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Fish Intersexuality as Indicator of Environmental Stress

Monitoring fish reproductive systems can serve to alert humans to potential harm

Stephen A. Bortone and William P. Davis

Mike Howell and Ann Black examined each diminutive fish in the minnow seine haul they had made in the tea-colored waters of a small coastal stream, Elevenmile Creek, in the westernmost, panhandle area of Florida in 1978. All the mosquitofish (*Gambusia affinis*) appeared to be males. At first, the researchers, then both at Samford University in Birmingham, Alabama, were not too surprised because they were aware that among mosquitofish populations there occurs some microhabitat segregation by sex (Martin 1975). Moreover, they knew differential mortality could also lead to unequal sex distributions (Snelson 1989). On closer inspection, however, the scientists noted an astonishing oddity.

Many of the apparently male fish (as distinguished by the presence of a gonopodium, an elongation of the anterior anal-fin rays that serves as a copulatory organ to inseminate females) bore what appeared to be a gravid spot, which in these live-bearing fish normally indicates females with internally developing young (Figure 1). Later, a laboratory analysis confirmed that many of the fish thought to be male were actually masculinized females (Howell et al. 1980). These masculinized female

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Intersexuality presents an opportunity for biologists to design testing procedures to evaluate detection of endocrine-disrupting agents

mosquitofish showed, in addition to a gonopodium, more subtle male secondary sex characters, including behavioral characteristics such as pursuit and gonopodial swinging.

Speculation on the potential causes of this masculinization ranged from genetic phenomena to environmental induction. The environmental hypothesis gained strength a few months later when a second population of masculinized fish was discovered in the Fenholloway River (Bortone and Drysdale 1981), another coastal Florida stream, approximately 300 kilometers east of Elevenmile Creek. The second group included masculinized females from two other live-bearing species in the family Poeciliidae (the least killifish, *Heterandria formosa* [Figure 2], and the sailfin molly, *Poecilia latipinna*) as well as masculinized female mosquitofish.

In both streams, the masculinized females occurred downstream of paper mills discharging kraft-mill effluent (KME), but they were not upstream of the paper mill discharge nor in any tributaries to the effluent-receiving streams. We observed in least killifish a higher degree of masculinization among females captured closest to a KME discharge point, and we saw proportionately reduced masculinization in samples collected downstream from the point of KME discharge (Figure 3). The phenomenon was not observed in nonpoecilid fishes inhabiting the streams, however.

Fish have long been known to display a wide range of variation in the expression of their sexuality (Atz 1964). Moreover, investigators specializing in the reproductive biology of fishes have noted a morphological plasticity of their expressed sex in response to changes in environmental factors (Reinboth 1980). These factors include temperature, pH, light intensity, and social conditions, among others. Careful examination of sexuality in fishes has the potential to allow us to measure the nature and extent of some types of environmental stress (Davis and Bortone 1992).

In this article, we examine how human modification of the environment through waste discharge has altered the sexual condition of fishes. We also examine the degree and nature of fish intersexuality relative to environmental stress. In addition, we show how these sexual alterations can serve as sentinels to alert resource managers to potential problems. Last, we indicate how this phenomenon can be used to direct future research.

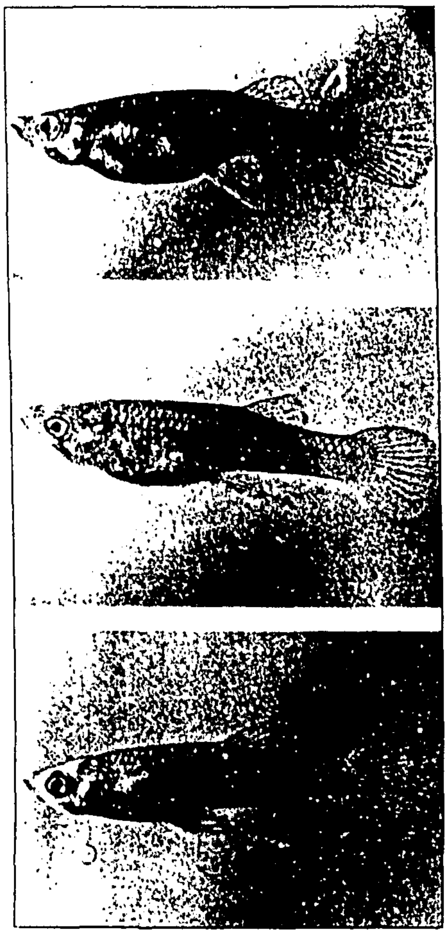

Figure 1. Normal female (top) and male mosquitofish (bottom). A masculinized female is pictured in the center, showing the gonopodial elongation of the anal fin.

A few words about sex

Fish species are extremely broad in the expression of their reproductive modes. These modes can vary between phylogenetic lineages and among members of the same family. The term *intersexuality* encompasses a broad range of sexual expression in which both male and female characters, either primary or secondary, are found in a single individual at some time during its life. *Hermaphroditism* describes a subcategory of intersexuality where the primary reproductive characters (testes and ovaries) are found in a single individual. The male and female characters can occur at the same time (as in the small sea basses; e.g., Fischer and Petersen 1987) or at different ages (i.e., *sex reversal*). Individuals can initially be male (described as *protandry*, as in the anemone fishes; e.g., Fricke and Fricke 1977) or female (termed *protogyny*, as in the groupers; e.g., Moe 1969). Masculinization, called *arrhenoidy*, is a subcategory of intersexuality where the presence of male secondary sex characters is observable in females. It should not, however, be conceptually confused with hermaphroditism, sex reversal, or "adaptive ambisexuality" (Reinboth 1988), where sex change is part of a species' normal reproductive mode.

Induced alterations of sexuality in fishes have been well documented (see review by Reinboth 1980). For example, salmon are known to attain sexual maturity earlier in their development when treated with gonadotropins (Funk and Donaldson 1972). In laboratory experiments pregnant-mare serum, chorionic gonadotropin, X-rays, and incomplete hypophysectomy have all been used to produce arrhenoidy in female swordtails. Additionally, more naturally occurring phenomena such as old age or parasitism have also been associated with masculinization among fishes, including guppies (Atz 1964).

The complex nature of paper mill effluent

The preparation of wood pulp for paper and cellulose manufacture separates cellulose fibers and lignum from the sugars, saps, and other components (including animal inhabitants) of tree stems. Different paper mills use variations of the pulping process depending on the materials being processed (e.g., pine or hardwoods) and the end product (e.g., bulk cellulose, brown kraft paper, and white paper).

Basic to all the pulping processes is separation and discharge of sugars, lipids, resins, and fatty acids that are the digestion by-products of kraft-mill operations. Typically, these waste products are entrained in heated waters that are directed to settling and aeration ponds. There they receive bacteriological treatment analogous to sewage treatment processes. Plants, especially pine trees, are potentially rich sources of phytosterols (Conner et al. 1976). However, owing to the structure of the complex resins and sugars, there is an inherent resistance to a rapid breakdown into humic and fulvic acids. The treatment processes also appear not to destroy phytosterols, some of which masculinize poeciliid fishes in the laboratory.

The complexity of paper-mill effluent presents a considerable challenge to specific identification of androgenic factors. On one hand, there is the presence of various phytosterols, which have been shown to induce masculinization under controlled conditions (Denton et al. 1985). Phytosterol action may be influenced by a variety of environmental conditions, including the seasons, tree species being pulped, degree of effluent treatment, and effluent dilution. We have chosen to assess phytosterol activity.

On the other hand, the chemical processes within a mill (e.g., chlorine, chlorine dioxide, or oxygen bleaching) may add to the complexity of the effluent and thus may influence the production of dioxin, which is known to be an endocrine disrupter. However, the literature has attributed feminization, rather than masculinization, to dioxin (Peterson et al. 1992). For this reason, and because dioxin exposure presents serious hazards, we have chosen not to assess dioxin effects.

Quantification of pulp-mill effluent is typically done through comparative effects of whole and diluted effluent samples. Because the masculinization response in test fishes commences in microbially degraded phytosterols after 15–20 days of exposure, experiments using effluent samples are cumbersome and highly variable. To carefully assess the androgenic effects of paper-mill effluents, it is preferable to have a direct connection to the effluent with continuous, flow-through exposure. Selectively filtered samples would allow fractionation analyses so that identification of components can be determined by their relative activity. No research support that we know of, to date, has been made available to conduct such experiments.

Intersexuality and paper-mill effluent

Fishes and other organisms occurring in aquatic habitats receiving

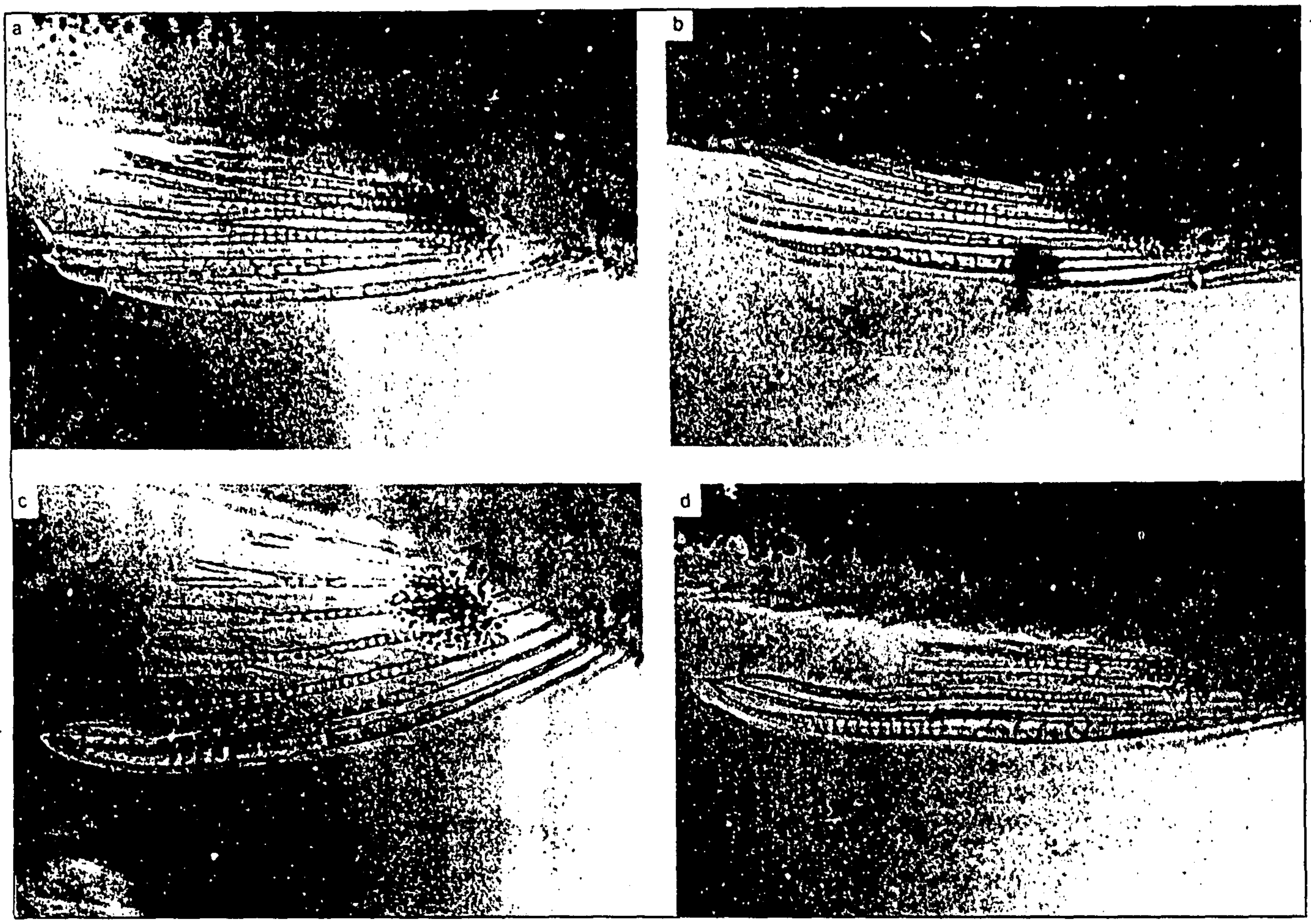

Figure 2. An array of anal fins from the least killifish. a. Normal female. b. Fish exposed to kraft-mill effluent; note the increased segmentation at the tips of the rays. c. Another example of a fish exposed to kraft-mill effluent with some elongation of the fin rays. d. Normal male gonopodium resembles this highly masculinized female fin.

pulp and paper-mill effluents exhibit a variety of biochemical, physiological, metabolic, and behavioral responses (McLeay et al. 1987, Owens 1991). Recent reports indicate that paper-mill effluents in natural waters can produce modifications in the life-history features of fishes including adverse schooling behavior in whitefish (Myllyvirta and Vuorinen 1989), changes in maturity and serum steroid levels in white suckers and lake whitefish (Munkittrick et al. 1992), and increased growth, biochemical changes, and higher levels of stress in juvenile coho salmon (McLeay 1979). Juvenile American eels (*Anguilla rostrata*) from Elevenmile Creek exhibited precocious male secondary sex characters (enlarged eyes and precocious testicular development; Caruso et al. 1988).

KME-induced male secondary sex characters in female mosquitofish occur under both field and laboratory conditions (Bortone et al. 1989, Davis 1989, Drysdale 1984, Drysdale and Bortone 1989, Rosa-Molinar and Williams 1984). We exposed female mosquitofish to stream water that had received naturally degraded KME for three weeks both in the field and in the laboratory. Several morphological characters were affected including anal-fin length, pre-anal length, dorsal-fin height, pelvic-fin height, interorbital width, eye diameter, and body depth. Each character changed to a more masculine state. These modified characters were statistically significant indicators of KME exposure (Bortone et al. 1989). After three weeks of exposure, the morphological characters of KME-treated adult females were intermediate to the normal male and female conditions.

Masculinization in female mosquitofish and other poeciliids is most readily observed in the increased segmentation and elongation of the third, fourth, and fifth anal rays (Howell and Denton 1989, Howell et al. 1980). These changes can be observed in fish exposed to such steroids as methyltestosterone (Turner 1960), androstenedione, androstanol, and spironolactone (Hunsinger and Howell 1991).

A hypothesis has been offered that bacterial processes associated with KME may be responsible for the arrhenoid condition among natural populations of mosquitofish. Denton et al. (1985) and Howell and Denton (1989) exposed mosquitofish to phytosterols (i.e., sitosterol and stigmastanol) mixed with a bacterium, *Mycobacterium smegmatis*. The masculinization response they observed (principally the elongation of the anal fin) was nearly identical to that we observed in a similar laboratory experiment exposing the least killifish to KME that had also been microbially degraded.

Phytosterols in tall (pine) oil can be microbially converted to C-19 sterols (Conner et al. 1976). These sterols include steroid compounds known to influence sex and reproduction. During 1974, for example, the more than 800,000 tons of tall oil produced in the United States could have yielded more than 20,000 tons of phytosterols (Conner et al. 1976). The amount discharged to the environment cannot be calculated, but even a small fraction could significantly harm fishes and other aquatic organisms.

Our studies indicate that reproductive systems in fishes are useful bioindicators for detection of substances that interfere with endocrine modulated processes. More specifically, live-bearing poeciliid fishes such as mosquitofish and least killifish may be useful to detect the presence of endocrine disrupters.

Poeciliid masculinization by KME may be due to a few, many, or specific combinations of endocrine-disrupter compounds that mimic or trigger an organism's receptors to its own steroidal androgens. It is likely that the masculinization response varies with exposure time, concentration, degree of microbial activity, fish species, and water conditions (e.g., temperature, pH, and conductivity).

Bioindicators for the duration and intensity of KME exposure

The field and laboratory studies conducted thus far represent only short-term observations of the masculinization response to KME; the results may not be indicative of what one might observe under long-term, continuous exposure. Rosa-Molinar and Williams (1984) noted the potential for diminished fecundity among arrhenoid mosquitofish that had been captured from streams receiving KME. However, most previous studies have been concerned with the morphological features of KME-exposed individuals and have not considered life-history traits that could ultimately affect an individual's fitness.

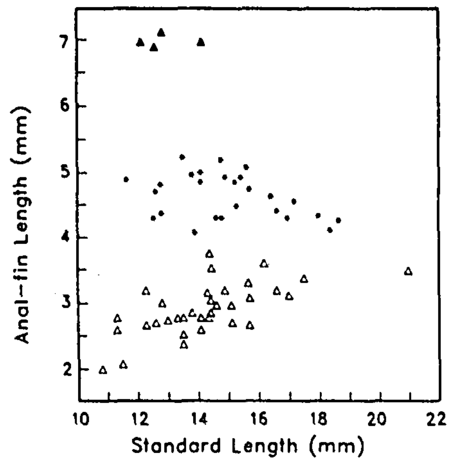

Figure 3. Scatter diagram of anal-fin length versus standard length from the least killifish, indicating the degree of anal-fin modification relative to the point of kraft-mill effluent discharge: fish collected immediately below the discharge point of kraft-mill effluent (dark triangles), fish from the farthest distance downstream (open triangles), and fish from mid-distance along the stream (asterisks).

Fish life-history traits, including the reproductive characteristics of poeciliid fishes, can vary relative to environmental conditions (e.g., Trexler 1988). Therefore, continuous exposure to KME might affect not only the sexual characteristics of female fishes but also their offspring's fitness and development. Long-term exposure may also have an impact on other population parameters such as mortality, growth, reproductive rates, and abundance. We strongly suspect that females masculinized by long-term (perhaps eight months to a year) exposure to water receiving KME suffer from impaired reproductive function.

Studies that have investigated the response of fish to continuous KME exposure have not reported any overt androgenic effects in other species. However, Munkittrick et al. (1992) noted testicular atrophy in males and abnormal oocytes in the ovarian tissue of females among KME-exposed whitefish. We believe the long-term effects of KME exposure in fishes will be found to include reduced embryo viability, developmental modifications, and neutering of female reproductive function. The effects may include responses to other potential KME components (e.g., dioxins, furans, and chlorinated lignins).

Hermaphroditism among KME-masculinized females appears to be rare (Bortone and Drysdale 1981). However, we may be observing intersexuality that could eventually lead toward facultative hermaphroditism. If fishes exposed to KME have reduced fitness and shortened life spans, they may not live long enough to achieve full hermaphroditism. Notably, the observation that highly masculinized females (as witnessed by the extreme elongation of the anal fin rays) are rare in our field collections might indicate higher mortality levels among modified fish.

At present, we have no way of measuring the exact amount of KME or its components present in a stream at any given site or time. We have noted, however, that the degree of masculinization varies with different conditions. Interestingly, large-size mosquitofish and least killifish were generally absent where the most masculined female fish were found—from streams having the highest relative amount of KME and from sites proximate to the KME discharge point. However, in streams with relatively low concentrations of KME, where poeciliid fish displayed less masculinization, the fish were larger.

During prolonged drought (three months or more), both mosquitofish and least killifish were smaller and less abundant in streams receiving KME than in other streams. The females exposed to KME during drought displayed more prominent anal-fin elongation compared with females exposed under other conditions. Drought conditions may concentrate the masculinization factor present in KME, or the process generating the masculinizing factor may be more effective under these conditions.

The degree of masculinization among female fish (when compared with normal male behavioral traits) decreased when fish were captured from the field and placed in aquaria free of KME. Furthermore, gonopodial and other morphological features showed no further development. Our unpublished observations and those of Hunsinger et al. (1988) confirm that KME-masculinized mosquitofish and least killifish can produce viable offspring after having been placed in aquaria free of KME. Although Larkin (1986) observed male reproductive behavior in female mosquitofish continuing as long as they were exposed to water containing microbially degraded phytosterols, we found that male reproductive behavior was not detectable among KME-masculinized female mosquitofish after they had been acclimated for two months to water lacking KME (Bortone et al. 1989).

Assessment of KME effluents requires long-term strategies to effectively define and evaluate effects on reproductive cycles and life-history alterations. To date, such in-depth assessments and evaluations have not been conducted. Therefore, we are not yet able to definitely state whether the observed masculinization is due to androgenic or, alternatively, antestrogenic stimuli. (Antestrogens are compounds that inhibit estrogenic processes and ovarian function.) We have described the effects as an androgenic stimulus because we observed production of embryos in females after removal from laboratory exposure to the microbially transformed phytosterols and because male juvenile American eels demonstrate accelerated testes development (Caruso et al. 1988) at a time in their life history when sex determination is not normally possible (Helfman et al. 1987).

Also, the hypothesis that masculinization is due to the presence of an androgenic stimulus in paper-mill effluent is supported even though there are often dioxins present in the effluent. Dioxins are antestrogens. Laboratory simulations using plant phytosterols in the absence of dioxins induced masculinized morphological and behavioral effects comparable to those observed in the poeciliid species exposed to KME in field studies.

These poeciliid fishes are dedicated invertebrate foragers. Piscivorous fish species feeding at higher trophic levels may be more vulnerable to uptake and accumulation of halorganics from their prey species. This masculinization may reflect the waterborne mode of exposure contrasted with the acquisition of a halorganic chemical body burden through the food web.

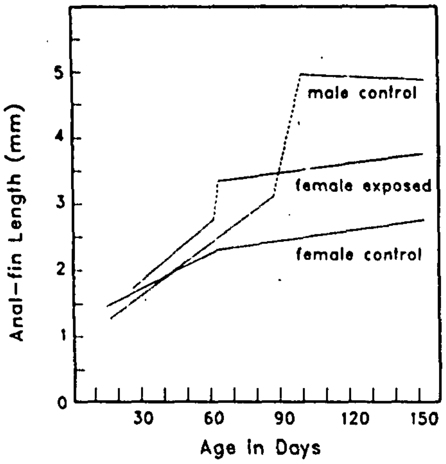

Figure 4. Results of continuous exposure of female mosquitofish to kraft-mill effluent (beginning one day after birth). The male and female controls were fish not exposed to kraft-mill effluent. (Redrawn from Drysdale and Bortone 1989.)

Degrees of masculinization as a bioindicator

Until other methods of detection and measurement other than the fish's morphological and behavioral response per se are developed, one is faced with an experimental tautology: heavily exposed fishes may be so severely modified that the female fish appears as a normal male and, therefore, undetectable by gross inspection in the field. Moreover, female fish may proceed through a complete series of intersexual steps in response to continued androgen exposure. This progression may be significant if exposure occurs during ontogenetic development. It could eventually lead to hermaphroditism. Clearly, both laboratory and controlled field exposures are needed, the former focusing on KME component extraction and the latter on multigeneration and long-term exposure effects.

There is additional experimental evidence indicating that different androgenic compounds produce differing degrees of morphological modification among fish species (Asahina et al. 1989), further confounding attempts to predict a fish's response to the complex chemistry of KME exposure. Males of the various poeciliid species mature at different sizes and age as a genetic or adaptive trait (Travis et al. 1989, Trexler and Travis 1990), which further underscores the need for a carefully conducted series of observations on fishes exposed to KME.

Although the mechanisms for sex determination among fishes are diverse (Angus 1989), sex expression may be adaptive. Genetically, sex determination has been described as polygenic in many fishes (Kosswig 1964). If the polygenic sex determination hypothesis is correct, there could exist multiple, genetically based and variable, responses to the exposure to endocrine disrupters. Regardless of whether or not the endocrine disrupters are acting through a genetic or environmental operand, we are alarmed at the potential problems they may cause to aquatic and other organisms.

To date, masculinization of poeciliid fish has been considered a scientific oddity. Concomitantly, its importance as a bioindicator has been unappreciated. Few studies have elucidated the potentially disruptive effects of hormones or hormonelike substances on natural populations in the field. Gibbs et al. (1991) found that populations of the American oyster drill (*Urosalpinx cinerea*) declined when the females apparently become masculinized (i.e., *imposex*) when exposed to tributyltin in nature.

After the initial observations of arrhenoidy in mosquitofish, we conducted different bioassays to determine if an individual had been exposed to KME in the coastal stream environment. The bioassays made use of the morphological and behavioral responses of females exposed to paper-mill effluent (Bortone et al. 1989). Drysdale and Bortone (1989) conducted a series of experiments that indicated that the induction of masculinization can occur early in the life history of these fishes (Figure 4). In another study, we noted the relationship between the degree of masculinization among female mosquitofish to the proximity of the paper-mill discharge point (Bortone and Drysdale 1981).

In the Fenholloway River, poeciliid fishes are virtually the only species present during times of drought, when KME is presumably more concentrated (judging by the dark-colored water). In a biotic region where high aquatic biodiversity is the norm, this stream is distinctly depauperate of fish species. Tributary streams, peripheral pools, and entering spring runs are rich in fish species absent from the contaminated portions. Additionally, the female poeciliids in these adjacent habitats show no sign of masculinization.

Poeciliids have the potential to serve as indicators of water quality to determine if and how fishes and other aquatic organisms (especially other vertebrates) are affected by effluent containing endocrine disrupters. Fishes in the family Poeciliidae are naturally distributed in the lowland and coastal areas of the temperate areas of the New World. Most species are tolerant of a wide range of environmental conditions and, therefore, are found in a broad range of habitats.

The mosquitofish is naturally distributed in a variety of habitats as well (Krumholz 1948), either as its eastern Atlantic slope form, *Gambusia affinis holbrooki*, or its Gulf of Mexico and Mississippi embayment form, *Gambusia affinis affinis*. Moreover, it has been introduced throughout the world as an ill-conceived aid for mosquito control. Its abundance, small size, broad distribution, and tolerance of a range of environmental conditions indicate that the mosquitofish is an excellent candidate to serve as a natural sentinel for detecting environmental stress. It may be useful at detecting stress caused by the addition of endocrine disrupters to the aquatic environment. Moreover, the fish can be especially useful when stress causes them to react through observable changes in reproduction, development, and other life-history features.

In the future, it is likely that long-term monitoring of secondary sex characters and life-history traits in poeciliid and other fishes will serve to detect the presence of endocrine disrupters. The advantage of such a detection system is that it recognizes the ecological impact these compounds may cause. Similarly, the life-history sentinel may prove to be a meaningful indicator of stress owing to disruption of endocrine modulations in the life cycle of many organisms.

Application of the model to other research areas

Endocrine disrupter has emerged as a generalized term to denote factors responsible for inducing in the critical timing of events in ontogenetic developmental sequences (Colborn et al. 1993). These alterations can affect morphology, physiology, and life-history traits. This concept has been brought together in the presentations edited by Colborn and Clement (1992) from a 1991 workshop (called "Chemically Induced Alterations in Sexual Development: The Wildlife/Human Connection") held in Racine, Wisconsin. Participants in the conference examined various examples of developmental alterations induced by environmental exposure to synthetic compounds. The focus of the workshop was on compounds that, after assimilation by parents, caused transgenerational exposure to offspring. The second-generation exposures were often at dose concentrations much higher than those typically occurring in the environment and produced various induced morphological or neurological modifications, reduced immune function, and altered reproductive function and behavior among all classes of vertebrates.

As more field and laboratory studies are reported, it is becoming increasingly clear that effects of endocrine disrupters are more common than one might at first suppose. Halorganic compounds and plasticisers are among 50 or more compounds that can induce similar responses. Their collective biological significance has been neglected and, thus far, notably absent from risk assessment analysis.

Fortunately, a considerable body of information has recently become available on the life history and basic biology of poeciliid fishes, especially mosquitofish (Larkin 1986, Meffe and Snelson 1989, Snelson 1989). The potential thus now exists for these fishes to serve as bioindicators for some types of endocrine disrupters. These baseline data can serve to help assess the impact of environmental stress on the varied life-history features of these coastal live-bearing fishes.

Through the efforts of Farr (1989), Travis et al. (1987), and Henrich (1988), information exists on the effectiveness of several experimental designs to measure the impact of environmental factors on the fitness of these poeciliid fishes. Moreover, there are several studies that have established the normal behavior of these fishes and make effective use of reproductive behavior as a way of assessing the impact of environmental stress on them (Itzkowitz 1971, Martin 1975, Schröder and Peters 1988). These studies on behavioral response, when coupled with the well-documented morphological response of live-bearing fishes to environmental stress (Bortone et al. 1989, Howell et al. 1980, Okada and Yamashita 1944, Riehl 1991, and Turner 1960), can provide reliable endpoint responses to potential endocrine disrupters.

Environmental masculinization of live-bearing fishes (including mosquitofish, least killifish, and sailfin mollies) may represent a unique form of intersexuality. Typically, androgens are aromatized in vertebrates, resulting in the feminization of males or antestrogen-induced female dysfunction. Wester et al. (1985) reported aromatization in the poeciliid guppy.

The masculinization of poeciliid females by androgens in KME represents only one example of a potent environmental sentinel among wildlife species. Concomitantly, it represents an opportunity to design testing procedures to evaluate the general application of the masculinization bioassay to the detection of endocrine-disrupting agents.

There is currently strong advocacy for initiating additional tests to detect potential endocrine disrupters in the procedures used to evaluate pharmaceuticals and regulate chemical discharge. Endocrine disruption of life history has a broader significance than traditional concepts of birth defects or teratogenesis. With the potential of transgenerational exposure and delayed responses, as in the impairment of immune systems, the effects of these compounds are particularly sinister.

Synthetically produced chlorinated and brominated organic compounds often demonstrate high levels of activity as endocrine disrupters. Perhaps the ability of some compounds to cause a high level of endocrine disruption was a factor contributing to their original empirical selection as effective pesticides. Toxicological testing has progressed from a focus on acute and chronic lethal effects to cancer induction on test species. Literature references of effects on offspring or adult life history and fitness have not typically been included in ecological risk-assessment procedures. However, reproductive toxicology is rapidly growing and moving from the laboratory toward field assessments that include life-history impact.

Developing a well-documented and well-referenced system to recognize the subtle changes in intersexuality in fishes represents a step in the right direction to better monitor the health of aquatic ecosystems. It may prevent unpleasant surprises, such as the recent reports of a global decline in reproductive success among amphibians (Phillips 1990, Vitt et al. 1990). Fish intersexuality, as a sentinel, may prove a reliable bioindicator to some forms of environmental stress.

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