirmed by Rosa-Molinar and Williams (1984). Both of these last two reports contend that some chemical, perhaps β-sitosterol from pines in the presence of aerobic/anaerobic sedimentary bacteria, could produce androgenic effects on Gambusia. It is reasonable to assume that if this type of mechanism can be demonstrated under laboratory conditions, then it is also likely to occur in nature where micro-organisms, Gambusia, and large quantities of plant sterols can come together under optimal conditions. It is well known that the quantity of plant sterols are greatly elevated in papermill effluents. The majority of the sitosterols are found in the resin fraction of conifers. These unsaponifiable neutral substances of wood extractives contain fatty alcohols and phytosterols, mainly

β-sitosterol and stigmastanol, in the approximate proportions 30:63:7 (Rydholm 1965). The removal of sitosterols during the pulping process presents great difficulty as they are only slightly hydrophilic at all pH levels. Papermill effluents released into settling ponds can possibly contain enough sitosterols and micro-organisms to produce androgens which are released into streams where Gambusia and other vertebrates are normally found. With the elevated temperatures that are prevalent at certain times of the year around southern-most papermills, it is feasible that androgens may be generated and released into the animal habitat, sometimes causing sexual modifications. Work is continuing in efforts to purify the respective phytosterols used in this study and to identfy the component(s) that presumedly is causing masculinization. Field tests will be greatly facilitated once these data are understood.

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