

REPRODUCTIVE AND TRANSGENERATIONAL EFFECTS OF METHYLMERCURY OR AROCLOR 1268 ON *FUNDULUS HETEROCLITUS*

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Abstract—This research determined the potential for methylmercury or Aroclor 1268 to disrupt reproduction and sexual differentiation in *Fundulus heteroclitus*. The research determined whether fish that are exposed to mercury or Aroclor 1268 survive and successfully reproduce; whether offspring of exposed fish hatch, survive, produce eggs, and fertilize them; and whether the second-generation offspring of exposed fish hatch and survive. *Fundulus heteroclitus* were exposed to mercury or Aroclor 1268 via contaminated food. Endpoints evaluated included survival, growth, fecundity, fertilization success, hatch success, larval survival, sex ratios, and the prevalence of gonadal abnormalities. In general, polychlorinated biphenyls were highly bioavailable and accumulated well through feeding. The only statistically significant effect observed as a result of treatment with Aroclor 1268 was an increase in growth in the offspring of exposed fish. Mercury was accumulated in a dose-dependent fashion via food exposures. Exposure to mercury in food increased mortality in male *F. heteroclitus*, which possibly occurred as a result of behavioral alterations. Increased mortality was observed at body burdens of 0.2 to 0.47 $\mu\text{g/g}$. Offspring of *F. heteroclitus* fed mercury-contaminated food were less able to successfully reproduce, with reduced fertilization success observed at egg concentrations of 0.01 to 0.63 $\mu\text{g/g}$, which corresponds with parent whole-body concentrations of 1.1 to 1.2 $\mu\text{g/g}$. Offspring of exposed fish also had altered sex ratios, with treatment at moderate concentrations producing fewer females and treatment at the highest concentration producing more females than expected. Alterations in sex ratios were observed at concentrations of less than 0.01 $\mu\text{g/g}$ in eggs or between 0.44 and 1.1 $\mu\text{g/g}$ in parents. Offspring of mercury-exposed fish also had increased growth in moderate treatments, when egg concentrations were less than 0.02 $\mu\text{g/g}$, or when parent whole bodies contained 0.2 to 0.47 $\mu\text{g/g}$. In summary, exposure to mercury reduced male survival, reduced the ability of offspring to successfully reproduce, and altered sex ratios in offspring. Both direct effects on exposed fish and transgenerational effects were observed.

Keywords—Mercury Polychlorinated biphenyls *Fundulus* Tissue concentrations Reproduction

INTRODUCTION

Bioaccumulative contaminants such as mercury and polychlorinated biphenyls (PCBs) are a serious environmental threat. Because of their common historical use, association with sediments, and persistence in the environment they are common contaminants in ports and harbors, where they are expensive to remediate. Because of their association with sediments and their tendency to bioaccumulate, lower trophic levels are exposed directly through consumption of sediment and detritus, whereas higher trophic level organisms are exposed through food consumption. The accumulation of these substances in fish tissue has resulted in fish closures and advisories to prevent adverse human health effects in many areas throughout the country. Exposure to mercury or PCBs has also been associated with adverse reproductive effects in fish and wildlife, and recently these chemicals have been implicated as endocrine disrupters [1].

Polychlorinated biphenyls are known to reduce hatching success and larval survival in fish. Exposure via contaminated water [2], sediment [3], and maternal transfer of PCBs to eggs [4] have resulted in toxicity to early life stages of fish. Adverse effects associated with bioaccumulation of PCBs have included reduced viable hatch in Baltic flounder [5], egg mortality in charr [6], reduced larval size in winter flounder [7], reduced egg hatchability in lake trout [8], and liver lesions in Atlantic

tomcod [9]. Recent studies have linked exposure to PCBs with depressed estradiol levels that could inhibit spawning [10]. Even after hormone treatments, fish from PCB-contaminated areas were less likely to spawn, took longer to spawn, and produced a higher proportion of abnormal larvae than fish from less contaminated areas [11]. Recent studies suggest that some PCB compounds could affect the process of sexual differentiation. Some PCB compounds can bind to estrogen receptors [12], may disrupt endocrine function [1], and alter sexual differentiation [13,14].

Mercury is also a reproductive toxin, a teratogen, and a neurotoxin [15]. Because mercury apparently acts through the inhibition of protein syntheses, a wide range of adverse effects has been observed. Mercury, like PCBs, is known to reduce spawning frequency, hatching success, and larval survival in fish [15]. Early life stages are thought to be the most sensitive to the effects of mercury. Reproductive systems of male fish may also be affected by exposure to mercury. For example, juvenile walleye developed atrophied testicles after eating methylmercury-contaminated food [16]. Exposed fish can become tolerant to the effects of mercury and this tolerance can be passed to offspring [17]. Methylmercury can also be directly toxic to fish embryos in water.

Many laboratory studies have demonstrated that PCBs or mercury can elicit adverse effects in fish. However, a great deal of uncertainty exists in applying these results to decision making because of differences between lab studies and field

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conditions in terms of the route of exposure examined (water versus food), PCB mixtures or individual congeners tested, extrapolation of results to other species, and specific conditions that control bioavailability at individual sites.

The overall goal of this study was to determine whether fish exposed to PCBs and mercury are capable of producing viable larvae. Elevated levels of mercury and PCBs have been found in salt-marsh sediments in the southeastern United States, where *Fundulus heteroclitus* is the dominant forage fish. The methods selected were intended to represent a realistic exposure pathway for accumulation of contaminants in both male and female fish through the consumption of contaminated food. The study design was not intended to separate whether any observed effects are due to exposure of females as compared to males. The study also did not include exposure of juvenile fish to contaminated food, water, or sediment and may therefore underestimate adverse effects to later generations.

This research had three specific objectives: to determine threshold tissue concentrations of mercury or Aroclor 1268 in adult *F. heteroclitus* that are associated with any observed reductions in survival, growth, fecundity, or fertilization success; to determine threshold tissue concentrations that are associated with a suite of adverse effects in offspring (F_1) of fish exposed to Aroclor 1268 or methylmercury; and to determine threshold tissue concentrations associated with reduced hatch success or larval survival in the second generation of offspring (F_2) of fish exposed to Aroclor 1268 or methylmercury.

MATERIALS AND METHODS

Adult F. heteroclitus contaminant exposure and observation

Adult *F. heteroclitus* were collected using baited minnow traps on February 25 and 26, 1997, and April 24, 1997, near the University of Georgia's Marine Institute on Sapelo Island, Georgia, USA. Fish were shipped by overnight courier to Seattle, Washington, USA, on March 5, 1997, and on April 25, 1997 (397 and 288 fish, respectively). Upon reaching Seattle, fish were acclimated to laboratory conditions in aerated 20‰ artificial seawater (Instant Ocean, Carolina Biological Supply, Burlington, NC, USA), and a 10:14 h light:dark cycle, which was similar to conditions at the collection site.

American Society for Testing and Materials protocol E-1241-92 on conducting early life stage toxicity tests with fish was used as a general guide for the test, although the procedures described there do not apply directly to determining adverse effects through food exposure. The exposure method for this study was based upon the methods of Boudou and Ribeyre [18] and Gutjahr-Gobell et al. [19].

Males and females were kept in separate tanks to prevent uncontrolled spawning. The acclimation period was between 10 and 61 d. Fish were then distributed to treatment tanks using a randomized block design. Each control treatment included 12 male and 12 female fish, whereas each contaminant treatment included 15 males and 15 females (to allow for interim chemical analysis to verify accumulation). Each 10-gallon glass tank held between five and seven fish.

Beginning on May 5, fish were fed contaminated food for at least six weeks until target concentrations in whole bodies were achieved. Although this exposure period was relatively short and exposure began with adult fish, some of which likely had already developed eggs, partitioning of PCBs to lipid-rich tissues was expected to occur rapidly. Methylmercury is read-

ily incorporated into tissues and has been demonstrated to accumulate quickly and depurate slowly [20].

Commercially available fish food (Nutra Fry, Moore-Clark, Vancouver, BC, Canada) was contaminated with solutions of methylmercuric chloride (Ultra Scientific, North Kingstown, RI, USA) or Aroclor 1268 (Aldrich Chemical, Milwaukee, WI, USA) dissolved in acetone. Food concentrations were selected that were expected to result in a range of tissue concentrations that bracket those found in the environment at contaminated sites.

The PCB-contaminated food was created as needed in 50-g portions (approximately once a week). Because of instability of the mercury stock solution, mercury-contaminated food was created in three batches (on May 5, May 13, and July 11, 1997) and frozen until needed. Dried food was spread in a thin layer in glass baking dishes and soaked in solvent. After a few minutes, various volumes of a stock acetone solution containing Aroclor 1268 or methylmercuric chloride were added to the dishes, with a total volume of 40 ml of acetone added to 50 g of food. The mixture was stirred and the solvent was evaporated at room temperature under a fume hood for at least 12 h. Food was added to the groups of fish twice a day in portions usually consumed within 10 min. This represents a balance of total consumption and sufficient time for all fish to have an opportunity to feed. Any uneaten food was removed from tanks after each feeding. Two groups of control fish were randomly established: one was fed uncontaminated food and one was fed food treated with solvent. A total of 10 groups was established. A 1-g subsample of food from each treatment group was collected weekly and composited for chemical analysis to verify exposure concentrations at the end of the feeding period.

Up to three composites of three fish from each female treatment group and one composite from each male treatment group were removed during the exposure period and chemically analyzed to verify that target concentrations were reached. Whole fish were frozen before shipment and packaged so that they remained frozen during shipment. Chemical verification for Aroclor 1268 was conducted by Analytical Resources (Seattle, WA, USA) using dual capillary column gas chromatography with electron capture detection (U.S. Environmental Protection Agency method 8081 modified by the use of method 3550 for tissue preparation). Verification analysis for methylmercury was conducted by Frontier Geosciences (Seattle, WA, USA) using the technique described by Bloom [21].

After target concentrations were reached in composites of whole fish, conditions suitable for breeding [22] were gradually established over a 7-d period. Fish continued to consume contaminated food during this period. Female fish were examined every few days for indications that they were ready to spawn. Fish were anesthetized in 15 mg/L ethyl-*m*-amino benzoate methanesulfonate before manual stripping of eggs was attempted. Males were selected randomly to be crossed with ripe females. In general, males were mated with only one female, unless the number of ripe females exceeded the number of available males, in which case, males were used again to create more than one spawning pair.

The weight and length of each fish was recorded at the first successful spawning attempt, or at the end of the test if the fish never successfully spawned. Eggs were manually stripped into dry petri dishes and mixed with milt stripped from the male. Between 10 and 50 additional eggs were collected from each female and frozen at -20°C in one composite per treat-

ment for chemical analysis. Each successfully spawning female fish was strip-spawned at least twice over several days. Fish were marked with fin clips and were returned to their tanks between spawning attempts. Mortality was recorded throughout the feeding and spawning period. Dead fish were removed immediately, weighed, measured, and frozen. Fecundity was determined by counting all eggs stripped over the entire spawning period. The first successful spawning occurred on June 19, 1997, and the last occurred on August 15, 1997.

Artificial seawater (20‰) was added to the egg–milt mixture after 30 min and eggs were rinsed after an additional 30 min [22]. Until they hatched, developing eggs were held at 22°C in petri dishes containing artificial seawater, which was replaced daily. Dead eggs were removed daily, recorded, and preserved in Stockard's solution, a mixture of 5% buffered formalin, glacial acetic acid, glycerin, and distilled water. Fertilization success was determined at the end of the test by examination under 16- to 40-power magnification for signs of cell division.

At the end of the test period (August 15, 1997), when most female fish had spawned at least twice, all fish were euthanized and chemically analyzed to verify body burdens in individual females and males. The U.S. Environmental Protection Agency Region IV Environmental Services Division (Athens, GA) conducted chemical analysis of dosed adult fish, eggs, and food using the same chemical analysis methods described above. Samples dosed with Aroclor 1268 and fish from control treatments were analyzed for lipid content to help interpret Aroclor 1268 concentrations.

Effects on F_1 offspring of exposed *F. heteroclitus*

After hatching, fish were held in 5-L polystyrene containers of aerated water with a salinity of 20‰, a temperature of 22°C, and a 16:8 h light:dark photoperiod. Until they were large enough to consume pelletized food, larvae were fed brine shrimp and water was changed daily. Later, the water was filtered and gently aerated and approximately one half of the volume in each tank was replaced twice each week. When juvenile fish outgrew 5-L tanks, they were transferred to 10-gallon aquaria. Fish were fed once each day with uncontaminated commercially available fish food. Temperature was monitored periodically in one replicate of each treatment group throughout the study period. Holding water salinity was verified to be within 2‰ of the target salinity before it was added to any tank. Tank filters were cleaned weekly and debris was siphoned from each tank daily. Dead larvae were preserved in 10% neutral buffered formalin.

Hatch success was determined as the percentage of hatched eggs of the total that were fertilized. Cumulative larval survival was determined according to the equation below. Larvae with obvious spinal or other severe deformities were considered nonviable for calculation of larval survival.

$$\text{cumulative larval survival} = \frac{(\text{No. live larvae}) - (\text{No. deformed larvae})}{\text{No. fertile eggs}} \times 100$$

This value provides an indication of the number or proportion of fertilized eggs that may be expected to survive to become juvenile fish.

About six months after fertilization, fish were examined periodically for signs that they were reaching sexual maturity. Examination and spawning methods were the same as those

described above. Female fish were crossed only with male fish from the same tank, which resulted in full-sibling crosses of the offspring of fish exposed to contaminated food. The first successful spawning occurred on July 13, 1998, and the last occurred on October 6, 1998.

Total length of F_1 offspring was measured as an indicator of growth at the end of the test. Sex and gonadal abnormalities were evaluated by gross examination of gonads under 40-power magnification. Gross anatomical structures in the gonad (the presence of ovarian lamellae and visible ovarian follicles in females, and the absence of these structures in males) were used to determine the sex of the fish. Gonadal abnormalities were determined through gross morphologic examination.

Effects on F_2 generation *F. heteroclitus*

Eggs from each breeding pair established were maintained as described above. The test was ended 3 d after the last egg hatched from each group to evaluate larval survival through yolk resorption.

Data analysis

To evaluate the results of all experiments, control and solvent treatments were compared using the nonparametric Mann–Whitney U test (Statview, Abacus Concepts, Berkeley, CA, USA) and combined for further analysis if means were not statistically significantly different. Hatch success, larval survival, juvenile growth, sex ratios (percent males), fecundity, and fertilization success between groups were compared using the Kruskal–Wallis test (Statview). Gonadal abnormalities and sex ratios for each treatment were compared using a chi-square test. Percent values were arcsin square-root transformed before analysis to stabilize variance [23,24]. In order to examine whether effects magnified between generations, t tests were used to compare results from one generation to the next. Significance values of 0.05 or less were considered to be significant.

RESULTS

Adult F. heteroclitus contaminant exposure and observation

Water quality parameters remained within acceptable limits throughout the duration of the test. Quality assurance and quality control analyses met control limits established by the individual chemical analysis laboratories. No contaminants were found in blank samples, surrogate recoveries for PCBs ranged from 48 to 123%, and spiked sample recoveries ranged from 76 to 200%. Analysis of standard reference materials for mercury indicated between 94 and 102% of certified concentrations. Spiked sample recoveries ranged from 97 to 114% and results of duplicate samples were within 14% of each other (relative percent difference).

Fundulus heteroclitus treated with mercury or PCBs accumulated whole-body concentrations in proportion to the dose received (Tables 1 and 2). No significant differences were found between control treatments in whole-body concentrations ($p > 0.07$), so controls were combined for further analyses. The highest mercury tissue concentration was different than control treatments. Aroclor 1268 concentrations in the two highest PCB treatments were elevated when compared to control samples.

Males treated with mercury seemed to be more aggressive than control males or males treated with Aroclor 1268. Male fish exposed to mercury were darker in color and exhibited either very aggressive behavior (chasing and biting other fish)

Table 1. Results of mercury exposure in *Fundulus heteroclitus*. Statistical comparisons were made to combined controls. Fecundity includes fish that did not spawn. Numbers represent mean \pm 1 standard deviation

Treatment	Control	Solvent	0.2 $\mu\text{g/g}$	0.5 $\mu\text{g/g}$	1 $\mu\text{g/g}$	11 $\mu\text{g/g}$	p value
Food ($\mu\text{g/g}$)	0.07 \pm NA ^a (n = 1)	0.06 \pm NA (n = 1)	0.50 \pm NA (n = 1)	1.9 \pm NA (n = 1)	5.6 \pm NA (n = 1)	54 \pm NA (n = 1)	
Female ($\mu\text{g/g}$)	0.08 \pm 0.02 (n = 10)	0.07 \pm 0.02 (n = 10)	0.21 \pm 0.05 (n = 11)	0.44 \pm 0.11 (n = 13)	1.1 \pm 0.17 (n = 11)	12 ^b \pm 3.7 (n = 10)	<0.0001
Eggs ($\mu\text{g/g}$)	<0.02 \pm NA (n = 1)	<0.02 \pm NA (n = 1)	<0.02 \pm NA (n = 1)	<0.02 \pm NA (n = 1)	0.01 \pm NA (n = 1)	0.63 \pm NA (n = 1)	
Male ($\mu\text{g/g}$)	0.06 \pm 0.01 (n = 9)	0.05 \pm 0.01 (n = 11)	0.20 \pm 0.05 (n = 10)	0.47 \pm 0.10 (n = 11)	1.0 ^b \pm 0.38 (n = 10)	11 ^b \pm 3.0 (n = 7)	<0.0001
Female survival (%)	91.7 \pm NA (n = 12)	100 \pm NA (n = 12)	60.0 \pm NA (n = 15)	100 \pm NA (n = 15)	100 \pm NA (n = 15)	93.3 \pm NA (n = 15)	0.009
Male survival (%)	100 \pm NA (n = 12)	91.7 \pm NA (n = 12)	86.7 \pm NA (n = 15)	52.3 ^b \pm NA (n = 15)	73.3 ^b \pm NA (n = 15)	60.0 ^b \pm NA (n = 15)	0.025
Weight (g) ^c	11.9 \pm 5.2 (n = 21)	10.9 \pm 4.5 (n = 24)	11.0 \pm 3.4 (n = 21)	11.9 \pm 3.2 (n = 23)	13.1 \pm 5.1 (n = 24)	11.4 \pm 4.9 (n = 22)	0.46
Fecundity (N)	87 \pm 72 (n = 12)	133 \pm 82 (n = 13)	88 \pm 40 (n = 12)	130 \pm 69 (n = 13)	142 \pm 86 (n = 13)	61 \pm 51 (n = 14)	0.059
Fertilization success (%)	57 \pm 32 (n = 10)	58 \pm 35 (n = 13)	76 \pm 27 (n = 12)	77 \pm 17 (n = 13)	63 \pm 35 (n = 14)	74 \pm 28 (n = 12)	0.22

^a NA = not analyzed.

^b $p < 0.05$.

^c Males and females combined.

or very submissive behavior (hiding behind filters or air lines and remaining very still). No differences in behavior of female fish were observed among treatments.

Mortality of adult fish was recorded and results are presented in Table 1 (mercury) and Table 2 (PCBs). Males exposed to the higher concentration of mercury in their diet had significantly higher mortality than controls, although differences were not significant when female and male mortality was combined.

Weights of control and solvent-treated fish were not significantly different from each other ($p = 0.64$), so these treatments were combined for further comparison to other groups. Male and female fish treated with mercury had similar final weights as control fish (Tables 1 and 2). No significant differences were observed between weights of PCB-treated fish and combined controls.

Control and solvent treatments were not statistically different from each other in fecundity ($p = 0.08$) or fertilization success ($p = 0.82$) and therefore were combined for further statistical analysis. Fecundity was highly variable, with some fish in many treatments (including the control treatment) producing no eggs and some fish producing in excess of 200 eggs. Fecundity was examined in two ways, by including fish that produced no eggs, and by excluding fish that did not spawn, because the lack of spawning could be an effect of contaminant exposure. Although not statistically significant, female fish exposed to the highest concentration of mercury produced fewer eggs than did females in other treatments both when including all females ($p = 0.059$) and when considering only fish that spawned (Table 1). Fish exposed to PCBs exhibited no difference in fecundity when compared to controls (for all females, $p = 0.66$ and for spawning fish only; Table 2). Fertilization success did not differ between combined controls and mercury- or PCB-treated groups. Other researchers have achieved fertilization success rates for *F. heteroclitus* of 63 to 95% [22,25].

Effects on offspring of exposed *F. heteroclitus*

Hatch success and larval survival of offspring of *F. heteroclitus* in control and solvent treatments were not significantly different from each other ($p > 0.48$) and were combined for further analysis. Offspring of mercury- or PCB-treated fish did not differ in hatch success or larval survival from combined controls (Tables 3 and 4).

Mean weights of offspring of control and solvent-treated fish were not significantly different ($p = 0.06$), so controls were combined for further statistical analysis. Weights of offspring of mercury-treated fish were significantly different than combined controls, with the medium and moderate mercury treatments having higher mean weights than those of other groups. Offspring of the three groups of fish treated with the highest concentrations of PCBs had significantly higher weights than combined controls.

When the percentage of male fish in spawning groups was compared using nonparametric analysis of variance, no significant differences were found between control and solvent treatments ($p = 0.2$), so these treatments were combined for further statistical analysis. Neither methylmercury treatments nor PCB treatments differed from combined control treatments in the percentage of male offspring produced (Tables 3 and 4). However, when overall sex ratios were compared using a chi-square test (by combining all fish in each group) the higher mercury treatments had a different sex ratio than expected (p

Table 2. Results of Aroclor 1268 exposure in *Fundulus heteroclitus*. Statistical comparisons were made to combined controls. Fecundity includes fish that did not spawn. Numbers represent mean \pm 1 standard deviation

Treatment	Control	Solvent	0.4 $\mu\text{g/g}$	2 $\mu\text{g/g}$	4 $\mu\text{g/g}$	15 $\mu\text{g/g}$	p value
Food ($\mu\text{g/g}$)	<0.010 \pm NA ^a (n = 1)	<0.013 \pm NA (n = 1)	0.71 \pm NA (n = 1)	3.3 \pm NA (n = 1)	7.5 \pm NA (n = 1)	32 \pm NA (n = 1)	
Female ($\mu\text{g/g}$)	0.01 \pm 0.002 (n = 10)	0.01 \pm 0.002 (n = 10)	0.34 (median) (n = 9)	1.3 \pm 0.63 (n = 10)	3.3 ^b \pm 1.6 (n = 10)	14 ^b \pm 6.8 (n = 9)	<0.0001
Eggs ($\mu\text{g/g}$)	NA	<0.047 \pm NA (n = 1)	<0.037 \pm NA (n = 1)	0.044 ^c \pm NA (n = 1)	0.071 ^c \pm NA (n = 1)	1.3 \pm NA (n = 1)	
Male ($\mu\text{g/g}$)	0.01 \pm 0.007 (n = 9)	<0.02 \pm 0.01 (n = 11)	0.42 \pm 0.008 (n = 9)	2.0 (median) (n = 10)	4.5 ^b \pm 1.4 (n = 10)	15 ^b \pm 6.2 (n = 9)	<0.0001
Female survival (%)	91.7 \pm NA (n = 12)	100 \pm NA (n = 12)	100 \pm NA (n = 15)	86.7 \pm NA (n = 15)	100 \pm NA (n = 15)	100 \pm NA (n = 15)	0.22
Male survival (%)	100 \pm NA (n = 12)	91.7 \pm NA (n = 12)	100 \pm NA (n = 15)	93.3 \pm NA (n = 15)	66.7 \pm NA (n = 15)	86.7 \pm NA (n = 15)	0.04
Weight (g) ^d	11.9 \pm 5.2 (n = 21)	10.9 \pm 4.5 (n = 24)	11.0 \pm 3.2 (n = 20)	15.4 \pm 7.3 (n = 22)	14.0 \pm 7.9 (n = 24)	12.4 \pm 5.3 (n = 19)	0.16
Fecundity (N)	87 \pm 72 (n = 12)	133 \pm 82 (n = 13)	108 \pm 99 (n = 11)	109 \pm 86 (n = 14)	161 \pm 224 (n = 12)	77 \pm 62 (n = 11)	0.66
Fertilization success (%)	57 \pm 32 (n = 10)	58 \pm 35 (n = 13)	70 \pm 34 (n = 8)	67 \pm 33 (n = 11)	65 \pm 29 (n = 13)	41 \pm 37 (n = 9)	0.57

^a NA = not analyzed.
^b p < 0.05.
^c Value is an estimate.
^d Males and females combined.

= 0.0004). The highest treatment had more females than expected (62%) and the moderate group had fewer females than expected (32%).

Gonadal abnormalities were compared in a subset of offspring from each treatment group using a chi-square test. No males were observed with gross gonadal abnormalities. Up to 17% of females examined had grossly abnormal gonads (with no observable follicles), but no statistically significant differences were found in the prevalence of abnormal female gonads between groups (Tables 3 and 4).

Control and solvent treatments were statistically different from each other in fecundity because of a large number of control fish that did not spawn (p = 0.009; Table 3). Therefore, control treatments were not combined for further analysis. The number of fish that did not spawn was compared between treatment groups and was not statistically significantly different (p > 0.05). Mean fecundity (including fish that did not spawn) ranged from 16 to 48. Significant differences in fecundity were found among the mercury- and PCB-treated groups, but with such a difference between the control (16) and solvent groups (37), results are difficult to interpret and differences may not be related to contaminant exposure.

Fertilization success did not differ between control and solvent treatments (p = 0.15), so these treatments were combined for further statistical analysis. Fertilization success was significantly lower in the offspring of fish treated with the highest concentrations of mercury (Table 3) as compared with the other treatments. Significant differences also were found between control groups and PCB groups in fertilization success (Table 4) with the lowest PCB treatment exhibiting lower fertilization success than controls and the moderate group exhibiting higher fertilization success than controls.

In order to examine whether fecundity or fertilization success differed between the F₀ and F₁ generations, the mean values for combined controls were compared using an unpaired t test. Fecundity was significantly less in the F₁ generation than in the F₀ generation (p < 0.0001), so test groups were not compared further for this endpoint. Reductions in fecundity in the F₁ generation may be a result of the shorter maturation time for this generation. However, fertilization success in combined control groups did not differ between generations. For fish exposed to mercury, fertilization success was significantly lower in the F₁ generation for all treatments except the group exposed to the moderate concentration. For fish exposed to PCBs, only exposure to the lowest concentration reduced fertilization success in the F₁ generation below that of the F₀ generation (p = 0.004).

Effects on F₂ generation *F. heteroclitus*

In the evaluation of effects on the second generation of offspring of *F. heteroclitus* exposed to mercury or PCBs, no difference was found between hatch success or larval survival in control and solvent treatments (p > 0.15), so these treatments were combined for further statistical analysis. Neither mercury nor PCBs affected hatch success or larval survival in this generation of fish (Tables 3 and 4).

Mean values of hatch success and larval survival were compared for individual treatment groups between the F₁ and F₂ generations. Hatch success was greater in combined controls from the F₂ generation when compared to the F₁ generation (p = 0.04), so this endpoint was not examined further. However, larval survival did not differ in control groups between generations. None of the groups exposed to mercury differed

Table 3. Effects of mercury exposure on later generations of *Fundulus heteroclitus*. Statistical comparisons were made to combined controls. Larval survival as of 3 d after last hatch. Numbers represent mean \pm 1 standard deviation

Treatment	Control	Solvent	0.2 $\mu\text{g/g}$	0.5 $\mu\text{g/g}$	1 $\mu\text{g/g}$	11 $\mu\text{g/g}$	p value
F ₁ hatch success (%)	59 \pm 27 (n = 9)	48 \pm 32 (n = 13)	70 \pm 22 (n = 12)	58 \pm 27 (n = 13)	45 \pm 29 (n = 14)	71 \pm 16 (n = 12)	0.09
F ₁ larval survival (%)	56 \pm 25 (n = 9)	46 \pm 31 (n = 13)	67 \pm 21 (n = 12)	51 \pm 25 (n = 13)	42 \pm 27 (n = 14)	64 \pm 19 (n = 12)	0.12
F ₁ juvenile weight (g)	1.84 \pm 0.94 (n = 78)	2.01 \pm 0.80 (n = 51)	1.84 \pm 0.83 (n = 74)	2.50 ^a \pm 0.83 (n = 56)	2.81 ^a \pm 1.2 (n = 30)	1.85 \pm 0.81 (n = 76)	<0.0001
F ₁ sex ratios ^b	1.44 \pm 1.02 (n = 6)	0.80 \pm 0.48 (n = 6)	1.59 \pm 0.68 (n = 6)	1.17 \pm 0.85 (n = 6)	1.90 ^a \pm 3.97 (n = 6)	1.96 ^a \pm 1.26 (n = 6)	0.0004
F ₁ females with abnormal gonads (%)	0 \pm NA ^c (n = 10)	8.3 \pm NA (n = 12)	0 \pm NA (n = 6)	13.3 \pm NA (n = 15)	0 \pm NA (n = 3)	0 \pm NA (n = 7)	0.64
F ₁ males with abnormal gonads (%)	0 \pm NA (n = 6)	0 \pm NA (n = 3)	0 \pm NA (n = 5)	0 \pm NA (n = 8)	0 \pm NA (n = 7)	0 \pm NA (n = 12)	
F ₁ fecundity (M)	16 \pm 26 (n = 57)	37 \pm 44 (n = 29)	33 \pm 46 (n = 50)	18 \pm 23 (n = 39)	48 \pm 68 (n = 21)	33 \pm 45 (n = 54)	0.04
F ₁ fertilization success (%)	47.8 \pm 29.3 (n = 27)	37.7 \pm 28.2 (n = 21)	35.0 \pm 28.4 (n = 32)	52.2 \pm 32.5 (n = 21)	43.1 \pm 38.3 (n = 15)	28.2 ^a \pm 25.7 (n = 33)	0.01
F ₂ hatch success (%)	60.3 \pm 23.7 (n = 26)	70.2 \pm 18.2 (n = 18)	65.4 \pm 25.8 (n = 28)	65.9 \pm 20.1 (n = 18)	61.2 \pm 32.2 (n = 12)	60.9 \pm 25.9 (n = 25)	0.96
F ₂ larval survival (%)	36.3 \pm 27.4 (n = 26)	35.0 \pm 24.1 (n = 18)	34.7 \pm 27.6 (n = 28)	41.8 \pm 27.7 (n = 18)	34.0 \pm 27.3 (n = 12)	39.6 \pm 30.0 (n = 25)	0.91

^a $p < 0.05$.

^b Females: males.

^c NA = not analyzed.

between generations in larval survival. However, the groups exposed to the two highest concentrations of PCBs had significantly lower larval survival in the F₂ generation than in the F₁ generation ($p < 0.006$).

DISCUSSION

This study sought to determine whether fish exposed to PCBs or mercury (and subsequent generations) can survive, develop normally, and reproduce successfully. A further goal was to identify any apparent tissue threshold concentrations for observed effects.

Fish were exposed to contaminants via food in order to achieve environmentally relevant tissue concentrations. The tissue concentrations achieved here were similar to those found at a hazardous waste site in Georgia, where whole-body *F. heteroclitus* contained as high as 1 $\mu\text{g/g}$ mercury and 65 $\mu\text{g/g}$ Aroclor 1268 [26]. Food concentrations used in this study were higher than those found in the environment. At the same waste site, fiddler crabs contained approximately 0.9 $\mu\text{g/g}$ mercury and 15 $\mu\text{g/g}$ Aroclor 1268 [26].

Although both contaminants are known to affect reproduction, they do so via different mechanisms. Mercury is primarily known as a neurotoxin, because brain cells seem to be particularly sensitive to its effects. Methylmercury binds tightly to sulfhydryl groups of proteins and can disrupt protein synthesis throughout the body [27]. Mercury disrupts thyroid and pituitary function [28], lipid metabolism, and production of steroid hormones [28]. Exposure to mercury disrupts cellular ion regulation and enzyme activity [27], and can cause a shift to anaerobic metabolism [29].

Polychlorinated biphenyls induce the cytochrome P450 enzyme system, which metabolizes xenobiotic and other compounds. The PCBs can also affect metabolism through inhibiting cellular sequestration of Ca²⁺ [30] and by inhibiting the action of functional groups on proteins in liver cells [31]. Some PCBs also bind to estrogen receptors [12].

In this study, PCBs were highly bioavailable to fish and accumulated in tissues in proportion to exposure concentrations. Exposure to PCBs via food resulted in increased growth in offspring. Numbers of fish in each treatment tank were similar, so this is unlikely to be a density-dependent effect. Increased growth in offspring may represent a stimulatory effect in response to the stress of low levels of contaminants. This effect has been noted in other studies. For example, Sheffrin et al. [32] theorized that increased growth in larval fathead minnows exposed to low levels of hexavalent chromium or *p*-cresol was due to increased cellular metabolism as a stress response. In that study, larvae had reduced DNA content but increased cell volume and total protein content.

Increased growth of offspring was observed when whole-body concentrations of parent fish were between 0.34 and 2.0 $\mu\text{g/g}$, and when eggs contained 0.044 $\mu\text{g/g}$. Applying egg concentrations as tissue thresholds for effects observed in offspring may be more appropriate, although it was not possible from this study to distinguish whether effects on *F. heteroclitus* were due to accumulations in male or female parents (or both), or whether observed effects were due to accumulations in eggs.

The only other significant effect of PCBs noted in this study was a decrease in larval survival between the F₁ and F₂ generations in the two groups treated with the highest concentrations of PCBs. Although larval survival did not differ from that of control groups, the combination of stressors (exposure

Table 4. Effects of Aroclor 1268 exposure on later generations of *Fundulus heteroclitus*. Statistical comparisons were made to combined controls. Larval survival as of 3 d after last hatch. Numbers represent mean \pm 1 standard deviation

Treatment	Control	Solvent	0.4 $\mu\text{g/g}$	2 $\mu\text{g/g}$	4 $\mu\text{g/g}$	15 $\mu\text{g/g}$	p value
F ₁ hatch success (%)	59 \pm 27 (n = 9)	48 \pm 32 (n = 13)	60 \pm 35 (n = 8)	57 \pm 33 (n = 10)	77 \pm 15 (n = 13)	72 \pm 29 (n = 8)	0.13
F ₁ larval survival (%)	56 \pm 25 (n = 9)	46 \pm 31 (n = 13)	49 \pm 33 (n = 8)	52 \pm 30 (n = 10)	65 \pm 22 (n = 13)	69 \pm 28 (n = 8)	0.3
F ₁ juvenile weight (g)	1.84 \pm 0.94 (n = 78)	2.01 \pm 0.80 (n = 51)	1.87 \pm 0.60 (n = 42)	2.83 ^a \pm 1.4 (n = 68)	2.39 ^a \pm 0.84 (n = 68)	2.39 ^a \pm 1.1 (n = 28)	<0.0001
F ₁ sex ratios ^b	1.44 \pm 1.02 (n = 6)	0.82 \pm 0.48 (n = 6)	0.86 \pm 0.49 (n = 4)	0.97 \pm 0.5 (n = 6)	1.25 \pm 1.23 (n = 6)	1.5 \pm 0.3 (n = 3)	0.53
F ₁ females with abnormal gonads (%)	0 \pm NA ^c (n = 10)	8.3 \pm NA (n = 12)		16.7 \pm NA (n = 6)	8.3 \pm NA (n = 24)	9.1 \pm NA (n = 11)	0.83
F ₁ males with abnormal gonads (%)	0 \pm NA (n = 6)	0 \pm NA (n = 3)	0 \pm NA (n = 2)	0 \pm NA (n = 7)	0 \pm NA (n = 11)	0 \pm NA (n = 13)	
F ₁ fecundity (N)	16 \pm 26 (n = 57)	37 \pm 44 (n = 29)	41 \pm 48 (n = 24)	23 \pm 37 (n = 43)	20 \pm 25 (n = 46)	24 \pm 23 (n = 19)	0.41
F ₁ fertilization success (%)	47.8 \pm 29.3 (n = 27)	37.7 \pm 28.2 (n = 21)	16.5 \pm 26.5 (n = 16)	46.5 \pm 40.7 (n = 29)	63.6 \pm 27.9 (n = 27)	56.8 \pm 26.8 (n = 19)	0.0003
F ₂ hatch success (%)	60.3 \pm 23.7 (n = 26)	70.2 \pm 18.2 (n = 18)	72.8 \pm 28.4 (n = 12)	58.1 \pm 31.2 (n = 21)	61.4 \pm 24.0 (n = 26)	58.0 \pm 23.0 (n = 13)	0.65
F ₂ larval survival (%)	36.3 \pm 27.4 (n = 26)	35.0 \pm 24.1 (n = 18)	55.3 \pm 35.7 (n = 12)	44.1 \pm 30.9 (n = 21)	27.4 \pm 22.8 (n = 26)	26.1 \pm 18.1 (n = 13)	0.22

^a p < 0.05.
^b Females: males.
^c NA = not analyzed.

Table 5. Aroclor 1268 tissue effects thresholds in *Fundulus heteroclitus*

	Aroclor 1268 ($\mu\text{g/g}$)				
F ₀ whole body ($\mu\text{g/g}$)	0.01–0.02	0.34–0.42	1.3–2.0	3.3–4.5	14–15
F ₁ egg ($\mu\text{g/g}$)	<0.047	<0.037	0.044	0.071	1.3
F ₀ effects					
Survival					
Weight					
Fecundity					
Fertilization success					
F ₁ effects					
Hatch success					
Larval survival					
Weight			×	×	×
Sex ratios					
Abnormal gonads					
Fecundity					
Fertilization success					
F ₂ effects					
Hatch success					
Larval survival					

to PCBs and spawning in the laboratory) possibly resulted in a significant effect. This suggests that effects may magnify in later generations. The PCB tissue effects thresholds from this study are summarized in Table 5.

Mercury was accumulated in an exposure concentration-dependent fashion after exposure through food. Exposure to mercury increased mortality in male *F. heteroclitus*. Mercury exposure seemed to cause an increase in aggression in some fish, and increased lethargy in other fish. The more lethargic fish in mercury-treated tanks did not survive. Some aggression was also noted in males from control tanks as fish sexually matured. Mortality associated with mercury exposure was not observed in female fish. Although this study was not designed to quantify behavioral effects, analysis of results of past studies suggests that the reduced male survival observed in this study likely was due to neurologic effects. Mercury exposure is known to disrupt neural function and a wide range of behavioral changes have been reported. For example, Rodgers and Beamish [33] noticed that 20 to 30% of rainbow trout fed 75 $\mu\text{g/g}$ methylmercury were darker and more lethargic than other fish in the same tank and than control fish. The darker fish would sometimes lose swimming ability and drift with the current in the tanks.

Increased male mortality was observed in this study at whole-body concentrations between 0.2 and 0.47 $\mu\text{g/g}$. Other surveys have indicated that tissue concentrations of 4 to 16 $\mu\text{g/g}$ in whole body of rainbow trout or brook trout were associated with behavioral effects [33–35].

Mercury exposure also may have decreased fecundity in this study but this difference was not statistically significant. Only a few references to the potential effects of mercury exposure on fecundity exist in the literature. Olsen [36] suggested that high levels of mercury in tissues might be responsible for low fecundity in school shark (*Galeorhinus australis*) near Australia. Female catfish exposed to mercury 180 d before spawning had more gonadal recrudescence, more postvitellogenic eggs, and impaired gametogenesis with more nonyolk eggs [37].

Offspring of *F. heteroclitus* fed mercury-contaminated food were less able to reproduce successfully than were control

Table 6. Mercury tissue effects thresholds in *Fundulus heteroclitus*

	Methylmercury ($\mu\text{g/g}$)				
	0.05–0.08	0.20–0.21	0.44–0.47	1.0–1.1	11–12
F_0 whole body ($\mu\text{g/g}$)	0.05–0.08	0.20–0.21	0.44–0.47	1.0–1.1	11–12
F_1 egg ($\mu\text{g/g}$)	<0.02	<0.02	<0.02	0.01	0.63
F_0 effects					
Survival			×	×	×
Weight					
Fecundity					
Fertilization success					
F_1 effects					
Hatch success					
Larval survival					
Weight			×	×	
Sex ratios				×	×
Abnormal gonads					
Fecundity					
Fertilization success					×
F_2 effects					
Hatch success					
Larval survival					

fish. Significantly lower fertilization success occurred in the offspring of fish treated with mercury, and fertilization success was significantly lower in the F_1 generation than in the F_0 generation. This suggests that the effect of mercury exposure may become more severe in later generations. Mercury exposure has inhibited fertilization success in fish in past studies [25,38]. The mechanisms for this were thought to include disruption of the micropyle of the egg or abnormalities in sperm structure or activity [25,38].

In this study, reduced fertilization success was observed at egg concentrations of 0.01 to 0.63 $\mu\text{g/g}$. Other studies have suggested that egg concentrations of mercury of 0.5 $\mu\text{g/g}$ in rainbow trout [39] and whole-body concentrations of 1.4 $\mu\text{g/g}$ in fathead minnows [40] inhibited larval survival and reproduction.

Mercury exposure also altered sex ratios in offspring, with moderate concentrations producing fewer females and highest concentrations producing more females than expected. Altered sex ratios could be a result of effects on sexual differentiation, or a result of differential mortality between males and females during early development. Alterations in sex ratios were observed at concentrations of less than 0.01 $\mu\text{g/g}$ in eggs or between 0.44 and 1.1 $\mu\text{g/g}$ in whole bodies of parent fish.

Offspring of fish exposed to mercury also had increased growth in moderate treatments when compared to controls. Mercury could affect growth by disrupting thyroid function, which controls metabolism, or by altering metabolic processes as a result of stress. This effect was observed when egg concentrations were less than 0.02 $\mu\text{g/g}$, or when parent whole-body concentrations were 0.2 to 0.47 $\mu\text{g/g}$.

In summary, mercury exposure resulted in increased mortality in males, reductions in the ability of offspring of exposed fish to reproduce successfully, and alterations in sex ratios. Both direct effects on exposed fish and transgenerational effects were observed. Mercury tissue thresholds for effects are summarized in Table 6. This study indicates that mercury and PCBs have the potential to adversely affect fish reproduction, and that adverse effects can carry forward to future generations. Relatively low tissue concentrations are associated with adverse effects.

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