Disturbed Sexual Characteristics in Male Mosquitofish (*Gambusia holbrooki*) from a Lake Contaminated with Endocrine Disruptors

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Previous laboratory studies have demonstrated that estrogenic and antiandrogenic chemicals can alter several sexual characteristics in male poeciliid fishes. Whether similar disturbances occur under field conditions remains to be confirmed. Lake Apopka, Florida, is contaminated with numerous chemicals, some of which possess endocrine-disrupting activity. Male mosquitofish (*Gambusia holbrooki*) were collected monthly from December 2000 through May 2001 from Lake Apopka and two nearby reference lakes, Orange Lake and Lake Woodruff National Wildlife Refuge. Selected sexual characteristics were compared temporally and among lakes during the collection period. Male fish from Lake Apopka had slightly shorter gonopodia and on average 32 and 47% fewer sperm cells per milligram testis, when compared with the fish collected from Orange Lake and Lake Woodruff, respectively. The testes weights increased markedly during spring, with significantly smaller testes in fish from Lake Apopka than from Orange Lake, but surprisingly, the smallest testes occurred in males obtained from the Lake Woodruff population. The highest liver weights were found in the Lake Apopka population. Whole-body concentrations of testosterone and estradiol varied among months; the peak testosterone concentration occurred in January and was significantly lower in male fish from Lake Apopka compared with Orange Lake. The intensity of male courtship behavior was highly correlated to body testosterone concentration, but no statistically significant differences in sexual behavior among the lakes were found. We conclude that sexual characteristics of relevance to male reproductive capacity are altered in the Lake Apopka mosquitofish population, and we discuss the presence of chemicals with antiandrogenic effects in Lake Apopka as a possible cause of the observed alterations. *Key words:* antiandrogen, courtship behavior, endocrine disruption, estradiol, mosquitofish, sex characters, sperm count, testosterone. *Environ Health Perspect* 111:695–701 (2003). doi:10.1289/ehp.6022 available via http://dx.doi.org/ [Online 23 January 2003]

Reproductive abnormalities have been observed in several wildlife populations living in polluted areas (Guillette et al. 1996; Howell et al. 1980; Jobling et al. 1998). In laboratory studies, it has been confirmed that environmental contaminants with endocrine-disrupting properties (EDCs) can disturb the development and expression of sexual characteristics in fish (Gimeno et al. 1996; Gray and Metcalfe 1997), amphibians (Hayes et al. 2002), reptiles (Crain et al. 1999; Willingham and Crews 1999; Willingham et al. 2000), birds (Feyk and Giesy 1998), and mammals (Gray et al. 1994, Sharpe et al. 1995). However, the extent to which the sexual characteristics and reproductive capabilities of natural populations are impacted by these EDCs is still not well understood.

Because of a chemical spill in 1980, Lake Apopka, Florida, is extensively polluted with dicofol, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT), and its metabolites 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (DDD) and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE). In addition, various pesticides, such as toxaphene, *trans*-nonachlor, dieldrin, and aldrin have been released into the lake from the surrounding farmland and are commonly found in the wildlife living in this area. A series of reproductive abnormalities have been reported in alligators living in this lake (Guillette et al. 2000), including reduced clutch size, altered gonadal morphology, smaller penis size, and altered hormone concentrations. Furthermore, elevated levels of environmental contaminants have been found in eggs and plasma of alligators living in Lake Apopka (Guillette et al. 1999; Heinz et al. 1991). It has been suggested that the elevated levels of contaminants in Lake Apopka cause these reproductive abnormalities (Guillette et al. 1994). However, the long generation time and large body size of alligators makes it a protracted and difficult task to verify the effects of chemical exposure in a controlled laboratory setting.

Mosquitofish and other poeciliid fishes have a much shorter generation time, and they are easily kept and handled in the laboratory. Furthermore, poeciliids have several readily measurable sexual characteristics that are affected by exposure to chemicals with estrogenic or antiandrogenic activity (Baatrup and Junge 2001; Bayley et al. 1999; Dreze et al. 2000; Haubruge et al. 2000; Toft and Baatrup 2001). Male mosquitofish have mature sperm cells in their testes year-round (Fraile et al. 1992), and they have an extended reproductive period dependent on photoperiod and temperature (Fraile et al. 1994; Koya

et al. 1998). Furthermore, mosquitofish are a very abundant freshwater fish in Florida, which makes it possible to collect specimens and compare the effects on fish living in reference and contaminated lakes. Sperm production and male courtship behavior is generally stimulated by androgens in fish (Borg 1994; Liley and Stacey 1983; Zentel 1988), but estrogens are also likely to influence the expression of these characteristics. Thus, testosterone is aromatized to estradiol locally in the brain and in the testes, where it is believed to be involved in the regulation of male behavior and testis function, respectively (Loomis and Thomas 1999; Nelson 2000; Pasmanik and Callard 1985). In many teleost fish, 11-ketotestosterone (11-KT) is a major androgen. Sperry and Thomas (1999a, 1999b) found two fish androgen receptor subtypes in Atlantic croaker (*Micropogonias undulatus*) and kelp bass (*Paralabrax clathratus*), which both have great affinity for testosterone, whereas 11-KT only binds one of these receptors. This indicates a major physiologic role for testosterone. Borg (1994) suggested that the production of 11-KT is very low or absent in poeciliid fishes (including mosquitofish) because very little or no 11-KT is produced in the testis or found in the blood of these fish. The testosterone level is usually similar in male and female teleosts (Borg 1994), but we found a significantly higher testosterone level in male mosquitofish (Toft et al. Unpublished data), suggesting that testosterone could play a key role in the control of male reproductive processes in these fish.

The purpose of this study was to compare male sexual characteristics in mosquitofish (*Gambusia holbrooki*) living in the contaminated Lake Apopka and several reference lakes. The study was conducted by monthly collections over 6 months to follow possible differences in the characteristics between lakes

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through a nonreproductive and a reproductive period. Furthermore, we wanted to compare these effects with previous observations of poeciliid fishes exposed to EDCs in the laboratory.

Materials and Methods

Animals and collections. Sexually mature male eastern mosquitofish (*Gambusia holbrooki*) were collected from three lakes in Florida using a 1-m² dip net. The criterion of hook development on the gonopodium (Angus et al. 2001) was used to determine if the fish were fully mature. The fish were collected monthly from December 2000 through May 2001 from the highly polluted Lake Apopka at the north shore near the mouth of the Apopka Beauclair Canal (28°67′N, 81°68′W) and from the reference site Orange Lake (29°46′N, 82°17′W). From March through May, Spring Garden Lake in the Lake Woodruff National Wildlife Refuge (29°12′N, 81°37′W) was included as a second reference site because of a very low water level in Orange Lake and the risk of dryout. The selection of the study sites was based on a study by Guillette et al. (1999) demonstrating high plasma concentrations of pesticides in alligators from Lake Apopka and low contaminant levels in animals from Orange Lake and Lake Woodruff National Wildlife Refuge.

At each of the monthly collections, approximately 60 male mosquitofish were collected from each lake. These fish were divided into two subsamples in the collections from January, February, and April, whereas three subsamples were made from the December, March, and May collections. The first subsample, consisting of 10–14 (mode = 12) individuals from each lake, was used for steroid measurements. To avoid steroid degradation in these fish, we killed them immediately after capture by covering them with ice until processing in the laboratory. Immediately after arrival in the laboratory, we measured the gonopodial and body lengths and body mass. Thereafter, the fish were frozen at –75°C until steroid determination by radioimmunoassay (RIA). The second subsample of 16–20 (mode = 20) fish was used for determination of sperm cell number, as well as for gonopodial and body lengths, and weights of the gonads, livers, and whole body. These measurements were performed within 24 hr after capture. This second group of fish were brought alive to the laboratory and killed by an overdose of 3-aminobenzoic acid ethyl ester (MS-222; Sigma Chemical Co., St. Louis, MO, USA) prior to measurements. Behavioral recordings were performed on a third subsample consisting of 11–23 (mode = 20.0) fish per lake. After behavioral recording, we determined body length, body weight, and length of gonopodium on these fish. All laboratory work was conducted in full compliance

with the guidelines of the University of Florida Institutional Animal Care and Use Committee. Fieldwork was conducted with permission from the Florida Game and Freshwater Fish Commission.

Length and weight measurements. The gonopodial lengths were measured as the length of fin ray 4 under a binocular microscope using an ocular micrometer. Body lengths, from the snout to the caudal peduncle, were measured to the nearest 0.5 mm using a ruler. Finally, the weights of the whole body, testis, and liver of the fish were determined to the nearest 0.1 mg.

Steroid determination. Whole-body extractions of steroids were performed to measure the amount of testosterone and 17β-estradiol in each fish. First, the fish were thawed on ice and each fish was homogenized in 3 mL distilled H_2O in glass tubes using a Polytron homogenizer (Kinematica AG, Littau, Switzerland). The steroids were extracted by adding 5 mL diethyl ether to each tube followed by vortexing for 1 min, then centrifuging at 1,500 × *g* for 4 min. Thereafter, the tubes were frozen in a methanol/dry ice bath at –30°C, and the ether layer containing the steroids was poured off into another set of tubes. The diethyl ether was then evaporated off under a flow of air. The extraction procedure was repeated twice to enhance the extraction efficiency. The extraction efficiency was tested by adding radiolabeled steroids to five fish homogenates per steroid and extracting these steroids using the above procedure. Steroid recoveries averaged 83% for estradiol and 111% for testosterone.

The amount of testosterone and 17β-estradiol in the collected fish was measured using a modification of a standard RIA protocol (Guillette et al. 1996). Briefly, we resuspended the extracted steroids in 500 µL borate buffer and added 100-µL aliquots of this suspension to two tubes per sample per measured steroid. To reduce nonspecific binding, we added 100 µL borate buffer with 0.75% bovine serum albumin (Fraction V; Fisher Scientific, Fair Lawn, NJ, USA) to the tubes for testosterone measurement, and 100 µL 0.95% bovine serum albumin for estradiol measurement. Thereafter, we added 200 µL antibody solution in a 1:20,000 final dilution for testosterone and 1:55,000 final dilution for estradiol (Endocrine Sciences, Calavasas, CA, USA). Finally, 100 µL radiolabeled steroid was added to each tube (12,000 cpm of [2,4,6,7, 16-³H]-estradiol at 37 MBq/mL or [1,2,6,7-³H]-testosterone at 37 MBq/mL). Both radiolabeled steroids were purchased from Amersham International (Arlington Heights, IL, USA). For standard curves, a serial dilution in 10 tubes from 1,600 pg/tube to 3.12 pg/tube for testosterone and from 800 pg/tube to 1.56 pg/tube for estradiol was prepared in

duplicate. All tubes were vortexed for 1 min and incubated 12–16 hr at 4°C. To separate bound and free radiolabeled steroids, 500 µL phosphate-buffered saline suspension containing 5% charcoal and 0.5% dextran (Sigma) was added, and immediately thereafter, the samples were centrifuged at 1,500 × *g* and 4°C for 30 min. Then, 500 µL from each tube was mixed with 5 mL scintillation cocktail and counted in a Beckman LS5801 scintillation counter (Beckman Coulter, Inc., Fullerton, CA, USA). The average intraassay coefficient of variance was 5.9% for testosterone and 4.1% for estradiol. The interassay variance was 20.9% for testosterone and 24.5% for estradiol, and steroid levels between assays were corrected accordingly. All chemicals for the RIA were purchased from Fisher Scientific, except when otherwise stated.

Sperm counts. The fish were stripped for sperm immediately after anesthetization with MS-222. The gonopodium was swung forward and the fish was stripped by gently stroking the abdomen with a tiny metal rod toward the gonopodium, hereby evacuating an ejaculate on a glass plate. The ejaculate consists of numerous spermatozeugmata (clusters of sperm cells), which were all collected with a
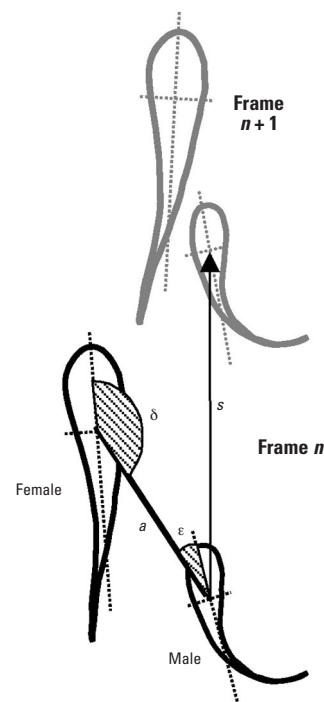

Figure 1. The male courtship behavior was automatically quantified using the DISPLAY vision system. The two oblong pixel assemblages represent the silhouettes of the female and the smaller male. After converting the elements into directed coordinate systems, the software can determine the distance between the animals (*a*) and the angular position of one fish relative to the longitudinal axis of the other (δ and ε). In addition, the swimming speed of the fish (*s*) can be determined by comparing successive frames.

Finn pipette and transferred to 100 μL 0.175 mM KCl solution to aid dispersal of spermatozoa. A Finn pipette was filled and emptied several times to ensure full dispersal of spermatozoa. Two samples of each sperm cell suspension were then transferred to an improved Neubauer Chamber hemocytometer (Paul Marienfeld, Bad Mergentheim, Germany), and after 10 min retention in a humid chamber, the number of sperm cells was counted under a microscope.

Sexual behavior. The field-collected male mosquitofish were acclimatized in two 40 × 20 × 26 cm aquaria per lake for a minimum of 3 days before the behavior recordings. These aquaria were filled to a depth of 7 cm with water collected from the lake of origin of the fish, and were kept under a 14:10 light:dark photoperiod at 21 ± 1°C. The fish were fed daily with TetraColor flake food (Tetra Werke, Melle, Germany), and the water was renewed every third day.

Prior to the behavioral measurements, individual male mosquitofish were acclimatized for 24 hr in separate 36 × 20 × 30 cm Plexiglas recording aquaria filled to a water depth of 7 cm. Immediately before the recording started, the male was paired with an adult female collected at the uncontaminated Boulware Spring, Gainesville, Florida (29°62′N, 82°31′W). This spring comes to the surface from the underlying aquifer, and the water contains very low (parts per trillion) to nondetectable concentrations of pesticides and nutrients (Gainesville Regional Utilities. Unpublished data). Each female was used only once per day.

During recording, the aquarium was placed on a sheet of glass 30 cm above diffusely lit white paper. The sides of the recording aquaria were opaque to prevent mirror images of the fish, whereas the bottom was transparent. When viewed from above, this arrangement resulted in clear dark silhouettes of the two fish against the light background, where the male was easily distinguishable from the much larger female (Figure 1). To exclude visual disturbances, the entire setup was enclosed in a metal frame covered with a blackout curtain. Five behavioral recordings, lasting 10 min each, were made per day.

The male mosquitofish courtship behavior was measured automatically employing DISPLAY software (Institute of Biological Sciences, University of Aarhus, Aarhus, Denmark), which records and analyzes complex behavior patterns in fish. The program has previously been used to identify guppy sexual behavior (Baatrup and Junge 2001). Briefly, the scenario was viewed by a CV-M10 progressive scan CCD camera (JAI, Inc., Copenhagen, Denmark) mounted 50 cm above the aquarium. The video signal from the camera was digitized by a DT3135

frame grabber (Data Translation, Inc., Marlboro, MA, USA) into a 768 × 576-pixel digital image, giving a 0.47-mm spatial resolution of the visual field. The frame grabber was interfaced with a personal computer.

Prior to the recording, appropriate size and gray-level ranges corresponding to the fish silhouettes were set in the software. These criteria were used for the conversion of each 8-bit gray-scale image into a binary (1 bit) image, where the pixel assemblages representing the fish silhouettes were stored in a frame file on disk for later analysis. During recording, an image was captured and processed approximately every 1/12 sec, so each 10-min frame file contained about 7,200 binary frames. These frame files, containing the time-series of fish contours, were subsequently analyzed by the DISPLAY program. In short, the two oblong pixel assemblages, representing the fish silhouettes, are converted into two coordinate systems by computing their principal axes or eigenvectors (Noble and Daniel 1988). For each frame it is now possible to determine the position and orientation of each fish relative to the other and the distance between them. Further, frame-to-frame comparisons enable calculation of speed and direction (relative to the body's longitudinal axis) of fish movements. With this information it is now possible to identify and quantify complex behavior patterns.

The sexual behavior of the western mosquitofish (*Gambusia affinis*) was previously described by Krotzer (1990). Using her description and our own observations, we configured the DISPLAY program to identify two distinct elements in the male's sexual behavior, the following behavior and the close-following behavior. When performing following behavior, the male swims behind the female, focused on her genital opening. Close-following behavior is a subset of the following behavior. Here the male is closer to the female genital opening and he swims faster. The following parameters were extracted from the approximately 7,200 frames in each frame file (Figure 1): a, the distance between the centroids of the two animals; δ, the angle between the female's longitudinal axis and a line connecting the centroids of the male and the female; ε, the angle between the male's longitudinal axis and a line connecting the centroids of the male and the female; and s, the speed of the male. To identify periods in the frame files with following behavior and close-following behavior, the search criteria for following behavior were as follows: a, < 50 mm; δ, > 100°; ε, < 30°; and s, > 20 mm/sec for a minimum of 0.1 sec; for close-following behavior, criteria were a, < 24 mm; δ, > 118°; ε, < 22°; and s, > 45 mm/sec for a minimum of 0.05 sec.

Statistics. To improve normality and homogeneity of variance, logarithmic transformations were performed on morphometric

parameters, whereas sperm counts per milligram testis were square root transformed and behavioral data were arcsine transformed. We used analyses of covariance (ANCOVA) to correct gonopodium length for body length, gonad weight, liver weight, testosterone, and estradiol for whole-body weight. The parameters were compared using two-way analysis of variance (ANOVA)/ANCOVA to determine differences between lakes and between months. Because we only collected data from Lake Woodruff in March–May, two separate two-way ANOVA/ANCOVA were performed, one comparing the combined data from all collection months from Lake Apopka and Orange Lake, and another comparing the three lakes from March–May. Differences between lakes at the separate monthly collections were analyzed by ANOVA/ANCOVA followed by Bonferroni test for multiple comparisons. Partial correlations were used to indicate relations between hormones and behavior corrected for body weight. We considered $p < 0.05$ statistically significant. All statistics were calculated using SPSS, version 10.0 (SPSS Inc., Chicago, IL, USA).

Results

Length and weight. The body length and weight (Table 1) varied among lakes during several of the collection months, but no consistent difference among the lakes was observed. Testicular weight also varied among lakes (Table 1). Male fish from the contaminated lake, Lake Apopka, had testes that were on average 8.7% smaller compared with male fish from Orange Lake throughout the collection months ($p = 0.001$). However, the smallest testes were found in fish from the other reference site, Lake Woodruff, where testicular weight was on average 44.1 and 50.5% lower compared with Lake Apopka and Orange Lake, respectively. In addition, between months there were marked differences, with a general increase in testis weight during the spring.

Overall, the largest livers were found in fish from Lake Apopka, whereas the smallest livers were found in fish collected from Lake Woodruff. In fish from Orange Lake, the liver weights were only statistically smaller compared with Lake Apopka in the February and May collections (Table 1). Liver weights also varied between collection months, with the greatest liver weights in January and the smallest in April.

The gonopodia were overall slightly shorter in fish from Lake Apopka compared with fish from Orange Lake and Lake Woodruff ($p < 0.05$). The difference in gonopodium lengths among lakes was never larger than 2.2%; in only two of the separate monthly comparisons were the differences among lakes significantly different (Figure 2).

However, large sample sizes between 28 and 60 (average 45) per lake per collection made it possible to distinguish these small differences between sites. The gonopodial lengths differed up to 10% between months, but these differences corresponded to the differences in body length of the animals and likely are due to sampling bias, not real morphologic alterations of the gonopodium.

Testosterone and estradiol. A peak in the body concentration of testosterone appeared in January with about 2,400 pg testosterone per gram body weight in fish from Orange Lake. This peak was markedly lower in fish from Lake Apopka (Figure 3A). From February, testosterone concentration decreased to between 1,000 and 1,600 pg/g in fish from all the lakes. During this period, the lowest testosterone concentration was found in animals from Lake Woodruff. At the May collection, the fish from Orange Lake and Lake Woodruff still contained about 1,100 pg/g, whereas those from Lake Apopka contained 1,600 pg/g.

The estradiol concentrations varied between 100 and 450 pg/g throughout the collection months, with the lowest estradiol concentration found in male fish collected in February and March; higher concentrations were observed before and after this period (Figure 3B). The estradiol concentrations were similar in fish from the three lakes.

Extreme steroid hormone levels of 5,800 pg/g testosterone and 2,038 pg/g estradiol in one fish from the December collection and 2,671 pg/g estradiol in one fish from the April collection were excluded from the analysis presented in Figure 3.

Sperm counts. Male mosquitofish contained mature sperm cells throughout the winter and spring. The total number of sperm cells per ejaculate, averaged over the collection months, were 1.9, 4.2, and 3.4 million in fish from Lake Apopka, Orange Lake, and Lake Woodruff, respectively. The markedly lower sperm cell number in fish from Lake Apopka was still evident when the sperm count was expressed as the number of sperm cells per milligram testis (Figure 4), which corrected for differences in testicular weights among lakes. Overall, the sperm counts were 32% and 47% lower in Lake Apopka when compared with Orange Lake and Lake Woodruff, respectively

($p < 0.001$). In addition, at all the monthly collections except for the March collection, the sperm counts were lower in fish from Lake Apopka compared with the reference sites. The May collection is particularly noteworthy, as the fish at this time of the year have reached full reproductive capacity. The ejaculates of males from Orange Lake and Lake Woodruff contained on average 1.1 and 2.6 million sperm cells per milligram testis, respectively, whereas the fish from the polluted Lake Apopka contained only 0.2 million sperm cells per milligram testis (Figure 4).

Behavior. The proportion of time spent on sexual behavior measured as following behavior and close-following behavior was markedly lower in the December collection ($p < 0.003$) than in the male fish collected in March or May (Table 2), demonstrating a much lower sexual activity during the winter period. The time spent on following and close-following behavior was similar in mosquitofish from the three lakes.

A high correlation existed between the mean testosterone concentration for male fish and the corresponding sexual behavior

Table 1. Body length and weight of whole body, gonad, and liver (mean ± SE) of the mosquitofish collected from Lake Apopka, Orange Lake, and Lake Woodruff.

	December	January	February	March	April	May
Body length (cm)						
Lake Apopka	2.09 ± 0.025[a]	2.02 ± 0.021[a]	2.11 ± 0.026[a]	2.08 ± 0.022[a]	2.06 ± 0.027[a]	2.06 ± 0.023[a]
Orange Lake	2.00 ± 0.027[b]	2.20 ± 0.032[b]	2.36 ± 0.028[b]	2.14 ± 0.025[a,b]	1.91 ± 0.037[a]	1.91 ± 0.025[b]
Lake Woodruff	NA	NA	NA	2.16 ± 0.021[b]	1.99 ± 0.027[a]	1.95 ± 0.021[b]
Body weight (g)						
Lake Apopka	0.134 ± 0.005[a]	0.143 ± 0.005[a]	0.159 ± 0.007[a]	0.146 ± 0.006[a]	0.165 ± 0.008[a]	0.157 ± 0.007[a]
Orange Lake	0.131 ± 0.004[a]	0.179 ± 0.008[b]	0.222 ± 0.008[b]	0.156 ± 0.006[a,b]	0.119 ± 0.007[b]	0.124 ± 0.005[b]
Lake Woodruff	NA	NA	NA	0.166 ± 0.006[b]	0.134 ± 0.006[b]	0.119 ± 0.004[b]
Testis weight (mg)						
Lake Apopka	1.991 ± 0.074[a]	1.946 ± 0.077[a]	4.107 ± 0.333[a]	4.748 ± 0.199[a]	4.162 ± 0.286[a]	4.176 ± 0.238[a]
Orange Lake	2.212 ± 0.074[b]	2.282 ± 0.074[a]	3.771 ± 0.323[a]	5.084 ± 0.196[a]	5.359 ± 0.283[a]	4.523 ± 0.234[a]
Lake Woodruff	NA	NA	NA	3.253 ± 0.199[b]	1.849 ± 0.269[b]	2.266 ± 0.239[b]
Liver weight (mg)						
Lake Apopka	1.540 ± 0.078[a]	2.947 ± 0.114[a]	2.526 ± 0.118[a]	1.533 ± 0.181[a,b]	1.528 ± 0.126[a]	2.560 ± 0.224[a]
Orange Lake	1.603 ± 0.078[a]	2.748 ± 0.114[a]	1.935 ± 0.114[b]	2.048 ± 0.178[a]	1.419 ± 0.117[a]	1.764 ± 0.213[b]
Lake Woodruff	NA	NA	NA	1.399 ± 0.180[b]	0.863 ± 0.109[b]	1.566 ± 0.218[b]

NA, not analyzed. Testis weight and liver weight are expressed as estimated marginal mean corrected for whole body weight within collection months. Different letters indicate significant difference ($p < 0.05$) between lakes.
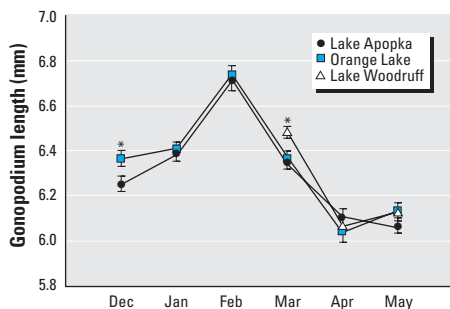

Figure 2. Only small differences in gonopodium length were found between the mosquitofish from Lake Apopka and the two reference lakes. Gonopodial lengths are expressed as mean ± SE gonopodium length corrected for standard length (estimated marginal means).

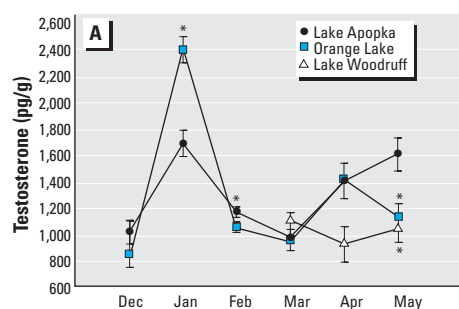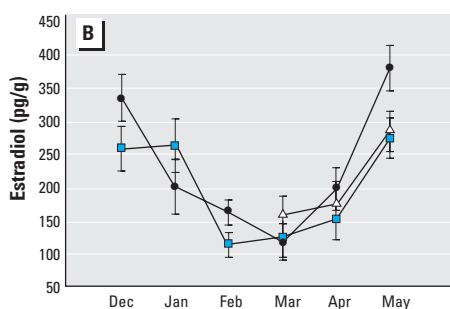*Differences from Lake Apopka ($p < 0.05$) in the monthly comparisons.

Figure 3. Whole-body concentrations of testosterone (*A*) and estradiol (*B*) in mosquitofish from the three lakes. Steroid concentrations are expressed as mean ± SE corrected for whole-body weight (estimated marginal means).

*Differences from Lake Apopka ($p < 0.05$) in the monthly comparisons.

observed in groups of fish collected monthly from the three lakes, when combined in a correlation analysis across lakes and months ($r = 0.81$; $p = 0.03$ for following behavior and $r = 0.86$; $p = 0.01$ for close-following behavior). In contrast, estradiol levels were not significantly correlated to the observed sexual behaviors ($r = 0.38$; $p = 0.40$ for following behavior and $r = 0.50$; $p = 0.25$ for close-following behavior). This suggests that testosterone, at least in part, controls male sexual behavior.

Discussion

In this study we analyzed selected sexual characteristics in male mosquitofish from a period of reproductive inactivity through a period of reproductive activity (December–May). All sex characteristics demonstrated monthly variation throughout winter and spring. Furthermore, we found lower sperm counts, reduced gonopodial lengths, and a lower testosterone peak in fish from the contaminated Lake Apopka. In addition, the livers tended to be enlarged in fish from this lake.

The testicular weights differed among the three lakes, with the smallest testes in fish from Lake Woodruff. We have no explanation for why fish from this lake had smaller testes. It is, however, interesting to note that even though the testes were smaller, both the sperm counts per milligram testis and the total number of sperm cells per ejaculate were higher in fish collected in Lake Woodruff compared with fish from Lake Apopka, whereas the total sperm cell number was similar in fish from Lake Woodruff and Orange Lake. This indicates that the available sperm cells were similar in the two reference lakes, but the sperm production (per milligram testis) was highest in fish collected in Lake Woodruff, intermediate in fish from Orange Lake, and lowest in fish from the contaminated Lake Apopka.

The livers tended to be larger in fish collected in Lake Apopka compared with the reference sites. Altered liver morphology and/or enzymatic activity is a typical response in fish after exposure to toxicants, as the liver is the major detoxification organ (Kime 1998). Increased liver growth is likely to be a nonspecific response to toxicants induced by increased demands on enzymatic activity in the liver. However, specific protein induction such as vitellogenin induction by estrogenic compounds could also have caused the increased liver weights.

The smaller gonopodia in fish from Lake Apopka are, on the other hand, likely to be caused by altered endocrine function. In castration studies it has been demonstrated that gonadal secretions are necessary for gonopodium development in mosquitofish, and that ethynyl testosterone restored the gonopodium (Turner 1947). Furthermore, gonopodium-like development can be induced in female poeciliids by administration of androgens (e.g., Angus et al. 2001; Rosa-Molinar et al. 1998; Turner 1941). Therefore, androgens are believed to be crucial for development of the gonopodium in poeciliids. The inhibition of gonopodium development could be either an antiandrogenic effect blocking androgen receptors, or an indirect effect on steroid hormone production and secretion, as suggested after the observed inhibited regeneration of the gonopodium in guppies exposed to the antithyroid agent thiouracil (Hopper 1965). Male mosquitofish collected in sewage-contaminated waters have reduced gonopodium length (Batty and Lim 1999), whereas females collected in androgenic pulp mill effluent have gonopodium-like anal fin enlargement (e.g., Bortone and Cody 1999; Howell et al. 1980; Parks et al. 2001). These reports and the present results indicate that gonopodium development is sensitive to chemicals in the environment, possibly through disruption of endocrine function, but in fish from Lake Apopka, the length of the gonopodium was only slightly shorter, and this difference in itself is unlikely to affect male reproductive capability. However, as noted for the reduction in phallus size in alligators of Lake Apopka (Guillette et al. 1996), the reduction in gonopodium size reported here represents a change in androgen action that is more than just a transitory alteration.

Marked seasonal variation in androgens is common in teleost fishes (Borg 1994), and often the testosterone level peaks just before the breeding season (Fostier et al. 1983), as was observed in this study. Mosquitofish have mature sperm cells in their testis year-round, but spermatogenesis and gonad weight vary considerably between seasons (Fraile et al. 1992). Stimulatory effects of androgens on spermatogenesis have been observed in poeciliids (Schreibman et al. 1986; Zentel 1988), and androgens, including testosterone, are therefore probably major regulators of spermatogenesis in the mosquitofish. Accordingly, the lower peak in testosterone in fish from Lake Apopka in January could, in part, explain the reduced sperm counts in these fish.

The concentrations of 17β-estradiol also varied during the 6 months of this study. 17β-Estradiol has only been measured in males of a few fish species, and it is often found in very low or undetectable concentrations (Fostier et al. 1983). As in previous studies, the concentration of estradiol was low in the fish we studied; in a few samples it was even below the level of detection. Estradiol is produced by aromatization of testosterone in the brain, pituitary, and gonads. It is involved in the regulation of sexual behavior and gonadotropin secretion (Callard 1983; Fostier et al. 1983) and probably spermatogenesis (Miura et al. 1999). Because this estrogen is locally produced and utilized, the whole-body level of estradiol does not necessarily correlate with the level in the target organs. Consequently, the present lack of correlation between whole body estradiol contents and behavior does not preclude a role of estrogens in male mosquitofish sexual behavior.

A markedly lower sperm cell number was found in the ejaculates of the Lake Apopka fish when compared with fish from the two reference lakes. Results from histologic examinations of testes from guppies exposed to 4-tert-octylphenol, bisphenol A, flutamide, or p,p′-DDE indicated that these compounds inhibit spermatogonial mitosis and/or the transformation of spermatogonia into spermatozytes, but do not inhibit the transformation of spermatids and spermatozoa into spermatozeugmata (Kinnberg and Toft 2003). Whether similar morphologic aberrations in
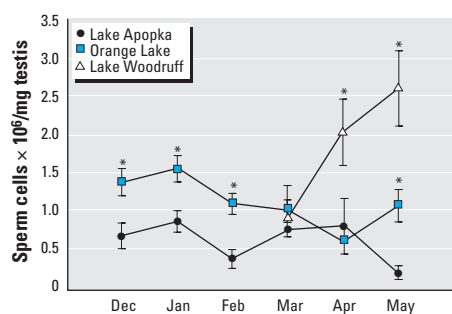

Figure 4. Significantly smaller sperm counts were found in the Lake Apopka fish when compared with the two reference lakes. Graph entries are expressed as mean ± SE stripped sperm cells per milligram testis.

*Differences from Lake Apopka ($p < 0.05$) in the monthly comparisons.

Table 2. Sexual behavior.[a]

	December	March	May
Following behavior			
Lake Apopka	6.61 ± 2.53	25.40 ± 7.94	47.72 ± 12.13
Orange Lake	4.45 ± 3.40	30.00 ± 8.57	12.57 ± 5.13
Lake Woodruff	NA	46.22 ± 14.95	19.70 ± 5.18
Close-following behavior			
Lake Apopka	0.30 ± 0.14	2.25 ± 0.96	4.18 ± 1.41
Orange Lake	0.01 ± 0.01	1.88 ± 0.70	1.18 ± 0.82
Lake Woodruff	NA	4.12 ± 1.91	1.27 ± 0.42

NA, not analyzed.
[a]Values represent mean ± SE of the measured sexual behavior expressed in permillage of total 10-min recording time.

spermatogenesis occur in the Lake Apopka mosquitofish is presently under investigation. However, it is reasonable to assume that the lower sperm production is caused by endocrine disruption, because reduced sperm counts in poeciliid fishes have previously been observed in laboratory studies after exposure to EDCs such as 4-*tert*-octylphenol, bisphenol A, tributyltin, vinclozolin, and *p,p′*-DDE (Baatrup and Junge 2001; Bayley et al. 2002; Haubruge et al. 2000; Toft and Baatrup 2001). The endocrine control of sperm cell production in fish has not been described in detail, but it has been demonstrated in Japanese eel (*Anguilla japonica*) that chorionic gonadotropin or 11-ketotestosterone injections induce complete spermatogenesis (Miura et al. 1991a, 1991b). In the mummichog (*Fundulus heteroclitus*), testosterone or pituitary homogenate also stimulated spermatogenesis (Cochran 1992). Miura et al. (1991b) proposed that gonadotropin stimulated Leydig cells to produce androgen, which subsequently activated a Sertoli cell–mediated completion of spermatogenesis. In addition, estrogens appear to play a role in spermatogenesis, as spermatogonial stem cell renewal in Japanese eel is increased after exposure to 17β-estradiol (Miura et al. 1999). Assuming a similar endocrine function in the mosquitofish, compounds with endocrine-disrupting activities could interfere with the gonadotropin or androgen/estrogen receptors or their production at one or several sites within the hypothalamo–pituitary–gonad axis, causing the observed alteration of spermatogenesis.

The time spent on sexual behavior by the mosquitofish from the EDC-polluted Lake Apopka was not statistically different from the cleaner reference sites. This was an unexpected result, as both estrogenic and antiandrogenic compounds have been demonstrated to suppress guppy courtship behavior (Baatrup and Junge 2001; Bayley et al. 1999, 2002). The sexual behavior of male fish can be removed by castration and restored by androgen administration, and male sexual behavior can be induced in female fish by exposure to androgens (Borg 1994; Liley and Stacey 1983). In addition, the high correlation between the testosterone level and the measured sexual behaviors in the present study suggests that testosterone is involved in the regulation of the sexual behavior in male mosquitofish. Therefore, the presence of antiandrogenic chemicals (e.g., *p,p′*-DDE) in Lake Apopka was hypothesized to alter the sexual behavior of these fish. In a parallel study (Toft and Guillette. Unpublished data), male mosquitofish were collected in a clean spring and exposed to water from Lake Apopka or two reference sites for 1 month. This resulted in a significant reduction in the sexual behavior in the group exposed to the water from Lake Apopka. Therefore, the fish living in Lake Apopka with chronic lifetime exposure to EDCs apparently possess either an induced or inherent defense mechanism against behavioral disturbances. Resistance to organochlorine exposure based on the concentration lethal to 50% (LC_{50}) has been observed in populations of mosquitofish living in contaminated areas (Moffett and Yarbrough 1972).

DDT and its degradation products (DDE and DDD) are known to biomagnify through the food web. A 27,000–85,000 times concentration of DDT or degradation products in mosquitofish, compared with surrounding water, was observed after 33 days of exposure in a model ecosystem (Metcalf et al. 1973). Likewise, the concentrations of DDT and degradation products in fishes from Lake Malawi, East Africa, were in the range of 100,000 times higher in tissues compared with the surrounding water (Kidd et al. 2001). In Lake Apopka, the DDT degradation product *p,p′*-DDE was the environmental contaminant found at the highest level in alligator serum in an extensive analysis of 46 organochlorine pesticides and polychlorinated biphenyls (Guillette et al. 1999). In addition, mosquitofish (U.S. Fish and Wildlife Service. Unpublished data) and Brown Bullhead (*Ameiurus nebulosus*) (Gallagher et al. 2001) from Lake Apopka contained elevated *p,p′*-DDE and toxaphene. Antiandrogenic properties of *p,p′*-DDE have been demonstrated in mammals (Kelce et al. 1995), but the binding of *p,p′*-DDE to fish androgen receptors varies among species and tissues (Sperry and Thomas 1999a; Wells and Van Der Kraak 2000). Still, it is likely that the reduced sperm counts observed in mosquitofish collected in Lake Apopka are caused by antiandrogenic contaminants. We cannot exclude the possibility that the differences observed in fish from these lakes are caused by genetic or other environmental differences. However, reference mosquitofish exposed to water from Lake Apopka, but not Orange Lake, were found to have a reduction in sperm counts, similar to the observations in this study (Toft and Guillette. Unpublished data). These data strongly indicate that compounds in the water are, at least in part, the cause of the observed effects.

In summary, we found depressed sexual characteristics in male mosquitofish from Lake Apopka when compared with fish from Orange Lake and Lake Woodruff National Wildlife Refuge. Specifically, the sperm counts were reduced in fish from Lake Apopka throughout the winter and spring. Contaminants with antiandrogenic action present in this lake are suggested to cause these differences. It is not known whether the observed results cause adverse effects at the population level, but no obvious difference in fish density was observed between the polluted and reference lakes. However, a reduction in sperm count and other sexual characteristics could reduce an individual fish's reproductive success in competition with unaffected animals. This needs to be examined and demonstrated in mosquitofish.

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