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CADMIUM EXPOSURES DURING EARLY DEVELOPMENT: DO THEY LEAD TO REPRODUCTIVE IMPAIRMENT IN FATHEAD MINNOWS?

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Abstract

The primary objective of this study was to determine whether cadmium (Cd) exposures during embryonic and larval development alter the reproductive performance, reproductive physiology, and sex ratio of fathead minnows (*Pimephales promelas*). Embryonic exposures were conducted by exposing adult female fathead minnows to 0, 25, or 100 µg/L Cd for 8 d prior to breeding. Larval exposures were conducted by exposing the larvae to waterborne Cd at 0, 12.5, 25, or 50 µg/L from 8 to 29 d posthatch (the time period associated with female sexual differentiation). Minnows from each exposure period were raised to maturity, at which time their reproductive success, secondary sexual characteristics, gonadosomatic index (GSI), plasma 11-ketotestosterone (11-KT) concentrations, and sex ratios were assessed. Results from the embryonic study reveal that Cd exposures alter the secondary sexual characteristics of male fathead minnows but do not alter reproductive performance, GSI, 11-KT concentrations, or sex ratios. Larval exposures, during the period of female sexual differentiation, significantly reduce the frequency of adult spawning and increase clutch size but do not alter fecundity, secondary sexual characteristics, GSI, or 11-KT. Subtle alterations in sex ratio were observed, indicating that larval Cd exposures may increase the proportion of females in an exposed population.

Keywords

Cadmium; Fathead minnow; Reproduction; Sex ratio; Early development

INTRODUCTION

The majority of studies examining the effects of cadmium (Cd) on fish have typically focused on Cd toxicity and disruption of calcium ionoregulation. Cadmium toxicity, as measured by lethality, has been well documented in freshwater fishes [1–3]. Furthermore, the primary mechanism of toxicity—disruption of calcium ionoregulation—has also been well established [4–7]. While several studies have clearly demonstrated the effects of exposures to lethal concentrations of Cd, fewer studies have investigated the effects of sublethal Cd exposures. Evidence exists that, at sublethal concentrations, Cd may act as a reproductive toxin and endocrine disruptor.

Cadmium has been shown to be responsible for a number of reproductive abnormalities in fish. For example, Das [8] found that Cd reduced the gonadosomatic index (GSI) of both male and

female Asian cyprinids (*Labeo bata*). Furthermore, spermatids and spermatozoa were absent in the testes of Cd-exposed males. Likewise, the ovaries of exposed females contained no mature oocytes and had a significantly higher proportion of atretic follicles relative to ovaries from unexposed females. Cadmium has also been shown to induce the production of vitellogenin in Atlantic croaker (*Micropogonias undulatus*) [9]. In addition, both male and female Japanese medaka (*Oryzias latipes*) exposed to Cd experience a significant reduction in gonadal steroid release relative to controls [10].

The ability of Cd to alter reproductive health may be, in part, related to its ability to activate both androgen and estrogen receptors. Several in vitro studies have shown that Cd has estrogenic and androgenic properties. For example, Cd stimulates the growth of MCF-7 cells, an estrogen-responsive breast cancer cell line [11], and binds to the estrogen receptor, noncompetitively blocking the binding of estradiol [12]. Using an estrogen receptor-dependent transcriptional expression assay and E-screen assay, Choe et al. [13] found Cd to be highly estrogenic. In addition, Cd activates the androgen receptor and blocks the binding of androgen to the receptor in studies conducted using mammalian androgen receptors [14].

Several studies have shown that exposure to endocrine active compounds during early developmental stages can cause organizational effects in the structure of gonadal tissue [15, 16]. Furthermore, it has been demonstrated that exposures to estrogenic or androgenic compounds during early life stages disrupt sexual differentiation, ultimately leading to altered sex ratios in the adult populations. For example, fathead minnow populations exposed 4.0 ng/L 17 α -ethinylestradiol (EE₂) from fertilization to 56-d-old had a female-to-male sex ratio of 84: 5 with ovotestes occurring in 11% of fish [17]. Likewise, Parrott and Blunt [18] found that exposure to EE₂ concentrations above 3.5 ng/L resulted in complete feminization of fathead minnow populations, whereas van Aerle et al. [19] found the presence of ovarian-like cavities in 64.3% of males exposed to EE₂ from 10 to 15 d old. In contrast, early life history exposures to various androgens produce predominantly male populations. For example, exposures to either 17 α -methyltestosterone or trenbolone acetate from 40 to 45 d old yield all male populations of black crappie (*Pomoxis nigromaculatus*) [20].

The primary goal of this study was to determine the consequences of Cd exposures during early life stages on various reproductive parameters in fathead minnows. The specific objective of this study was to determine whether embryonic and larval Cd exposures impair reproductive success, alter sex specific characteristics, or skew the sex ratio of the exposed population.

MATERIALS AND METHODS

Fish maintenance

All the fish used in these experiments were produced by the University of Nebraska at Omaha fathead minnow colony according to the protocols outlined in Peake et al. [21]. Once the larvae hatched, they were transferred via pipette from 1-L hatching chambers to 2-L beakers containing 1.8 L of water. Each beaker contained 75 larvae to maintain a density of approximately 41 larvae/L. This density was justified based on U.S. Environmental Protection Agency guidelines suggesting a maximum density of 150 larvae/L for larvae up to 28 d old [22]. After three weeks, fish were transferred to 40-L aquaria containing 30 L of water. After five weeks, the fish were gradually switched from their larval diet (1-d-old *Artemia* nauplii twice daily) to high-protein flake food (Aquatic Ecosystems, Apopka, FL, USA). The density of fish in each 40-L tank was gradually reduced to between 15 and 20 fish over the time preceding initiation of the breeding studies.

Throughout all the experiments, minnows were maintained in aerated, dechlorinated, heated (26°C) tap water and were kept under a photoperiod of 16:8 h light:dark. Static renewal of

water in the beakers or aquaria occurred daily. Renewal water had the following water quality (mean $\pm$ standard error): pH, 7.2 ± 0.4 ; conductivity, 628 ± 9 μ S; hardness (expressed as mg/L CaCO_3), 173 ± 4 ; alkalinity (expressed as mg/L CaCO_3), 98 ± 4 and Ca, 38.8 ± 1.2 mg/L.

Embryonic exposures

Embryonic exposures occurred by way of maternal transfer in which Cd-exposed females transfer Cd to their offspring in a dose-dependent fashion (see *Results* section). Figure 1 shows the experimental design for minnows in the embryonic exposure experiment. Adult females were exposed to 0, 25, 50, or 100 μ g/L Cd for 8 d. Following the exposure, individual females were paired with unexposed males. Two pairs were maintained in each 40-L aquaria and were separated by a plastic divider. Pairs were provided with a breeding substrate (12-cm-long sections of polyvinyl chloride tubing 8 cm in diameter, split in half lengthwise) on which eggs were laid. Breeding substrates with adhered eggs were removed daily and placed in a 1-L beaker maintained in a warm-water bath at 26°C. Unfertilized and fungused eggs were removed daily and the eggs hatched in 3 to 4 d. On hatch, larvae were maintained according to the fish maintenance guidelines outlined previously.

Larval whole-body Cd concentrations

To ensure that the maternal transfer of Cd from female to offspring occurred, a subset of 8-d old larvae from each maternal exposure dose were sacrificed for determination of whole-body Cd concentrations. Individual larvae were digested in concentrated nitric acid [23] and diluted with nanopure water. Larvae were analyzed for Cd using graphite furnace atomic absorption spectrometry.

Larval exposures

Figure 1 shows the larval exposure regimes. Larvae were subjected to 0, 12.5, 25, or 50 μ g/L Cd for 21 d beginning at 8-d old. This exposure period corresponds with the time period in which females sexually differentiate [24]. Larvae were maintained in 2-L beakers for the first 14 d of the exposure and then transferred to 40-L aquaria containing 15 L of water for the remainder of the exposure. Following the exposure, fish were transferred to clean aquaria and were maintained according to the protocol described previously.

21-d breeding assay

On reaching maturity (>4 months), the reproductive performance of minnows from the 0-, 25-, and 100- μ g/L maternal exposures and minnows from the 0-, 12.5-, 25-, and 50- μ g/L larval exposures was assessed. To accomplish this, 20 pairs of minnows from each exposure concentration were subjected to a 21-d breeding assay modified from a protocol developed by Ankley et al. [25]. Pairs were maintained in 40-L tanks heated to approximately 26°C. Each tank contained two pairs separated by a plastic divider. Spawning events were recorded daily. Spawning substrates with adhered eggs were removed from each tank every morning and placed in 1-L beakers maintained in a warm-water bath at 26°C. The number of eggs on each substrate was counted on collection. The number of fertilized eggs was determined by counting the number of eggs with eye spots at 2 to 3 d postspawn. When larvae began to hatch (~ 4 d postspawn), they were transferred to 2-L beakers. The number of hatched larvae was recorded, and the mortality of hatched larvae was monitored for 48 h posthatch.

At the termination of the 21-d breeding study, male minnows were sacrificed for plasma collection, assessment of secondary sexual characteristics, and determination of gonadal condition (GSI). Fish were anesthetized, weighed, and bled. To collect blood, the caudal peduncle was severed, and blood was collected with heparinized micropipettes that were then centrifuged to separate the plasma. Plasma was aspirated into a heparinized microcentrifuge

tube and stored on ice until processing was complete. Plasma samples were then placed in the -80°C freezer. Secondary sexual characteristics were assessed by counting the number of nuptial tubercles and measuring the intraocular distance and head width of each male. Because head width and intraocular distance are positively correlated, an intraocular distance index was calculated as the intraocular distance/head width. Intraocular distance is a measurement that has been successfully used as a male secondary sexual characteristic in fathead minnows [26]. Both male and female gonads were removed and weighed for determination of GSI.

Plasma 11-etotestosterone quantification

Plasma samples were analyzed for 11-ketotestosterone (11-KT) using a commercially available fathead minnow 11-KT enzyme-linked immunosorbent assay (Cayman Chemical, Ann Arbor, MI, USA) following the manufacturer protocol. Sample concentrations were measured using a Dynatech MR5000 microplate reader managed by the computer software BioLinx Version 2.0 (Dynatech Labs, Chantilly, VA, USA).

Determination of sex ratio

Minnows not used in the 21-d breeding study were raised to maturity so that the sex ratio of the exposed sample populations could be compared to that of the unexposed sample populations. Following euthanasia, sex was determined by direct examination of the gonads of each individual.

Cd analysis

During the experiments, a water exchange ($>30\%$ of total volume) was conducted daily. A stock solution of CdCl_2 was used to manually dose all the experimental tanks. Water samples (125 ml) were collected daily from each aquarium during the maternal exposures. Each water sample was acidified with nitric acid to a pH of <2.0 and stored at 4°C . Actual Cd concentrations were determined by flame or graphite furnace atomic absorption spectrometry according to previously established methods [27]. The actual Cd concentrations during the maternal exposures averaged $86.7\text{ }\mu\text{g/L}$ (87% of nominal) in the $100\text{-}\mu\text{g/L}$ exposure aquarium and $20.8\text{ }\mu\text{g/L}$ (83% of nominal) in the $25\text{-}\mu\text{g/L}$ exposure aquarium and were below the detectable limit of $0.025\text{ }\mu\text{g/L}$ in the $0\text{-}\mu\text{g/L}$ aquarium. The actual Cd concentrations during the female sexual differentiation exposures averaged $50.8\text{ }\mu\text{g/L}$ (102% of nominal) in the $50\text{-}\mu\text{g/L}$ exposure beakers, $32.4\text{ }\mu\text{g/L}$ (130% of nominal) in the $25\text{-}\mu\text{g/L}$ exposure aquarium, and $11.9\text{ }\mu\text{g/L}$ (99% of nominal) in the $12.5\text{-}\mu\text{g/L}$ exposure beakers and were below the detectable limit of $0.025\text{ }\mu\text{g/L}$ in the $0\text{-}\mu\text{g/L}$ beakers.

Statistical analyses

Differences in reproductive endpoints between exposure groups were tested using one-way analysis of variance (ANOVA; Statview 5.0, SAS Institute, Cary, NC, USA). When the assumption of homogeneity of variances was met, ANOVA was followed by Newman–Keuls multiple comparison tests. When the assumption was not satisfied, data were analyzed using the Welch ANOVA (JMP 5.0, SAS Institute) followed by Kruskal–Wallis multiple comparison tests. The chi-square test (with Yates correction for continuity) was used to determine whether the sex ratio of Cd-exposed groups was different from the expected sex ratio of 50:50 (male:female). Statistical significance throughout the experiments was assumed at $p \leq 0.05$.

RESULTS

Embryonic Cd exposure

Maternal transfer of Cd—The whole-body Cd concentrations of 0-d-old larvae produced by females exposed to 0, 25, 50, and $100\text{ }\mu\text{g/L}$ are shown in Figure 2. Larvae produced by

females exposed to 100 µg/L Cd have significantly higher whole-body Cd concentrations than larvae produced by females exposed to 0, 25, and 50 µg/L Cd (ANOVA, $p < 0.01$).

Reproductive success—Cadmium exposures during embryonic development did not have negative impacts on any of the reproductive parameters measured (Table 1). The average percentage of pairs spawning per day ranged from 16.8 to 20.2, but no significant differences between the groups were detected. Average clutch size was between 183.1 and 195.8 eggs per clutch, while fecundity ranged from 30.8 to 36.5 eggs per female per day. Average fertilization and hatching success of each exposure group was greater than 74 and 94%, respectively. Of the reproductive parameters measured, only offspring mortality was significantly different between the groups (ANOVA, $p = 0.015$). Specifically, the offspring of minnows maternally exposed to 25 and 100 µg/L Cd had significantly lower mortality than offspring from unexposed minnows.

Secondary sexual characteristics, GSI, and 11-KT concentrations—The intraocular distance index of male minnows maternally exposed to 25 µg/L Cd was significantly greater than that of males maternally exposed to 0 or 100 µg/L Cd (ANOVA, $p = 0.0043$); however, no significant differences in the number of breeding tubercles (Table 2) were detected. No significant differences in male GSI, female GSI, or male plasma 11-KT concentrations were detected.

Sex ratio—The sex ratio of minnows maternally exposed to 25 µg/L Cd was 60.2:39.8 and was not significantly different from the sex ratio of unexposed minnows (Table 3, $p = 0.21$). Similarly, minnows maternally exposed to 100 µg/L Cd had a sex ratio of 60:40, which was not significantly different from the sex ratio of unexposed minnows ($p = 0.25$).

Larval Cd exposure

Reproductive success—The reproductive success of minnows exposed to Cd during female sexual differentiation is shown in Table 4 and Figure 3. Sample sizes were smaller for the group exposed to 50 µg/L because it was difficult to identify male fish from this group because of a lack of obvious secondary sexual characteristics. This ultimately led to same-sex pairing in four of the pairs used in the breeding assay. Individuals that were in the same sex pairings were excluded from any additional analysis.

Significant differences in the percentage of pairs to spawn per day were detected (ANOVA, $p = 0.025$). Pairwise comparisons revealed that the percentage of pairs to spawn per day was significantly lower among minnows exposed to 50 µg/L Cd relative to minnows exposed to 0, 12.5, and 25 µg/L Cd. Approximately 17% of pairs spawned per day in the 0-, 12.5-, and 25-µg/L groups, while only 10.6% of pairs spawned per day in the 50-µg/L group. Significant differences in the average clutch size of minnows exposed during female sexual differentiation were detected (ANOVA, $p = 0.023$). Specifically, the average clutch size of minnows exposed to 50 µg/L Cd was significantly greater than that of minnows exposed to 12.5 or 25 µg/L Cd but did not differ from that of unexposed minnows. Fecundity ranged from 14.1 to 21.0 eggs per female per day; however, no significant differences between the groups were detected. Significant differences in the fertilization success of minnows exposed during female sexual differentiation were detected (Welch ANOVA, $p < 0.01$). Nonparametric multiple comparisons tests revealed that the fertilization success of minnows exposed to 12.5 mg/L was significantly lower than that of minnows exposed to 0 and 50 mg/L Cd (Table 4). No significant differences in hatching success or offspring mortality were detected. Average hatching success of each group was greater than 96%, respectively, while offspring mortality was less than 12.1%.

Secondary sexual characteristics, GSI, and 11-KT concentrations—No significant differences were detected in male secondary sexual characteristics, male GSI, female GSI, or male plasma 11-KT concentrations between minnows exposed to 0, 12.5, 25, or 50 µg/L Cd during female sexual differentiation (Table 5).

Sex ratio—The sex ratios of minnows exposed to 12.5 and 25 µg/L Cd were not significantly different from the sex ratio of unexposed minnows ($p = 0.22$ and 0.12 , respectively; Table 3). At 50 µg/L Cd, 59.4% of the exposed fish were female; however, the statistical significance of this result proved difficult to assess, as the p value was exactly 0.05.

DISCUSSION

The primary objective of this study was to determine if Cd exposures during embryonic and larval development impair reproductive success, alter sex-specific characteristics, and skew the sex ratio of the exposed population. Results of this study indicate that Cd exposures during embryonic development at the concentrations tested do not adversely affect reproductive success or alter sex ratios. However, embryonic exposures increase the intraocular distance of adult males maternally exposed to 25 µg/L Cd. Larval exposures to Cd during the period of female sexual differentiation do not cause alterations in sex-specific characteristics but significantly reduce the frequency of spawning and fertilization success.

Cd exposures during embryonic development

Maternal transfer of Cd—Results of this study show that Cd is maternally transferred from a female to her offspring (Fig. 2), as 0-d-old larvae from females exposed to 100 µg/L Cd have significantly higher whole-body Cd concentrations than larvae from unexposed females. This finding is not surprising considering that cadmium has been shown to bind to vitellogenin [28]. Furthermore, Lin et al. [29] showed that Cd-exposed female tilapia (*Oreochromis mossambicus*) maternally transfer Cd tolerance to their larvae. Specifically, larvae from Cd-exposed females had a higher tolerance to Cd than larvae produced by unexposed females. The maternal transfer of Cd ions from mother to offspring found in this study may help explain the increase in Cd tolerance found in the study by Lin et al. [29].

It appears that Cd may be maternally transferred in a dose-dependent manner. However, the lack of significance between the whole-body Cd concentrations of larvae from females exposed to 25 and 50 µg/L relative to controls does not support this. While the maternal transfer of Cd may be dose dependent, it is also possible that at lower doses (i.e., 25 and 50 µg/L) females do not transfer Cd to their larvae because it is bound by a metal-binding protein such as methallothionein. Presumably, once a threshold concentration is reached, the female would begin to maternally transfer the Cd to her offspring. Regardless of whether maternal transfer of Cd is a dose-dependent or threshold response, it remains an important mechanism by which females can transfer potentially hazardous metals to their offspring.

Reproductive success—The maternal transfer of Cd to developing embryos did not produce any negative impacts on reproductive performance. Other studies investigating the reproductive consequences of Cd exposure have yielded similar results. Foran et al. [30] found that Japanese medaka exposed to Cd in ovo did not experience impaired reproductive success relative to controls. Similarly, Tilton et al. [10] found no significant differences in the reproductive success of adult medaka exposed to Cd. However, Cd-exposed medaka did experience a significant reduction in plasma estradiol concentrations, ovarian steroid release, testicular steroid release, and male GSI. The results of these studies suggest that the reproductive performance of fish exposed to Cd as embryos is protected despite biochemical and physiological alterations of the fish.

Secondary sexual characteristics, GSI, and 11-KT concentrations—No significant differences in male GSI, female GSI, or male plasma 11-KT concentrations between the Cd-exposed and unexposed minnows were detected. Males maternally exposed to 25 µg/L Cd had an average of 16.4 tubercles, while unexposed males had an average of only 13.6; however, no significant differences in the number of breeding tubercles were detected. Males maternally exposed to 25 µg/L Cd had a significantly greater intraocular distance index than males maternally exposed to 0 or 100 µg/L Cd. An increase in the intraocular distance index suggests that Cd exposures during embryonic development may have a subtle masculinizing effect. This increase in intraocular distance may indicate that Cd has androgenic activity in vivo. This is possible considering that Cd has been shown to bind to and activate the androgen receptor [14].

Sex ratio—Results of this study show that Cd exposures during embryonic development do not alter the sex ratio of exposed populations, suggesting that embryonic exposures to Cd do not have an impact on sexual differentiation.

Summary—When all the results from the embryonic exposure experiment are considered together, it appears that Cd exposures during embryonic development do not have much of an effect on reproduction with regard to the endpoints measured in this study.

Cd exposures during female sexual differentiation

Reproductive success—Cadmium exposures during female sexual differentiation did not have a significant impact on fecundity, hatching success, or offspring mortality. Similar findings have been documented for Japanese medaka. Specifically, medaka exposed to 1, 5, or 10 µg/L Cd from 2 to 16 d old did not experience any alterations in fecundity [30]. However, in the current study the percentage of pairs spawning per day (spawning frequency), an endpoint not reported in Foran's study, was significantly lower among minnows exposed to 50 µg/L Cd relative to minnows exposed to 0, 12.5, or 25 µg/L Cd. Similarly, adult minnows exposed to Cd experience a significant reduction in spawning frequency relative to controls [31]. Brion et al. [32] also found similar results for young fish exposed to 17β-estradiol (E₂). Specifically, zebrafish exposed to E₂ from 21 to 42 d old experienced a decrease in the number of spawning events relative to controls. Brion et al. [32] hypothesized that this reduction in spawning rate is related to a disruption of mating behavior and not necessarily a result of impaired gonadal function.

Minnows exposed to 50 µg/L Cd during female sexual differentiation also produced significantly larger clutches than minnows exposed to 12.5 or 25 µg/L. This increase in clutch size is likely related to the increased length of time between spawning events. Presumably, females given a longer period between spawning events would produce an increased number of mature oocytes.

Exposure to Cd during female sexual differentiation affected the fertilization of minnows. Specifically, exposure to 12.5 mg/L Cd during female sexual differentiation caused a significant decrease in fertilization success relative to controls. This reduction in fertilization success may be due to a reduction in sperm quality or alterations in male breeding behaviors. Das [8] found that following exposure to Cd, male *Labeo bata* experienced a significant decline in the number of primary and secondary spermatogonia and primary and secondary spermatocytes relative to unexposed males. Furthermore, spermatids and spermatozoa were completely absent in the Cd-exposed males. While it is possible that the reduction in fertilization success observed in this study is due to a decline in the number and/or quality of sperm, this seems somewhat unlikely given the fact that minnows exposed to 25 and 50 µg/L Cd did not experience alterations in fertilization success relative to controls. If the number of

viable sperm decreases because of cellular injury and testicular necrosis, as suggested by Das [8], one would expect that minnows exposed to 25 and 50 $\mu\text{g/L}$ would suffer the same adverse effects as minnows exposed to 12.5 $\mu\text{g/L}$. However, this is not the case. It seems more likely that this effect is related to alterations in endocrine-mediated, male breeding behaviors. This would potentially explain why adverse effects are seen at lower doses only.

Secondary sexual characteristics, GSI, and 11-KT concentrations—No significant differences in male secondary sexual characteristics, male GSI, female GSI, or male plasma 11-KT were detected. The lack of significant differences in these parameters suggests that Cd exposures during female sexual differentiation do not directly impair gonadal function.

Sex ratio—The sex ratio of minnows exposed to 50 $\mu\text{g/L}$ Cd was 41:59 (M:F), while the sex ratio of unexposed minnows was 51:49. As stated previously, the statistical significance of this result was difficult to interpret, as the p value was exactly 0.05. Interestingly, when the 21-d breeding study began, fish from the control and 50- $\mu\text{g/L}$ Cd groups were the same age; however, it was very difficult to identify obvious males from the 50- $\mu\text{g/L}$ group for use in the breeding study. As such, four of our breeding pairs proved to contain two females. Therefore, it seems as though the population became progressively more female with increasing Cd dose and also that the males in general became more cryptic with respect to secondary sexual characteristics. Given this anecdotal evidence and the near significant skew in sex ratio, it is reasonable to suspect that Cd may be acting to feminize exposed populations. However, further studies featuring larger sample sizes are needed to definitively determine whether Cd significantly skews the sex ratio of exposed populations and to determine whether the subtle effects on sex ratio is ecologically relevant.

Summary—Exposure to Cd during female sexual differentiation has a significant impact on reproduction. One effect of Cd was a decrease in spawning frequency, along with a concomitant increase in clutch size. We hypothesize that these two factors taken together indicate an alteration in minnow behavior rather than an alteration in reproductive physiology. Furthermore, the reduction in the fertilization success of minnows exposed to 12.5 $\mu\text{g/L}$ Cd may also be indicative of behavioral changes. Fathead minnows perform complex breeding behaviors, and if Cd is altering neural function, spawning frequency and fertilization success can significantly decrease. The fact that fecundity is not altered by this reduction in spawning frequency lends further support to this hypothesis, as gonadal function (e.g., egg viability) does not appear to have been altered by Cd exposure.

Reproductive effects in these fish are interesting, particularly given the methods that were employed in selecting breeding pairs. Currently, no genotypic method is available to segregate male and female fathead minnows; therefore, the only way to proceed with the breeding studies was to choose fish on the basis of their secondary sexual characteristics. Selecting for sex in this manner was not flawless, as experimental conditions existed in which it was very difficult to identify unambiguous males. Nevertheless, for the most part, the most masculine males and the most feminine females from each experimental population were selected for use in the breeding study. While this undoubtedly biased the results of this study, selecting males and females on the basis of secondary sexual characteristics is a common practice in the literature investigating the reproductive success of fish exposed to various toxic substances [17,30,33–35]. The fact that the results of this study reveal any changes in reproductive performance is accentuated by the fact that the individuals selected for inclusion in this study were the least likely individuals from their respective populations to exhibit impaired reproductive performance. A more comprehensive, random selection of individuals for inclusion in the experiments may very well have led to additional effects on reproductive performance.

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Fig. 1.

Exposure regime for minnow in the embryonic exposure and female sexual differentiation experiments. Dashed line indicates time periods when exposures to Cd occurred, while solid line indicates time periods when minnows were maintained in uncontaminated water. GSI = gonadosomatic index; KT = ketotestosterone.

Fig. 2.

Whole-body Cd concentrations of 0-d-old larvae produced by females exposed to 0, 25, 50, and 100 $\mu\text{g/L}$ Cd. Data are expressed as mean $\pm$ standard error. Uppercase letters denote a significant difference from controls.

Fig. 3. Spawning frequency (top panel), average clutch size (middle panel), and fecundity (bottom panel) of minnows exposed to 0, 12.5, 25, and 50 $\mu\text{g/L}$ Cd during female sexual differentiation. Error bars represent positive standard error. Numbers in brackets indicate sample size. Uppercase letters denote significant differences.

Table 1

Reproductive success of minnows produced by females exposed to 0, 25, and 100 µg/L Cd. Data are expressed as mean $\pm$ standard error. Numbers in parentheses indicate sample size. Uppercase letters denote significant differences

Reproductive parameter	Maternal exposure concn.		
	0 µg/L	25 µg/L	100 µg/L
Pairs spawning/d (%)	16.8 $\pm$ 2.1 (21)	17.9 $\pm$ 1.8 (21)	20.2 $\pm$ 1.7 (21)
Clutch size (no. eggs/clutch)	195.8 $\pm$ 15.3 (17)	185.5 $\pm$ 13.7 (14)	183.1 $\pm$ 10.2 (19)
Fecundity (no. eggs/female/d)	30.8 $\pm$ 4.8 (19)	32.3 $\pm$ 5.2 (17)	36.5 $\pm$ 4.0 (20)
Fertilization success (%)	83.0 $\pm$ 3.2 (68)	74.0 $\pm$ 4.6 (63)	79.2 $\pm$ 3.5 (84)
Hatching success (%)	94.7 $\pm$ 1.0 (63)	95.6 $\pm$ 0.9 (53)	95.1 $\pm$ 1.2 (73)
Offspring mortality (%)	19.7 $\pm$ 4.2 (63) A	9.7 $\pm$ 3.0 (53) B	7.3 $\pm$ 2.3 (73) B

Table 2

Secondary sexual characteristics, gonadosomatic index (GSI), and 11-ketotestosterone (11-KT) concentrations of minnows produced by females exposed to 0, 25, and 100 µg/L Cd. Data are expressed as mean ± standard error. Numbers in parentheses indicate sample size. Uppercase letters denote significant differences

Parameter	Maternal exposure concn.		
	0 µg/L	25 µg/L	100 µg/L
Intraocular distance index	0.78 ± 0.01 (20) A	0.81 ± 0.01 (19) B	0.76 ± 0.01 (20) A
Number of breeding tubercles	13.6 ± 1.3 (20)	16.4 ± 0.7 (19)	15.8 ± 0.9 (20)
Male GSI	0.018 ± 0.001 (20)	0.020 ± 0.002 (19)	0.017 ± 0.001 (20)
Female GSI	0.095 ± 0.010 (19)	0.102 ± 0.008 (17)	0.107 ± 0.008 (19)
Plasma 11-KT concentrations (ng/ml)	28.3 ± 7.7 (19)	26.4 ± 5.5 (19)	16.8 ± 2.5 (20)

Table 3
Sex ratio of sample fathead minnow populations subjected to embryonic and larval Cd exposures. Sample size is indicated by *n*

		Exposure concn.				
% Sex ratio as		0 µg/L	12.5 µg/L	25 µg/L	50 µg/L	100 µg/L
Embryonic exposures	Male	53.2	—	60.2	—	60.0
	Female	46.8	—	39.8	—	40.0
	<i>n</i>	94	—	93	—	85
Larval exposures	Male	51.0	46.0	44.5	40.6	—
	Female	49.0	54.0	55.5	59.4	—
	<i>n</i>	182	155	155	96	—

Table 4

Reproductive success of minnows exposed to 0, 12.5, 25, and 50 µg/L Cd during female sexual differentiation. Data are expressed as mean ± standard error. Numbers in parentheses indicate sample size. Uppercase letters denote significant differences

Reproductive parameter	Larval exposure concn.			
	0 µg/L	12.5 µg/L	25 µg/L	50 µg/L
Fertilization success (%)	84.9 ± 2.7 (68) A	68.5 ± 3.8 (74) B	82.1 ± 2.6 (63) A,B	85.2 ± 4.1 (33) A
Hatching success (%)	98.9 ± 0.5 (68)	96.3 ± 1.9 (74)	96.5 ± 1.4 (63)	97.5 ± 1.0 (33)
Offspring mortality (%)	3.2 ± 1.3 (57)	12.1 ± 3.7 (51)	5.9 ± 1.9 (59)	9.9 ± 4.4 (30)