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Laboratory Induction of Intersexuality in the Mosquitofish, *Gambusia affinis*, Using Paper Mill Effluent

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Intersexual traits have been described in a wild population of mosquitofish in a northwest Florida (USA) stream which receives effluents from a paper/kraft mill (Howell et al. 1980). Adult female Gambusia affinis taken as far as 15 km downstream of the discharge from the kraft mill effluent (KME) were phenotypically masculinized (arrhenoid), exhibiting a range from imperfect to well-developed examples of gonopodial anal-fins and exhibiting elements of male reproductive behavior. Male G. affinis from the same area precociously developed secondary male sex characters. Subsequently, surveys of another Florida stream receiving paper mill effluent in a separate drainage 400 km distant, revealed the same condition in the least killifish, Heterandria formosa, the sailfin molly, Poecilia latipinna, as well as mosquitofish (Howell et al. 1980, Bortone and Drysdale 1981).

Previous studies demonstrated that development of male secondary sex characters in G. affinis and other poeciliids may be induced in both sexes by treatment with various androgens (e.g., Grobstein 1940; Turner 1941a, 1941b, 1942, 1960). These and other studies illustrate how poeciliids phenotypically respond to exogenous androgens. Genetic information for development of the gonopodium and other structures are perhaps latent in female poeciliid fishes in the absence of androgens.

To test the hypothesis that masculinization of natural Gambusia affinis populations was induced by a KME-borne androgen or androgen-like substance, newly hatched laboratory-reared offspring were experimentally exposed to KME-receiving streamwater. Parental G. affinis stocks were collected from streams not known to receive KME. Development of secondary sex characters was recorded and compared between KME-exposed fish and non-KME exposed fish. Statistically significant differences in size or rate of development of secondary sex characters between the treatment groups was considered justification for rejection of the null

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hypothesis, that KME factors exerted no influence upon induction of masculinization of G. affinis.

MATERIALS AND METHODS

Adult male and female G. affinis stocks were collected from streams receiving no KME in Escambia and Santa Rosa Counties in northwest Florida (USA). Adult fish were maintained in aquaria residing in an enclosed temperature-regulated (25 ±2°C) chamber with fluorescent lighting (12 hr light/12 hr dark). Fish were fed twice daily with Tetramin dry flake food. During the 10-wk acclimation period, normal reproductive behavior was observed and several females gave birth to apparently healthy offspring.

Gravid females were tansferred to separate 3-L glass containers, which served as breeding aquaria. In these breeding aquaria, water was gently aerated and polyamide netting seperated newborn offspring to protect them from cannibalism by adults. Young (1-3 day old) fish were removed and placed in aquaria containing streamwater that received KME (4-L glass containers with 3-L of aerated water collected from Elevenmile Creek [Escambia County, Florida, USA] at a site 3.6 km downstream from the point of KME discharge) or non-KME receiving streamwater (in similar sized aquaria as the exposure aquaria above) and daily fed identical portions of live brine shrimp (Artemia) nauplii.

Fish were measured for standard length (SL) and anal fin length (AFL: distance from the terminus of the distal-most ray segment to the point of attachment of the proximal-most segment and its corresponding interhemal spine) using a dissecting microscope with substage illumination and ocular micrometer. To facilitate their handling while being measured, fish were anesthetized in a 0.10 g/L solution of tricaine methanesulphonate (MS-222, Sandoz Pharmaceuticals, Hanover, New Jersey, USA) in aquarium water. In treatment and control aquaria, water was completely replaced every 2-3 days with their respective KME-receiving or non-ME receiving streamwater.

Variation in the dependent variables, SL and AFL, were analyzed for their relationship to the independent variables: sex, KME-exposure or non-KME exposure, and age in days. Data were subjected to multiple linear regression and analysis of variance statistical analyses.

RESULTS AND DISCUSSION

Mean standard length (SL) for the four treatment groups were similar during the first few weeks of life. There were no significant differences in mean SL at 8 d of age ($p > 0.05$) among the treatment groups but a progressive divergence occurred with age. Males (regardless of their expsure to KME or not) increased in SL from 9.5 mm at 8 d of age to 17 mm at 100 d of age. The mean SL then remained between 16.8 and 17.7 mm. Mean SL among females increased from 9.5 mm at 8 d of age to 17 mm at 116 d of age and

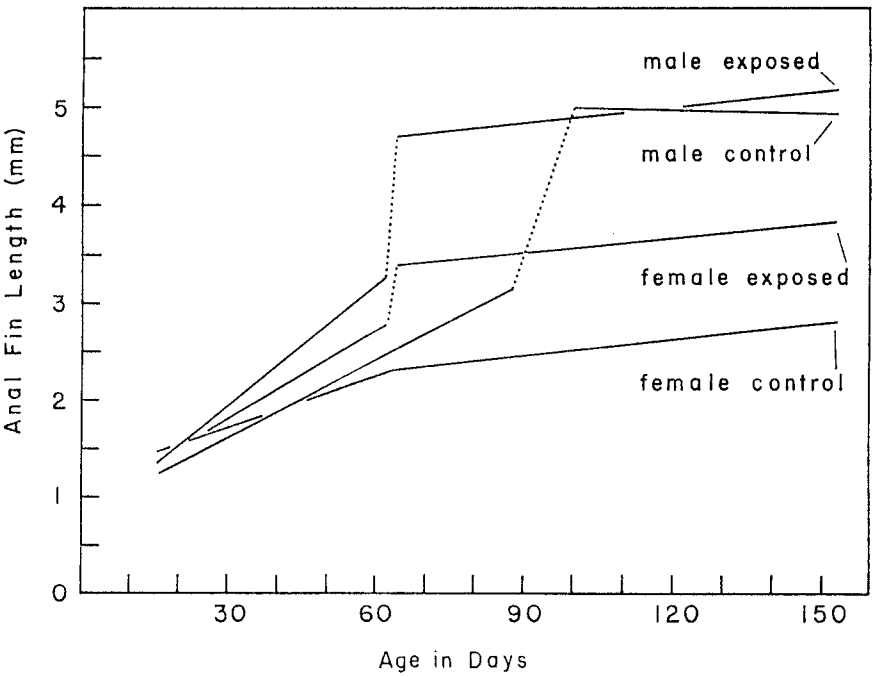

Fig. 1. Relationship between age in days and anal fin length of mosquitofish under the four treatment categories. Interrupted lines connect the slopes between the linear growth phase and the plateau phase.

remained at this size until the experiment was terminated when fish attained 153 d of age. KME-exposed fish were consistently longer than non-KME exposed fish for both sexes after 39 d of age ($p < 0.00001$).

After 100-116 d of age, mean SL and the age-SL slope relationship differed markedly among the four treatment groups. Differences among slopes of the age-SL relationship were compared using Students's t-test. During the linear phase of growth (prior to 116 d of age), slopes of both groups of males and the female KME-exposed group varied from 0.069 to 0.075 and the probability of equality between slopes was high ($p > 0.20$). The age-SL slope for control females was 0.064, and this was only significantly different from the age-SL slope of the male, KME-exposed group ($p < 0.001$). After 100-116 d of age, the age-SL slopes varied from 0.0005 among male controls to 0.021 among female controls. All p-values for tests of equality among slopes for the age-SL relationship after 100-116 d of age were greater than 0.20.

The mean anal fin length (AFL) of male, non-KME exposed fish increased from 1.29 mm at 8 d of age to 3.34 mm at 88 d of age (Fig. 1). Between 88 and 100 d of age the mean AFL increased to 5.22. Following this increase, the mean AFL among male controls

remained between 4.50 and 5.25 mm and did not fluctuate significantly ($p > 0.05$).

The AFL of KME-exposed males increased from 1.3 mm at 8 d of age to 3.17 mm at 63 d of age. Subsequent to 65 to 67 d of age the mean AFL in KME-exposed males increased dramatically to 4.74 mm. After 88-100 d of age the AFL had increased to 5.00 mm among the KME-exposed males and remained at approximately 5 mm for the duration of the experiment. The mean AFL among control and KME-exposed females increased from 1.31 mm at 8 d of age to 2.11 mm and 2.79 mm at 63 d of age for the control and exposed females, respectively. After this age, the mean AFL among KME-exposed females increased rapidly, attaining a mean length of 3.32 mm at 70 d of age. At the conclusion of the experiment, the mean AFL of control females had increased to only 2.81 mm.

Because of the dramatic and obvious differences in growth by age, anal-fin growth rates were analyzed as a ratio to SL separately for growth prior to and after 63 d of age. Differences in anal-fin growth rates between the four treatment groups during the early-growth phase were all highly significant ($p < 0.00005$), except between the male control and female KME-exposed groups ($p > 0.20$). During the later-growth phase, none of the differences in anal-fin growth rates between the four treatment groups was sufficiently large to be considered significant ($p > 0.05$).

Both sexes of G. affinis reared in KME contaminated streamwater from Eleven Mile Creek exhibited significant morphological differences compared with the respective sexes of control fish reared in uncontaminated water. The results of this study generally agree with the reported effects of exogenous hormone administration to poeciliids (e.g., Turner 1960).

Somatic growth patterns (as indicated by SL) were similar among fish in the four treatment groups. Prior to 100-116 d of age fish length increased in an approximately linear fashion and remained essentially unchanged thereafter. Although differences in body length between control and exposed groups were not large at any given age, the differences became statistically significant at least by the end of the study. Why KME-exposed fish were longer than their respective male or female controls is not clear. The possibility of increased food ingestion coupled with increased aggressive behavior (i.e., more active foraging as was found by McLeay and Brown [1974] in coho salmon) is improbable in the present study since all fish were provided equal amounts of food as well as being isolated to avoid interaction with conspecifics.

Growth rates for the four treatment groups were similar except that growth rates among the control females were significantly less than the growth rate among KME-exposed males during the linear phase of growth (<116 d of age). These rate differences are consistent with the KME-androgen hypothesis: with a source of androgen or androgen-like compounds, females would exhibit the growth characteristics of male fish.

With regard to the KME-androgen hypothesis as an explanation of apparent differences in body growth in G. affinis, the literature is somewhat contradictory. Moshen (1959) concluded that the effects of exogenous androgen administration (water-borne methyltestosterone, 0.03 mg/fish) inhibited somatic growth in poeciliids. A similar effect was obtained by Clemmens et al. (1966) by feeding methyltestosterone (20-30 mg/kg diet) to guppies, Poecilia reticulata. In contrast, lower doses of 17α-methyl-testosterone (1-10 mg/kg diet) to carp, Cyprinus carpio, significantly increased somatic growth over controls (Lone and Matty 1980). Coho salmon fed 17α-methyl-testosterone (1 mg/kg diet) grew faster and weighed more than controls (Fagerlund et al. 1979). It seems likely that for poeciliids, doses of steroids such as testosterone in excess of the amount naturally required to induce masculinization will lead to growth retardation.

Differences between KME-exposed and non-KME exposed fish with regard to their anal fin length were evident. Normal elongation of the anal fin among male G. affinis occurs in three phases: an early phase (< 88 d old); a rapid growth phase (88-100 d old); and a late phase (> 100 d old). During the first 8-63 d of life, anal fin length in the four treatment groups increased with age in an approximately-linear fashion although slight differences existed between the groups. Anal fin growth rates differed in ways which are explainable in terms of the KME-androgen hypothesis. Assuming that endogenous androgens provide a single "androgen dose", and the KME substance provides an additional (but exogenous) "androgen dose", one would expect the highest anal fin growth rates to occur among KME-exposed males (with two doses), lower but similar rates among female KME-exposed fish and male control fish (each with one dose), and the lowest rate among female controls (with no dose). The relatively early growth rates calculated from data in the present study agree well with those predicted above and could indicate that the presumed androgenic component of KME was exerting its effect on gonopodial (i.e., sexual) development in conjunction with naturally synthesized, endogenous testosterone. Early anal fin growth rates of male control and female exposed groups were nearly identical.

The biological and ecological mechanisms operating to produce the observed intersexual condition in G. affinis exposed to KME both in the field and the laboratory are yet to determined. The KME-androgen hypothesis introduced by Howell et al. (1980) cannot be rejected by the present study. Presumably, the compound (or compounds) in KME could mimic either: (1) a pituitary hormone-releasing hormone (most likely LH-RH); (2) a gonadotropic hormone (GtH); (3) a biochemical precursor of the active androgen; or (4) the androgen in its active form (e.g., testosterone). After uptake by the fish, the suspect compound could engage the biochemical pathways involved in the synthesis of sex steroids and ultimately produce the androgenic and anabolic effect. Given the variety and complexity of physiological processes involved in normal gonopodial differentiation in G. affinis (Turner 1960), it

is unlikely that the suspect compound acts at a level of biochemical control more specific than the sex steroids. In-vivo biotransformation of an effluent-borne testosterone precursor to a physiologically-active androgen could also account for the observed precocious male and arrhenoid female poeciliids. Alternatively, a testosterone sterol precursor in KME might become transformed to the active androgen by microorganisms existing in the effluent, or stream sediment (Denton et al. 1985).

Numerous microbiological transformations of steroid compounds are recognized, including those with adrenocortical and gonadal hormones as end-products (Prescott and Dunn 1959). Rosa-Molinar and Williams (1984) suggested that β-sitosterol was being transformed by anaerobic microorganisms to compounds including androstenedione. β-Sitosterol is a component of the by-product of the kraft paper process (Merck Index 1976). Recently, Denton et al. (1985) and Howell and Denton (1988) were able to induce masculinization in female G. affinis by exposing them to the degradation products resulting from microbacterial action on the plant sterols: β-sitosterol and stigmastanol.

The present research has provided evidence that the agent responsible for the intersex condition of poeciliid fishes is an androgen, androgenic-like compound, or androgen precursor borne by the water and perhaps later modified by microbial action in the environment. A rather obvious androgen bioassay procedure is suggested by this study, and by previous work of a similar nature (Bortone et al. 1989). Relatively inexpensive long-term monitoring of aquatic systems for androgens or steroids could be established with captive breeding colonies of poeciliids. The need for such a bioassay at this time is obscure. However, given the increasing knowledge of the ecological effects of various pollutants and the increasing costs associated with sampling and laboratory analysis, a qualitative bioassay such as this might be of some practical use in the future.

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