THE EFFECTS OF CHRONIC EXPOSURE TO ENVIRONMENTALLY RELEVANT LEVELS OF WATER-BORNE CADMIUM ON REPRODUCTION IN FATHEAD MINNOWS

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ABSTRACT

Cadmium (Cd) is a priority pollutant in ecosystems worldwide. It is highly toxic to aquatic organisms including fish at fairly low concentrations. Numerous studies have investigated the influence of Cd exposure on fish, but few of them have considered how environmentally relevant levels of Cd affect reproduction, particularly reproductive behaviour. To assess the toxicity of Cd on fish reproduction, breeding fathead minnows (*Pimephales promelas*) were exposed to water-borne Cd for 21 days at four different concentrations (0, 1, 2.5 and 5 μg/L, respectively) based on a standard short-term reproductive assay and reproductive performance as well as behaviour were examined during or at the end of the exposure period. The results showed that Cd accumulated in a dose-dependent manner in the livers and ovaries of female fish. Brood size and mean egg production were significantly reduced in Cd-exposure treatment groups. When fertilized eggs were incubated in the water containing 2.5 μg/L or higher Cd, there was delayed hatching, but at the same time there was greater synchronous hatching after hatching started. Hatching success of Cd-exposed eggs also declined compared to the control. No significant difference was observed among treatments in adult fish survival, the number of breeding attempts, fertilization success, egg size, plasma β-estradiol levels of female, larval deformities, reproductive behaviour, gonadosomatic index or liver somatic index.

The results of this study demonstrate that Cd is able to impair reproduction of fathead minnow at the concentration as low as 0.64 μg/L. It is harmful to both breeding fish and their offspring. The traditional endpoints used in standard reproduction assay (e.g. egg production and brood size) are probably more sensitive than behavioural endpoints, but the traditional method of interpreting reproductive impairment may underestimate toxic effects. The findings of this study have important implications for understanding the effects of chronic Cd exposure in metal-impacted feral fish populations. It can be applied to the protection or restoration of fish populations in Cd contaminated aquatic systems.

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LIST OF ABBREVIATIONS

11-KT	11-Ketotestosterone
AIC	Akaike's Information Criterion
ANOVA	Analysis of variance
ATPase	Adenosine triphosphatase
℃	Degree celsius
Ca	Calcium
$CaCO_3$	Calcium carbonate
Cd	Cadmium
DMT1	Fe^{2+}-divalent metal transporter-1
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
E2	β-Estradiol
Fe	Iron
GSI	Gonadosomatic index
Hg	Mercury
HNO_3	Nitric acid
LC_{50}	Lethal concentration that kills 50% of the test population
LSI	Liver somatic index
Mg	Magnesium
MS-222	Tricaine mesylate
Na	Sodium
$NaHCO_3$	Sodium bicarbonate
OECD	Organisation for Economic Co-operation and Development
PVC	Polyvinylchloride
ROS	Reactive oxygen species
SE	Standard error
T	Testosterone

USA	United States of America
U.S. EPA	United States Environmental Protection Agency
Vtg	Vitellogenin
Zn	Zinc

PREFACE

Chapter 1 is a general introduction into the research topic, while Chapter 4 is a general discussion. Chapter 2 – 4 have been rewritten and submitted to “Journal of Hazardous Materials”.

CHAPTER 1. INTRODUCTION

1.1 General Information of Cadmium

Cadmium (Cd) is a soft, malleable, bluish white metal that is chemically similar to two other stable metals in group 12, zinc (Zn) and mercury (Hg) (Morrow, 2010). Pure Cd ore does not exist. Cd is usually found in greenockite or zinc ores and therefore is a byproduct of zinc refining process (Morrow, 2010). Cadmium-sulfide (CdS) has been used as a pigment for a long time, because of its ability to produce orange, red and brilliant yellow colours (Morrow, 2010). Early uses of Cd also include the corrosion protection coatings on iron and steel (Smith *et al*., 2000). More recently Cd can be seen as an ingredient of plastic stabilizers, which are used to retard the degradation processes of polyvinylchloride (PVC). Cd is usually detected in window or door frames, water or drain pipes made of PVC (Jennings, 2005). The consumption of Cd is continually growing. Nickel-cadmium (NiCd) rechargeable batteries were developed in the early of 20th century (Morrow, 2010) and cadmium-telluride (CdTe) is one of the key components of solar cells and solar panels (Zayed & Philippe, 2009). In addition, Cd also exists in alloys and as an impurity in non-ferrous metal, iron and steel, fossil fuels, cement and phosphate fertilizers (Morrow, 2010).

Even though Cd is a naturally existing chemical in the environment in minor amounts, the concentrations of Cd in air, soil and water can be dramatically increased as a result of human activities. In natural freshwaters, Cd typically occurs at concentrations of less than 0.1 μg/L, but in impacted environments, the concentrations can be several micrograms per liter or greater (U.S. EPA, 2001). Cd may enter aquatic systems through phosphate fertilizers, non-ferrous metal production, iron and steel industries, atmospheric deposition discharged from industrial operations or leakage of landfalls and contaminated sites (Morrow, 2010; OECD, 1994). Cd has no known metabolic roles or any benefits to metabolism in higher organisms. Since it can lead to death at much lower concentrations than other metal ions (Faucher *et al*., 2008), Cd is treated as an environmental hazard. Its supplies and applications have been restricted by multiple regulations, e.g. RoHS directive (Restriction on Hazardous Substances directive, took effect on

1 July 2006) and REACH Regulation (Registration, Evaluation, Authorization and Restriction of Chemicals Regulation, came into force in 1 June 2007) in Europe (RoHS, 2003; REACH, 2006).

Cd is known as a carcinogen, mutagen and immunotoxin to many animals including humans (Bertin & Averbeck, 2006; Filipic *et al*., 2006; Giaginis *et al*., 2006; Hutchingson & Manning, 1996; Zelikoff *et al*., 1995). It affects cell proliferation, differentiation, apoptosis and other cellular activities, and causes numerous molecular lesions that would be relevant to carcinogenesis (Filipic *et al*., 2006). Cd also induces oxidative DNA damage. Given that the proteins involved in DNA repair systems are sensitive targets of Cd toxicity, exposure to Cd could increase the risk for tumor formation in humans through interfering and inhibiting DNA repair processes (Bertin & Averbeck, 2006; Filipic *et al*., 2006; Giaginis *et al*., 2006). Due to its adverse effects on health following to the tendency to accumulate in specific tissues, chronic exposure to Cd contaminated air, water or food results in human kidney failure and bones losing mineral density. Bones subsequently become softer and weaker, which causes pain in the joints and the back, and also increases the risk of fractures and deformities. One of the most publicized cases of Cd poisoning in the world occurred in Toyama Prefecture, Japan. Itai-itai disease, occurred when the residents in that area consumed the rice grown in Cd contaminated irrigation water resulting in many of them, particularly middle aged and elderly women, suffering severe renal tubular dysfunction and osteomalacia (Inaba *et al*., 2005; Nakagaw *et al*., 1990).

1.2 Known Effects of Cadmium on Fishes

Aquatic organisms are sensitive to Cd contaminated water at fairly low concentrations. Based on the criteria of the United States Environmental Protection Agency (U.S. EPA), at hardness of 120 mg/L, the dissolved Cd concentration that is believed to protect 95% examined freshwater species in a 24-h exposure is 2.5 μg/L (U.S. EPA, 2001). Fish, which may be able to tolerate higher Cd concentrations than aquatic invertebrates, still easily become the victims of Cd toxicity. Cd can be absorbed by fish from both diet and water. The primary uptake routes of Cd in fish are the gills and intestine. The apical uptake of water-borne Cd^{2+} at fish gills occurs via a

lanthanum-sensitive voltage-independent epithelial Ca^{2+} channel (ECaC) located in the mitochondria-rich (MR) chloride cells (Galvez *et al*., 2006) or the Fe^{2+}-divalent metal transporter-1 (DMT1) (Bury & Grosell, 2003). The basolateral extrusion of Cd^{2+} is believed to occur via the Ca^{2+}-ATPase as well as Na^{+}/Ca^{2+} exchanger (McGeer *et al*., 2012; Verbost *et al*., 1989). The dietary Cd is mainly absorbed in fish gastrointestinal track (stomach and intestine), although the transport mechanisms are not well characterized as the gills. Existing evidence indicates that in fish stomach and anterior intestine, Cd^{2+} uptake seems to occur via L-type voltage-gated Ca^{2+} channels (Larsson *et al*., 1998) which are different from voltage-insensitive Ca^{2+} channels in the gills, whereas in mid and posterior intestine, Cd^{2+} transport is not directly Ca^{2+} sensitive. Cd uptake in the intestine may also rely on DMT1 and the copper transporter, CTR1 (Elisma & Jumarie, 2001; Lee *et al*., 2002). The basolateral mechanism of gastrointestinal Cd^{2+} transport is still unclear but probably relates to the high-affinity Ca^{2+}-ATPase and Na^{+}/Ca^{2+} exchanger (Schoenmakers *et al*., 1992). The additional uptake routes of Cd include the skin and olfactory rosette in which transport mechanisms may be different than that in the gills (McGeer *et al*., 2012). After being absorbed by fish, Cd remains in uptake tissues or enters circulation and accumulates in other tissues, like liver, kidney, gonads (testes and ovary), even in the olfactory bulb. Cd in these tissues inevitably affects their normal functions (Mathews & Fisher, 2009; Rani, 2000; Scott *et al*., 2003; Tepe & Turkmen, 2008; Tilton *et al*., 2003).

Acute exposure to water-borne Cd at high concentrations will lead to gill epithelium hyperplasia and necrosis (McGeer *et al*., 2012). At lower concentrations, the primary effects of chronic Cd exposure are disruption of ion homeostasis (particularly Ca^{2+} regulation) and generation of reactive oxygen species (ROS), which can be linked to multiple physiological impacts and performance impairments following Cd exposure (U.S. EPA, 2001). As an immunotoxin to fish, Cd was reported to reduce the respiratory burst of kidney phagocytes (Hutchingson & Manning, 1996). An exposure to 2 μg Cd/L has been shown to result in reduced macrophage-mediated immune function in fish, and this would translate into a reduced ability to fight off bacterial or fungal infections as well as other diseases (Zelikoff *et al*., 1995).

Chronic sub-lethal exposure to Cd degrades tissue ultra-structure in organs that accumulate high concentrations (e.g. gills, liver and kidney). Gill lamellae hypertrophy and hyperplasia, hepatic vacuolization, and glomerular and tubular damage in the kidney were observed in silver barb (*Puntius gonionotus*) exposed to 60 μg Cd/L for 60 days (Wangsongsak *et al*., 2007, cited in McGeer *et al*, 2012). A 47-day exposure to 10 μg Cd /L resulted in vertebral deformities in carp (Muramoto, 1981). Cd is reported as an endocrine disruptor. It reduced the plasma cortisol and thyroxine (T_4) levels of rainbow trout exposed to sub-lethal concentrations of 25 μg Cd /L for a month, which probably was related to inhibited growth in fish. However, plasma cortisol levels increased at low dose exposure (10 μg Cd /L) in the same experiment, which indicated the complex effects of Cd on endocrine system (Richard *et al*, 1998). Whole-organism impacts like reduced survival and growth, and impaired reproduction, have also been reported for fish exposed to Cd at low concentrations (McGeer *et al*, 2012).

Even if a contaminant is not lethal at low concentrations, it may still alter normal behaviour so that fish are unable to respond appropriately in a specific ecological context. Scott and Sloman (2004) used "ecological death" to describe this phenomenon which may occur after exposure to toxicants at quite low concentrations. As a contaminant in water, Cd inevitably influences fish behaviour. Most of the studies focusing on behavioural effects concern olfactory-mediated behaviour. These studies show that fish in sub-lethal Cd environments have reduced chemical cue perception, which inhibits fish from reacting to stimuli with correct behaviour. Alterations in anti-predator responses (e.g. escaping, hiding and shoaling), prey detection, or intraspecific interactions can lead to indirect mortality. Kusch *et al*. (2008) found that a 50-day exposure to 2 μg Cd/L made zebrafish (*Danio rerio*) take longer to respond to alarm cue, and their anti-predator behaviour was totally eliminated when they were exposed to 20 μg Cd/L. The most likely reason for this result was olfactory lesions following the deposition of Cd within the olfactory sensory neurons (Blechinger *et al*., 2007; Faucher *et al*., 2008; Kusch *et al*., 2008; Scott *et al*., 2003). Cd is a calcium (Ca) antagonist at the level of gills. Since Ca ions play a preponderant role in signal transduction mechanisms in neuromast hair cells in the fish lateral line system, many behaviours of fish in which the lateral line system is involved, like

predator and prey detection, obstacle avoidance, rheotaxis and intraspecific interactions, are affected when fish are exposed to water-borne Cd (Faucher *et al*., 2008).

1.3 Known Effects of Cadmium on Fish Reproduction

Previous studies demonstrated that Cd exposure reduced gonadosomatic index (GSI) of both female and male Asian cyprinids (*Labeo bata*); spermatids and spermatozoa were absent in the testes of Cd exposed males and high proportion of atretic follicles were found in the ovaries of exposed females (Das, 1988, as cited in Sellin & Kolok, 2006). Even at a concentration of 9.3 μg Cd/L, extended exposure (up to 90 weeks) of adult rainbow trout (*Oncorhynchus mykiss*) resulted in delayed oogenesis (Brown *et al*., 1994). Pelgrom *et al*. (1995) indicated that Cd could accumulate in mature female tilapia (*Oreochromis mossambicus*) ovaries after they were exposed to 5 μg/L water-borne Cd over six days. In experiments by Allen (1995) showed that the average Cd concentration in the gonads of steindachner (*Oreochromis aureus*) was significantly higher than the control level after exposure to 0.1 mg Cd/L for one week. The ovaries had a higher accumulation of Cd than the testes, but the testes appeared to be particularly vulnerable to Cd (Allen, 1995). High Cd accumulation in ovaries might result in Cd depositing in the eggs, which likely implicates a direct burden for the young fish (Pelgrom *et al*., 1995).

Cd can affect fish reproduction through influencing the hormones involved in reproductive performance. Previous studies have shown that Cd could alter the normal levels of vitellogenin (Vtg) and sex steroids in fish blood plasma (Sellin & Kolok, 2006; Thomas, 1989; Tilton *et al*., 2003), and reduced the β-estradiol (E2) stimulated transcription of an estrogen-responsive reporter gene (Le Guevel *et al*., 2000). Cd is also known to activate both androgenic and estrogenic receptors (Sellin & Kolok, 2006). The mechanism may be similar to that reported by Stoica *et al*. (2000), which stated that Cd mimicked the effects of E2 in breast cancer cell lines and activated estrogen receptor-alpha (ER-α) through interacting with the hormone binding domain.

Additionally, Cd is one of the primary causes of larval deformities, such as head and eye hypoplasia, hypopigmentation, pericardial edema, yolk sac abnormalities, altered axial

curvature, reduction of pigmentation and tail malformation (Jezierska *et al*., 2009; Nguyen & Janssen 2002; Williams & Holdway, 2000). These deformities probably result from reduction of egg swelling (too little space for the embryo to move, see Jezierska *et al*., 2009), which may occur when fertilized eggs are exposed to Cd at low concentrations (0.001-0.05 mg/L) as observed in common carp, *Cyprinus carpio* (Witeska *et al*., 1995). Data from other studies indicate that fish embryonic malformations induced by Cd may be mediated through ectopic expression of developmental regulatory genes (Cheng *et al*., 2000). Cd can also cause delayed hatching or premature hatching. Witeska *et al*. (1995) found that incubating common carp fertilized eggs in the water containing 0.001-0.05 mg Cd/L resulted in extending embryo development time (delayed hatching). On the other hand, exposure to 0.1 mg Cd/L promoted premature hatching in rainbow trout (*Oncorhynchus mykiss*) eggs (Woodworth & Pascoe, 1982). A similar result was reported by Jezierska *et al*. (2009), in which the hatching period of common carp eggs was shortened in 0.2 mg Cd/L.

Extending or shortening development time of fish embryos may reduce hatching success or increase the mortality of new hatchlings (Gonzalez-Doncel *et al*., 2003; Jezierska *et al*., 2009; Williams & Holdway, 2000; Witeska *et al*., 1995; Woodworth & Pascoe, 1982). Romhough and Garside (1982) reported that water-borne Cd inhibited the growth of embryos and alevins of Atlantic salmon (*Salmo salar*) at the concentration of 0.47 μg Cd/L, and led to significant increase in mortality of alevins at the concentration of 8.2 μg Cd/L.

Successful reproduction of fish also requires appropriate performance of behaviour, including spawning site selection, territorial defence of spawning sites, nest building, courtship, spawning and post-fertilization investment (such as nest cleaning, guarding, and fanning behaviour, see Scott & Sloman, 2004). Fish reproduction is easily disrupted by Cd which potentially alters the timing or occurrence of appropriate reproductive behaviour, resulting in disruption of mate selection, reduction of fertilization and the offspring survival in a natural setting (Scott & Sloman, 2004). However, few studies have examined the effects of Cd on fish reproductive behaviour. Benoit *et al*. (1976) indicated that brook trout (*Salvelinus fontinalis*, particular males), which were exposed to 3.4 μg Cd/L for 24 weeks, became hyperactive at the

onset of courtship and then died quickly. Spehar *et al*. (1978) described a similar result in male flagfish (*Joudanella floridae*) experiment just before spawning. However, these fish were exposed to mixtures of Cd and Zn, and the fish became hyperactive during feeding as well. Bluegills (*Lepomis macrochirus*) exposed to 80 μg Cd/L were also reported to become hyperactive at the onset of spawning, but there was no indication that the female were excluded from these effects in the experiment (Eaton, 1974). Given that reproductive behaviour is a key component in fish reproduction success and that there is less knowledge relating to the effects of Cd on it, it is necessary to evaluate the alterations of reproductive behaviour of fish exposed to environmentally relevant water-borne Cd, and in particular, to comprehensively evaluate behavioural endpoints with other reproductive endpoints.

1.4 Test Species: Fathead Minnow

The fathead minnow (*Pimephales promelas*) has a relatively cosmopolitan distribution and is a representative of the ecologically-important and ubiquitous Cyprinidae family. It is one of the most extensively tested fish species in the world and has been used in short term lethality, early life-stage, and full life-cycle tests. Also, there are a number of regulatory programs, such as water quality criteria derivations, using fathead minnow as a model species. One important advantage to using this species in testing is that many academic, industry and consulting laboratories are familiar with this species (Ankley *et al*., 2001; Jensen *et al*., 2001).

Fathead minnows are relatively easy to culture in a laboratory (Denny, 1987). They have a rapid life cycle, and the timing of the reproductive cycle can be effectively controlled through using temperature/photoperiod manipulation (Ankley *et al*., 2001). Under favorable conditions, fathead minnows achieve their reproductive maturity within 4 to 5 months of hatch. Adult fathead minnows are sexually dimorphic. At maturity, males weigh 4 to 5 g, and females weigh 2 to 3 g. Mature males with secondary sexual characters, which include nuptial tubercles on the snout, spongy pad from nape to dorsal fin and black body coloration with two light-colored vertical bars, making them easily distinguishable from females. Females can produce clutches of 50 to 150 eggs every 3 to 4 days if they are in optimal lab conditions. Fertility rate of each

brood generally exceed 95%. Embryos typically hatch within 4 to 5 days at 25 ℃, and the hatching rate is in the range of 95 to 98% (Ankley *et al*., 2001; Denny, 1987; Jensen *et al*., 2001). Typical GSI for reproductively active fathead minnows ranges from 8 to 13% for females and 1 to 2% for males (Ankley *et al*., 2001). The concentrations of Vtg and sex steroids, such as E2, testosterone (T) and 11-ketotestosterone (11-KT) in blood plasma can be used as indicators to detect endocrine disruption effects. Vtg is a phospholipoglycoprotein precursor to egg yolk protein that normally occurs in sexually active females. The production of Vtg is controlled by interaction of estrogens, particularly E2, with the estrogenic receptors. Males also have the capacity to produce Vtg in response to stimulation with estrogenic receptor agonists (Ankley *et al*., 2001). In female blood plasma, E2 and T are easily measured, while 11-KT normally is non-detectable. The concentrations of 11-KT in mature males blood plasma are higher than T, and the concentration of E2 is very low but still measurable (Ankley *et al*., 2001; Jensen *et al*., 2001). In female fathead minnows, the concentration of E2 cyclically varies according to spawning interval. A significant peak in plasma E2 is observed on day 1 post-spawn, followed by a gradual decline in the concentration of estrogen. No other plasma sex steroid has such obvious cyclical variation in female fathead minnow (Jensen *et al*., 2001). Consequently, fathead minnow is an attractive model species for reproduction tests.

In the breeding season, spawning fathead minnows, particularly males, display a series of behaviours. Breeding males are territorial, and actively defend their nest sites against other males and intruders (Ankley *et al*., 2001). Before spawning, male fish spends some time in the nest site and cleans the underside of the nest using its head tubercles in a scraping action and pulling pieces of algae and associate debris off the nest surface with its mouth (Cole & Smith, 1987; Denny, 1987). Then the male approaches a female, nudges or bumps the female's flank region with its snout, and leads or pushes the female to its nest site (Cole & Smith, 1987). After arriving, the male and female swim back and forth beneath the prepared nest site and roll on their sides to emit gametes. Close lateral contact and body vibration between the male and female characterize the act of spawning (Ankley *et al*., 2001; Denny, 1987). After sufficient stimulation, the male presses the female upward, resulting in the female's urogenital region

contacting the substrate, with a concomitant release of eggs. Milt is released at this time as the pair terminates with an abrupt separation. This behaviour occurs intermittently until an increase in male aggression drives the female away (Ankley *et al*., 2001; Denny, 1987). This series of behaviour could be used as important indicators of successful reproduction and make fathead minnow an excellent experimental model for examining fish reproductive behaviour.

1.5 Research Hypothesis and Objectives

The present study focused on the effects of chronic, sub-lethal Cd exposure on reproductive performance and behaviour of fathead minnow. Cd concentrations tested in this experiment were chosen based on previous studies. Sherman *et al*. (1987) reported that the 96-h LC_{50} of Cd for fathead minnows was 4.39 mg/L (hardness 141 mg $CaCO_3$/L, alkalinity 86 mg $CaCO_3$/L, pH 8.3), and Cd had lower toxicity in higher pH or hardness water. A 32-day fathead minnow early life stage toxicity test conducted by Spehar and Fiandt (1986) showed that under flow-through conditions using water with hardness measured as 43.9±1.0 mg $CaCO_3$/L, chronic value (calculated as the geometric mean of the highest no effect and lowest effect concentrations) for fathead minnows was 10.0 μg Cd /L. The water used in the current experiment was a little harder than that used in the two experiments mentioned above (Saskatoon tap water [hardness 157 mg $CaCO_3$/L, alkalinity 109 mg $CaCO_3$/L, pH 7.9, dissolved organic carbon 2.5 mg/L]), therefore Cd levels tested in this experiment (1, 2.5 and 5 μg/L) were much lower than the lethal level for fathead minnows (U.S. EPA, 2001).

The goal of my work was to test the hypothesis that environmentally realistic concentrations of Cd would affect the reproductive performance and behaviour of fathead minnows, and that behavioural endpoints would be more sensitive than traditional reproductive endpoints (e.g. egg production, brood size and fertilization success) which were widely used in standard toxicity assessments (e.g. U.S. EPA reproductive assays). To test this hypothesis, fathead minnows were exposed to water-borne Cd (1, 2.5 and 5 μg/L) for 21 days, and their reproductive behaviour (male courting behaviour, time spent in the nest and time devoted to nest maintenance) as well as reproductive performance (egg production, the

number of breeding attempts, brood size fertilization success, egg size, the number of days required by eggs to hatch, hatching success and larval deformities) were evaluated. The endpoints evaluated in this study also included accumulation of Cd in two target tissues (liver and ovary), the effects of Cd exposure on breeding fish survival, morpho-metrics (gonadosomatic index and liver somatic index) and E2 levels in female blood plasma. According to previous studies, I predicted that Cd would significantly accumulate in female liver and ovary, and reduce the egg production, fertilization success, hatching success and the survival of breeding fish. Cd would also increase the proportion of deformed larvae. Hatching time, morpho-metrics and the level of female blood plasma E2 would be changed in Cd exposure. It was difficult to predict how Cd would affect these endpoints based on the previous studies, given conflicting results. For the behavioural endpoints, there is no study that could be used as a direct reference. However, considering that Cd could inhibit fish perception including olfactory, it was reasonable to predict that Cd would alter the reproductive behaviour. For example, male fish may have difficulty locating female fish in breeding condition, or females may respond male courtship inappropriately.

CHAPTER 2. MATERIALS AND METHODS

2.1 Fish Maintenance

This research was conducted in an aquatic laboratory of the Department of Biology at the University of Saskatchewan. Fathead minnows used in this study were purchased from commercial supplier (Osage Catfisheries, Inc. MO, USA). Fish were maintained at approximately 20 ℃, in six holding tanks containing filtered dechlorinated water. Photoperiod was adjusted to a 16-h light: 8-h darkness (16L:8D) cycle. All fish were fed commercial tropical fish flakes (Rolf C. Hagen Inc., QC, Canada) in the morning and flakes with dried bloodworms (OmegaSea Ltd., AK, USA) in the afternoon. The conditions of the fish were checked daily. Water quality was checked by measuring the following parameters: total ammonia, total chlorine, total hardness, total alkalinity and pH. 10%-20% water was changed in each tank when required.

Fathead minnows were acclimated in holding tanks for two weeks after arriving at the aquatic laboratory. The mortality of these fathead minnows was less than 5% of total population during the two-week acclimation period, which indicated that this batch of minnows were healthy and acceptable for experimentation (OECD, 2009).

2.2 Pre-exposure Phase

The experiment was carried out in two phases: the pre-exposure phase (without Cd exposure) and the exposure phase (Cd exposure). The purposes of the pre-exposure phase were to establish reproductive capacity of paired fathead minnows which were then used in the exposure phase, and to provide tank-specific baseline data for statistical comparison after initiation of Cd exposure (Ankley *et al*., 2001). The length of the pre-exposure period was 14-day.

In the pre-exposure phase, 90 pairs (1 female + 1 male) of fathead minnows were randomly placed into 9 L-experimental tanks (1 pair per tank). Each tank contained an artificial nest, a 10 cm long piece of PVC pipe (10 cm diameter) that was cut lengthwise. When being transferred from holding tanks to experimental tanks, each fathead minnow was measured for fork length and body weight to make sure that the length of female fish was no more than 80% of that of its

male partner. A minimum of 20% difference in body length between male and female were chosen to increase the likelihood that reproduction would occur in any given replicate tank (Pollock *et al*., 2008). All experimental tanks were maintained at around 25 ℃, 16L:8D photo period, and 1 L water from each tank was changed every two days. Paired fish were fed twice daily, with commercial tropical fish flakes (Rolf C. Hagen Inc., QC, Canada) at 9:00 a.m. and flakes with dried bloodworms (OmegaSea Ltd., AK, USA) at 4:00 p.m.

Fish health and the nesting object in each tank were checked every day at 10:20 a.m. If eggs were present, the nest was photographed and the eggs were counted using ImageJ (Schneider *et al*., 2012). Fish did not receive any treatment for disease, and dead fish were not replaced during the pre-exposure phase. Seven tanks were randomly chosen to check water quality daily (different tanks were chosen in each day until all tanks were measured, then the cycle was repeated). Measurements included: temperature, dissolved oxygen (DO), conductivity and pH, using YSI probe (YSI Incorporated, OH, USA). Tanks were siphoned every day to remove residual food and fecal materials.

2.3 Criteria for Selection of Test Fish

At the end of the pre-exposure phase, 60 pairs of fathead minnows were selected for use in the exposure phase. The criteria for selection of test pairs were: 1) both male and female survived and were in healthy conditions, 2) regular spawning occurred at least two times in the immediately preceding 7 days, and 3) egg production exceeded 15 eggs per female per day (U.S. EPA, 2002). A one-way ANOVA was performed when all pairs were assigned to treatment groups to ensure there was no significant difference between treatments with regard to egg production before experimental conditions were applied.

2.4 Experimental Conditions and Procedures

Fifteen pairs of fathead minnows were assigned to each of four treatments: control (0 μg Cd/L), low (1 μg Cd/L), medium (2.5 μg Cd/L) and high (5 μg Cd/L) Cd exposure levels (n=15 per treatment). Water in each tank was replaced by experimental water according to the Cd

concentration requirement of each treatment at the beginning of the exposure phase. Aside from the changes of Cd concentration in water, fish maintenance and water quality test were identical to that in the pre-exposure phase. The number of tanks that were chosen to check water quality daily was extended to 8 (2 tanks per treatment chosen randomly). Different tanks were checked each day until all tanks had been checked, then the cycle was repeated. Five tanks were randomly chosen to record breeding behaviour between 10:00 to 10:20 hr each day using tape-based video cameras. Different tanks were chosen from each treatment every day, when all the tanks had been recorded, the cycle was repeated. Water was sampled from five randomly chosen tanks of each treatment (1 ml per tank) every two days to determine the water-borne Cd concentration. The length of the exposure phase was 21-day.

In order to score fertilization success (number of fertilized eggs/total number of eggs x 100) accurately, nesting objects with eggs in any morning were transferred to 4 L incubation tanks with conditions identical to those of the parents. After one-day incubation, these nesting objects were photographed in order to measure the egg production, fertilization success and egg size by ImageJ afterwards (Schneider *et al*., 2012). Eggs from all broods were assumed to be in approximately the same stage of development, as the eggs were collected at the same time every day and fathead minnows are known to spawn in the morning (Ankley *et al*., 2001). Unfertilized eggs were opaque and were easily differentiated from viable eggs after one-day incubation. ObjectJ, a plug-in of ImageJ, was used to analyze egg size. This program can provide the diameter of selected circle in images directly. Egg size measurements were taken for a sub-sample of 10 eggs for each brood. Where broods were smaller than 10 eggs, all available eggs were measured.

After photographs were taken, 15 fertilized eggs were randomly chosen from one brood of each treatment per day to continue incubating in a 2 L-incubation tank (one brood per tank) with conditions identical to those of their parents. When eggs hatched, larvae were maintained in incubation tanks until all viable eggs hatched. Larvae were then counted to determine hatching success (number of eggs that hatched/number of fertilized eggs x 100) and fixed in 10% formalin to score deformities. Such deformities measurements included: spinal curvature

(kyphosis, lordosis and scoliosis), craniofacial malformation (head and jaw malformation), pericardial and yolk sac edema. These data were presented as a percentage (number of deformed larvae/total number of larvae x 100). The number of days required by the eggs to hatch was also recorded.

At the end of the exposure phase, all surviving adult fish were sacrificed by MS-222 (100 mg/L buffered with 200 mg $NaHCO_3$/L), and female blood was collected subsequently by caudal puncture. Since the volume of blood collected from each female was small, blood from every 3-4 fish in the same treatment group was pooled together to form one sample for hormone examination. Livers and gonads were removed by dissecting and stored in a -20 ℃ freezer for Cd accumulation analysis. The body weight, fork length and weights of liver and gonad were also measured for each fish.

2.5 Analysis of Reproductive Performance

First, mean egg production of each replicate tank was evaluated. It was calculated as the total eggs produced by each female being divided by the duration of the pre-exposure (14 days) or the exposure phase (21 days), and expressed as eggs/female/day. The purpose of measuring egg production in this way was to compare it between the pre-exposure and exposure phases, and also to allow for comparisons with other studies which had experimental periods of different lengths. The mean egg production provides a measurement of the overall reproductive potential of breeding pairs over time.

Reproductive performance of experimental fish was then analyzed by the average brood size per female. The total number of eggs produced by each female was divided by its number of breeding attempts during the exposure phase to get the average brood size (expressed as eggs/female). This endpoint represents an estimate of average productive output of a breeding pair.

The third measurement of reproductive performance was the cumulative egg production. The number of days in the exposure period (21 days) was used as the replicate in the cumulative egg production analysis. The cumulative eggs counted in each treatment group for each day was

divided by the number of female fish which were capable of breeding in this treatment group at that time. This endpoint was expressed as cumulative eggs/female/day. Due to the mortality of fish, the number of potential breeding pairs in each treatment group reduced during the 21-day exposure period. Cumulative egg production provides an estimate of reproductive potential of each treatment group while compensating for adult mortality.

Cumulative number of breeding attempts during the exposure phase and average number of breeding attempts per replicate tank were also analyzed. These calculations were performed to determine whether there was any difference between treatments in the number of fish that were able or willing to breed under the experimental conditions.

Besides egg production, average fertilization success and egg size of each brood during the exposure period were measured. Average fertilization success was presented as the number of fertilized eggs/the total number of eggs x 100, and this endpoint leads to a measurement of the quality of sperm and eggs. It also provides an estimate of spawning behaviour. Average egg size was calculated by the mean diameter of 10 eggs randomly chosen in each brood after one-day incubation, and it provides an estimate of the quality of egg as well.

2.6 Analysis of Egg Hatching

Each day, 15 fertilized eggs were randomly chosen from one brood of each treatment group, and then transferred into a 2 L incubation tank with identical conditions as those of their parents (one brood per tank). Eggs were held in the incubation tanks until all eggs hatched. All incubation tanks were checked twice a day, at 8:00 a.m. and 8:00 p.m., to record the length of hatching period of each brood. The measurements include the number of days required by all eggs, by the first egg in each brood and by the rest of eggs in this brood to hatch. When the hatching of all eggs was completed, larvae were counted and collected, and then the hatching success of each brood was calculated as the number of larvae/the number of fertilized eggs at the beginning of incubation x 100. The length of the hatching period and the hatching success provide estimates of embryo development and the activity and distribution of chorionase.

2.7 Analysis of Larval Deformities

Larvae were collected from incubation tanks and preserved in 10% formalin for the deformity analyses. A dissecting microscope was used to observe larvae samples and 5 larvae were randomly chosen from each brood to be photographed by QCapture program (Qimaging Corporation, BC, Canada). These pictures were used to score larval deformities according to different categories and degrees of severity. The deformity categories measured in this research included spinal curvature (kyphosis, lordosis and scoliosis), craniofacial malformation, pericardial and yolk sac edema. Subsequently, these larvae were scored by the degrees of severity for various deformities. Three degrees were used: 1) *negligible* referred to the larvae that had no deformity or only slight spinal curvature or/and slight edema; 2) *moderate* referred to the larvae that had craniofacial deformities or marked deformities of spine or edema and 3) *severe* referred to the larvae that had two or more severe deformities co-occurred. The criteria to distinguish the degrees of severity of deformity were whether these deformities would affect larval development and whether they were likely to lead to larval death. Deformities scored as negligible did not affect or only slightly affected larval development. Moderate deformities would affect larval development and probably result in death. Larvae that had deformities in the severe category died immediately after they hatched. All these endpoints were calculated as the number of deformed larvae/the number of larvae chosen to be assessed x 100. The percentage of new hatchlings with deformity is a measurement of embryo development.

2.8 Analysis of Reproductive Behaviour

Reproductive behaviour was monitored using tape-based video cameras and recorded in a subset of 5 tanks (one per treatment, and an additional randomly chosen tank) between 10:00 to10:20 hr. Each day, cameras were moved to record a different subset of tanks until all pairs had been recorded, and then the cycle was repeated. Due to adult mortality during the exposure period, some tanks were not recorded while others may have been recorded as many as four times. When tanks were recorded multiple times, I used the average score from each tank in the statistical analysis.

Videos were transformed to digital format and then scored for reproductive behaviour with the aid of JWatcher program. This program allowed the accurate recording of multiple sorts of behaviour simultaneously, including the number of occurrence and the length of duration for certain behaviour, via a specialized keypad. It also enabled slow-play of the videos, which allowed for the accurateness of scoring discrete behaviour. Moreover, this program can provide summaries of the mean number of occurrence and the proportion of duration time for all behaviour analyzed.

Behavioural observations included the number of spawning activities, the number of times the male courted the female outside or in the nest, the percent time spent in the nest by female or by male, and the percent time spent on maintaining the nest by male. Male courting female in the nest referred to male nudging or bumping the female's flank region with its snout when they were both beneath the nesting objects. Male courting female outside the nest included the male approaching and butting the female and then pushing or leading the female toward the nest. Male maintaining the nest were characterized by cleaning the underside of the nest using its head tubercles in a scraping action and pulling pieces of algae and associate debris off the nest surface with its mouth. All these are typical reproductive behaviours of fathead minnows which have been reported by previous studies (Cole & Smith, 1987; Denny, 1987). Except for the number of occurrence, the duration of any specific behaviour was calculated as the percentage of time rather than absolute value in order to control for the different length of videos.

2.9 Analysis of Cadmium Concentrations in Tissues and Water

The gonad and liver from all surviving adult fish at the end of the exposure period were weighed and stored in -20 ℃ freezer. Female liver and ovary were used to measure the Cd accumulation in these two organs. Tissues were digested in five volumes of 1 N HNO_3 at 60℃ for 48-h and then centrifuged at 15,000 g for 4 min. The supernatant was suitably diluted with 0.2% HNO_3 and measured for Cd concentration by graphite furnace atomic absorption spectrophotometry (AAnalyst, PerkinElmer CT, USA). In addition, water samples collected from each treatment group during the exposure period were used to measure the water-borne Cd concentration by

graphite furnace atomic absorption spectrophotometry. Water samples from the 1 µg Cd/L treatment group were acidified with HNO_3, and water samples from 2.5 and 5 µg Cd/L treatment groups were diluted before measurement. The quality control and assurance of the Cd concentration analyses were maintained using appropriate method blanks and certified standards for Cd (Fisher scientific Canada), and validated with certified reference material (Dolt-3; National Research Council, Canada). The % recovery of Cd in the reference material analysis was 96.4%.

2.10 Analysis of Blood Plasma β-Estradiol and Morpho-metrics

Blood samples from female fish were centrifuged at 4 ℃ at 15000 g for 3 min to separated blood plasma for E2 level analysis. This analysis was conducted using an Estradiol Enzyme Immunoassay (EIA) kit, (Oxford Biomedical Research, Inc. MI, USA). According to the manufacturer's instructions, plasma samples were ether extracted and then diluted to make sure that the measured volume fell within the reliable boundaries of the standards. Samples and standards were incubated in wells of a coated microplate with estradiol-HRP conjugate (estradiol horseradish peroxidase conjugate) for 1 hour at room temperature. After rinsing with wash buffer, TMB (3, 3', 5, 5'-Tetramethylbenzidine) substrate was added to the wells. The plate was then developed for 30 minutes at room temperature and read using a microplate reader (VersaMax ELISA Microplate Reader, Molecular Devices, LLC, CA, USA) at 650 nm afterwards. Additionally, gonads and livers from both female and male fish were used to evaluate liver somatic index (LSI=[liver weight/total body weight] x 100) and gonadosomatic index (GSI=[gonad weight/total body weight] x 100).

2.11 Statistical Analysis

One-way ANOVA was used when the data met the assumptions for parametric test. To test the assumptions required by parametric analysis, a quantile-quantile plot (Q-Q plot) and a histogram graph were used to determine normality, and Bartlett's test was used to assess homogeneity of variance. If the assumptions for parametric test could not be met, data were

transformed. Power or arcsine square-root transformations were used when it was necessary. A Tukey post-hoc test was used to determine which treatments were different from the control. For egg production, pairwise t-test was also used to compare mean egg production of each treatment between the pre-exposure and exposure period. Pearson's Chi-squared test was used to determine whether adult fish survival was different among treatments. A two-sample Kolmogorov-Smirnov test was used to compare Cd-exposure treatments to the control in cumulative egg production and cumulative breeding attempts analyses. Stepwise deletion of saturated general linear model was used to determine if a significantly different water quality parameter had effects on the changes in reproductive performance. Saturated models were simplified using stepwise deletion based on residual deviance and/or AIC (Akaike's Information Criterion). Statistics of all endpoints were analyzed using R language (R Core Team) and all tests were conducted using two-tails predictions with an alpha value of 0.05.

CHAPTER 3. RESULTS

3.1 Water Quality

Water temperature, DO, pH and conductivity during the 21- day exposure phase were analyzed by averaging daily measurements of each tank (n=15). The result of one-way ANOVA showed a significant difference in water temperature among treatments ($F_{(3, 56)}$=7.83, P<0.001). Tukey post-hoc tests revealed that the temperature of the 1 μg Cd/L treatment did not significantly differ from the control (P>0.6), but the temperatures in both the 2.5 and 5 μg Cd/L treatments were significantly higher than the control (control vs. 2.5 μg Cd/L: P<0.001; control vs. 5 μg Cd/L: P<0.008). Despite of the statistically significant difference in temperature, the actual magnitude of the difference was quite small (0.91 ℃). Conductivity of the 2.5 and 5 μg Cd/L treatment groups were also significantly higher than the control (ANOVA: $F_{(3, 56)}$=5.971, P<0.002; Tukey post-hoc test: control vs. 2.5 μg Cd/L: P<0.004; control vs. 5 μg Cd/L: P<0.02). The difference between highest (2.5 μg Cd/L treatment) and lowest (control) mean value of conductivity was 14.5 μS/cm. Temperature + conductivity were used as explanatory variables to create saturated general linear models for each significant response variable (see below) in order to determine if these two water quality variables were responsible for the significant changes in reproductive performance. As a result, conductivity was the only significant explanatory variable for brood size (ANOVA: $F_{(3, 47)}$=7.418, P<0.01), and neither of these two water quality parameters was responsible for the significant change in mean egg production (ANOVA: $F_{(3, 38)}$=5.024, P<0.04). No significant difference in pH or DO was observed among treatments (ANOVA: DO: $F_{(3, 56)}$=1.1386, P>0.3; pH: $F_{(3, 56)}$=1.1348, P>0.3, Table 3.1.1.).

Cd concentrations in water were analyzed by the water samples taken every other day (n=11). The mean Cd concentration of the control water was not shown because it was below the detection limit (0.1 μg/L). After power transformations, a one-way ANOVA showed that water-borne Cd concentrations of other three treatments were significantly different from each other ($F_{(2, 42)}$=798.81, P<0.001; Tukey post-hoc test: all P<0.001, Table 3.1.1.).

Table 3.1.1 The water quality parameters in different experimental treatments. Data are presented as mean ±SE. Nominal Cd concentrations are showed in parentheses.

Water quality parameter	N	control (0 μg/L)	Low (1 μg/L)	Medium (2.5 μg/L)	High (5 μg/L)
Temperature (℃)	15	25.36 ±0.13^{a}	25.6 ±0.20 ^{a,c}	26.27 ±0.10 ^{b}	26.08 ±0.15^{b,c}
DO (%)	15	83.58 ±0.89	82.16 ±0.91	79.76 ±1.74	82.51 ±1.11
pH	15	7.94 ±0.03	7.90 ±0.02	7.88 ±0.03	7.93 ±0.02
Conductivity (μS/cm)	15	476.4 ±2.4 ^{a}	480.2 ±3.1 ^{a,c}	490.9 ±3.1 ^{b}	488.9 ±2.6 ^{b,c}
Cadmium (μg/L)	11	N.D.^{a}	0.64 ±0.02^{b}	2.16 ±0.08^{c}	4.36 ±0.11^{d}

$^{a, b, c, d}$ Different letters indicate significant differences determined by ANOVA followed by Tukey post-hoc test ($P<0.01$)

N.D.: Not Detectable.

3.2 Fish Survival

The survival of female fish during the whole exposure phase was measured for each treatment, and statistical analysis did not show any significant difference among the treatments (Pearson's Chi-squared test: $X^2_{(3)}=4.6154$, $P>0.2$). Mean (±SE) female survival in the 0, 1, 2.5 and 5 μg Cd/L treatment group were 93% ± 4.5%, 82% ± 6.4%, 93% ± 4.5%, and 100% ± 0%, respectively. Only one male fish which was in the 1 μg Cd/L treatment group died in the exposure period.

3.3 Cadmium Accumulation in Tissues and Morpho-metrics

Cd accumulation in female liver and ovary were analyzed from all tanks, where the female survived until the end of the exposure phase (liver: n=13 for control and 2.5 μg Cd/L, n=11 for 1 μg Cd/L, n=12 for 5 μg Cd/L; ovary: n=8 for control, n=7 for 1 μg Cd/L, n=10 for 2.5 μg Cd/L, n=12 for 5 μg Cd/L). Cd accumulation in the liver and ovary increased significantly with the increase of Cd concentration in the water (one-way ANOVA: liver: $F_{(3, 45)}=90.673$, $P<0.001$; ovary: $F_{(3, 33)}=204.53$, $P<0.001$). Tukey post-hoc tests revealed that Cd accumulation in each treatment group significantly differed from others (all $P<0.05$, Figure 3.3.1. & 3.3.2.).

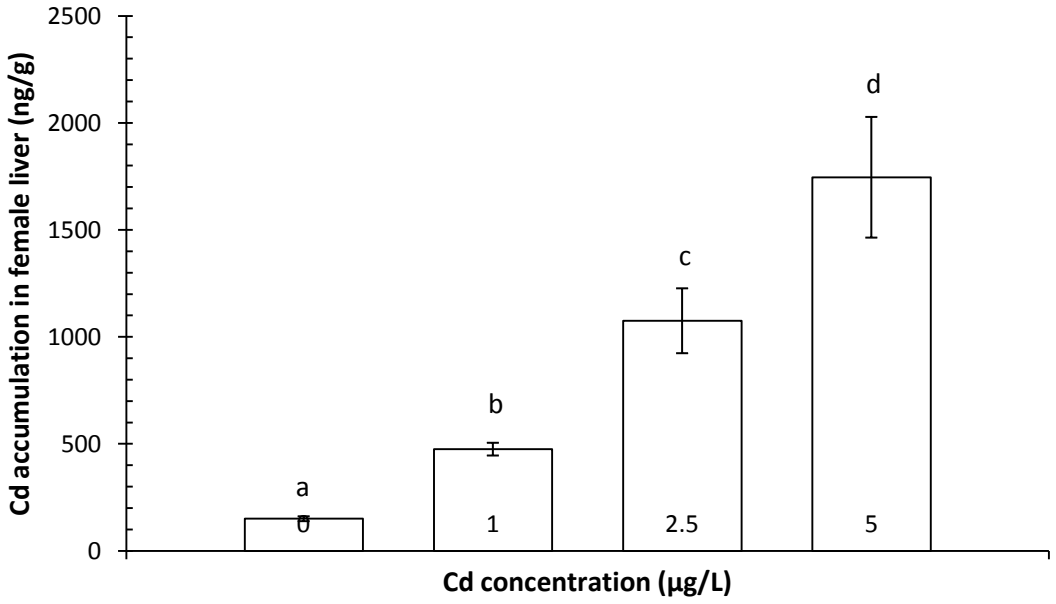

Figure 3.3.1 Cd accumulation in liver tissue of female fathead minnows after a 21-day exposure to 0 (control), 1, 2.5 and 5 µg/L of water-borne Cd. Data are presented as mean ±SE (n=11-13). Different letters denote significant differences determined by one-way ANOVA followed by Tukey post hoc test ($P<0.05$).

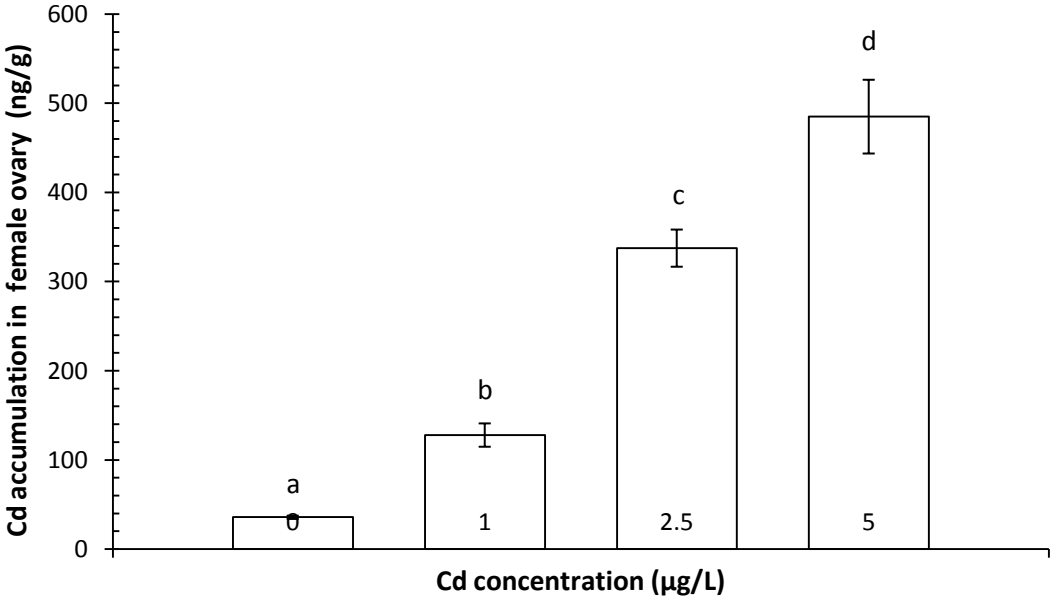

Figure 3.3.2 Cd accumulation in ovary tissue of female fathead minnows after a 21-day exposure to 0 (control), 1, 2.5 and 5 µg/L of water-borne Cd. Data are presented as mean ±SE (n=11-13). Different letters denote significant differences determined by one-way ANOVA followed by Tukey post hoc test ($P<0.05$).

GSI and LSI of both female and male fish were analyzed using all the adult fish that survived until the end of exposure phase (female: n=13 for control and 2.5 and 5 μg Cd/L, n=11 for 1 μg Cd/L; male: n=15 for control and 2.5 and 5 μg Cd/L, n=14 for 1 μg Cd/L). Neither GSI nor LSI of either sex was found to differ among treatments (female: GSI: $F_{(3, 46)}$=0.2384, P>0.8; LSI: $F_{(3, 46)}$=1.0913, P>0.3; male: GSI: $F_{(3, 55)}$=0.9412, P>0.4; LSI: $F_{(3, 55)}$=1.1711, P>0.3, Table 3.3.1.).

Table 3.3.1 GSI and LSI for female and male fathead minnows in different experimental treatments after a 21-day Cd exposure. Data are represented as mean±SE. Sample sizes are shown in parentheses.

FEMALES	**0 μg/L (n=13)**	**1 μg/L (n=11)**	**2.5 μg/L (n=13)**	**5 μg/L (n=13)**
GSI%	12.40±0.83	12.67±1.96	11.77±1.17	13.00±1.25
LSI%	2.45±0.23	2.01±0.18	2.55±0.20	2.34±0.25
MALES	**0 μg/L (n=15)**	**1 μg/L (n=14)**	**2.5 μg/L (n=15)**	**5 μg/L (n=15)**
GSI%	1.55±0.15	1.77±0.14	1.82±0.13	1.60±0.12
LSI%	1.78±0.18	1.79±0.12	2.14±0.19	1.85±0.12

3.4 Reproductive Performance

Mean egg production and the number of breeding attempts were analyzed from all pairs of fish that produced eggs and both adults survived until the end of the exposure phase (n=12 for control, n=8 for 1 μg Cd/L and 2.5 μg Cd/L, and n=14 for 5 μg Cd/L treatment; samples that cannot be measured accurately was excluded). The results of one-way ANOVA showed that there was no significant difference in mean egg production among treatments, neither in the 14-day pre-exposure period nor 21-day exposure period (one-way ANOVA: pre-exposure period: $F_{(3, 38)}$=0.7281, P>0.5, data are not shown; exposure period: $F_{(3, 38)}$=2.0145, P>0.1, Figure 3.3.1.). However, the comparison of mean egg production during the exposure period indicated that fathead minnows in Cd-exposure groups (1, 2.5 and 5 μg Cd/L) appeared to

produce fewer eggs than the control fish (Figure 3.4.1.), and the statistical analysis showed a significant difference in the mean egg production between all three Cd-exposure groups combined (n=30) and the control (n=12) (one-way ANOVA: $F_{(1, 40)}$=5.024, P<0.04, Figure 3.4.2.). Additionally, significant differences were observed when comparing mean egg production between the pre-exposure and exposure phase in each treatment (paired t-test: all P<0.001). Mean egg production decreased in all Cd-exposure groups during the exposure phase while in the control, it increased during the same period (Figure 3.4.3.). No statistically significant difference was found in the number of breeding attempts among treatments (one-way ANOVA: $F_{(3, 47)}$=1.2629, P>0.2, Figure 3.4.4.).

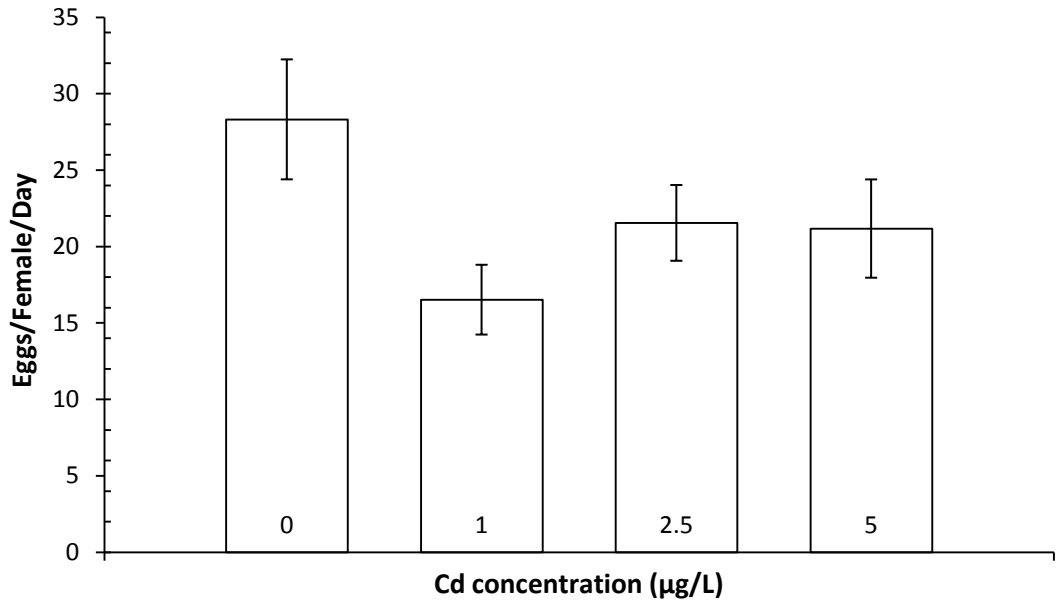

Figure 3.4.1 The mean egg production of each treatment during a 21-day exposure to 0 (control), 1, 2.5 and 5 µg/L of water-borne Cd. Data are presented as mean ±SE (n=8-14).

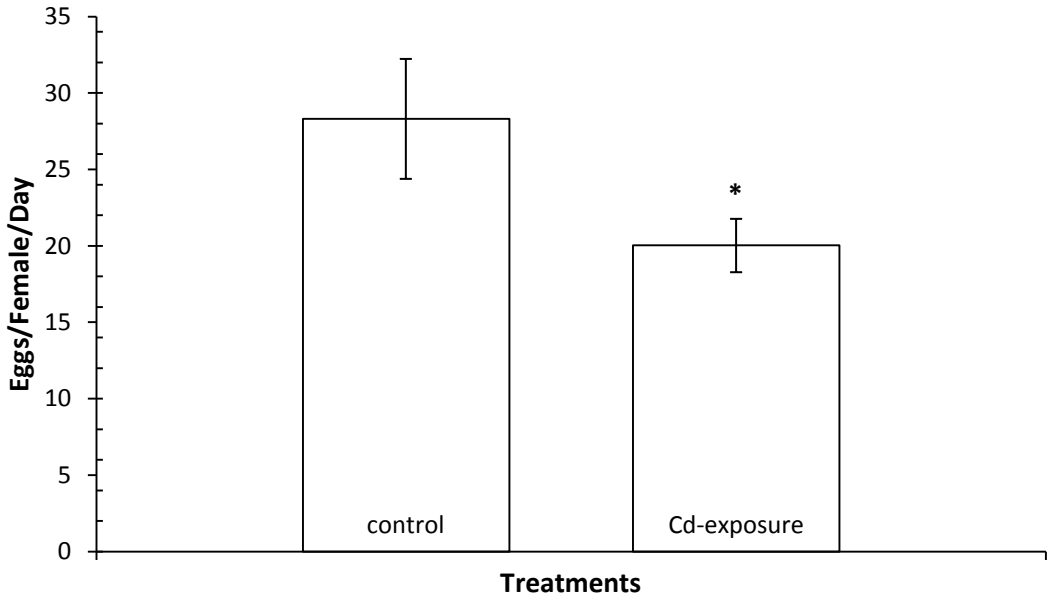

Figure 3.4.2 The mean egg production of the control and all three Cd-exposure treatments combined during a 21-day exposure phase. Data are presented as mean ±SE (n=12, 30). Asterisk denotes significant difference determined by one-way ANOVA (P<0.04).

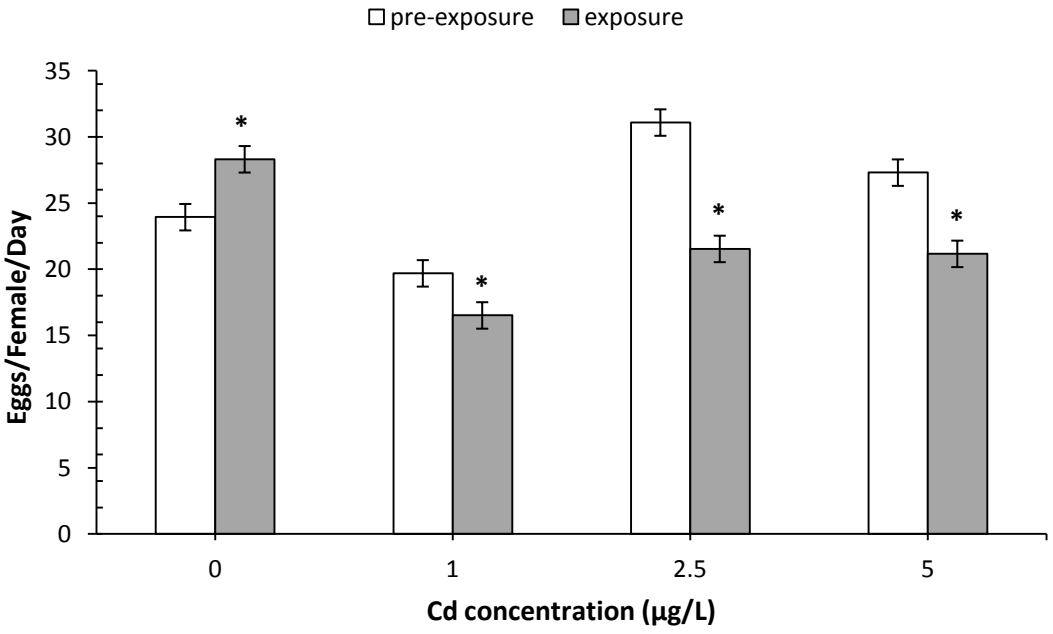

Figure 3.4.3 The mean egg production of 0 (control), 1, 2.5 and 5 µg Cd/L treatments during a 14-day pre-exposure phase and a 21-day exposure phase. Data are presented as mean ±SE (n=8-14). Asterisk denotes significant difference between the two periods determined by paired t-test (P<0.001).

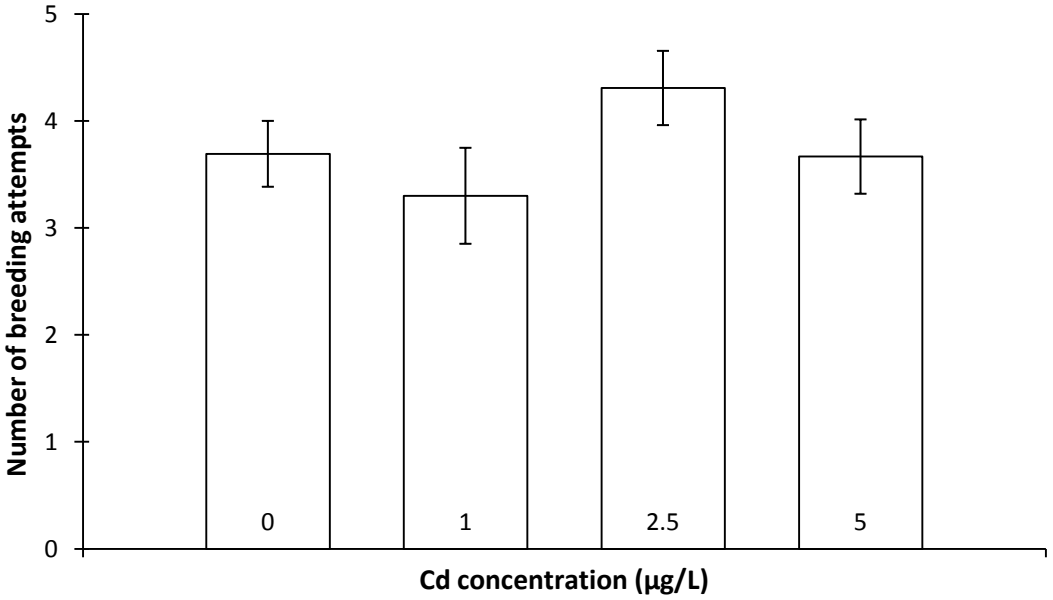

Figure 3.4.4 The average number of breeding attempts of each pair of fathead minnows over a 21-day exposure to 0 (control), 1, 2.5 and 5 µg/L of water-borne Cd. Data are presented as mean ±SE (n=8-14).

Fertilization success and brood size were assessed for all pairs of fish that produced eggs and both adults survived until the end of the exposure period (n=13 for control and 2.5 µg Cd/L, n=10 for 1 µg Cd/L, and n=15 for 5 µg Cd/L). A one-way ANOVA showed that neither fertilization success nor brood size significantly differed among treatments (fertilization success: $F_{(3, 47)}$=0.7121, P>0.5; brood size: $F_{(3, 47)}$=2.7796, P>0.05, Figure 3.4.5. & 3.4.6.). However, the comparison of brood size showed a clear trend of decrease with the increase of Cd concentrations in the water. Moreover, a significant difference was found when comparing the brood size of all the Cd-exposure groups combined (n=38) with the control (n=13), (one-way ANOVA: $F_{(1, 49)}$=7.418, P<0.01, Figure 3.4.7.).

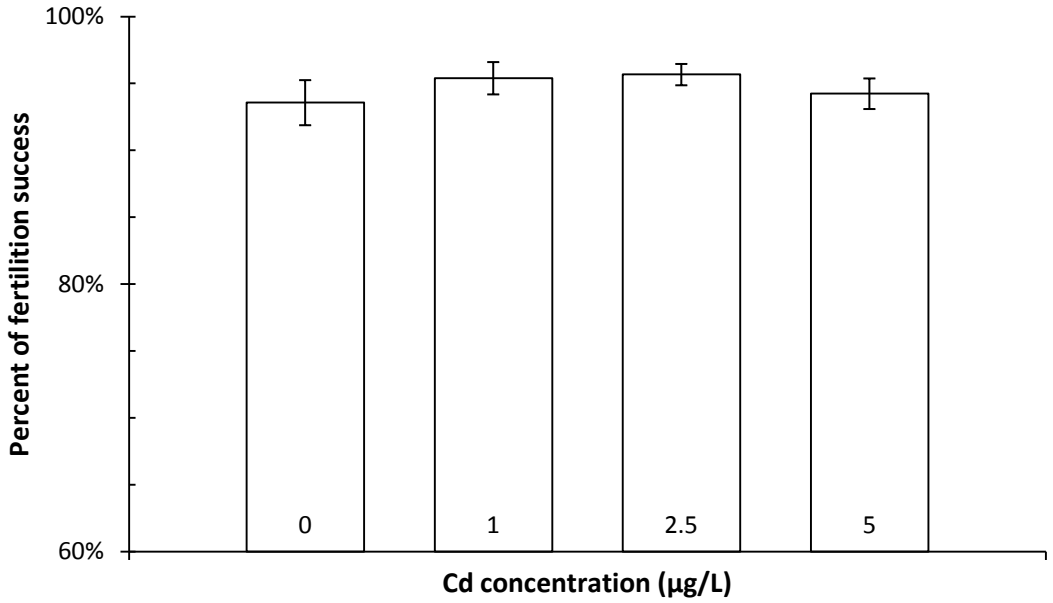

Figure 3.4.5 Percent of fertilization success for the eggs laid by fathead minnows over a 21-day exposure to 0 (control), 1, 2.5 and 5 µg/L of water-borne Cd. Data are presented as mean ±SE (n=10-15).

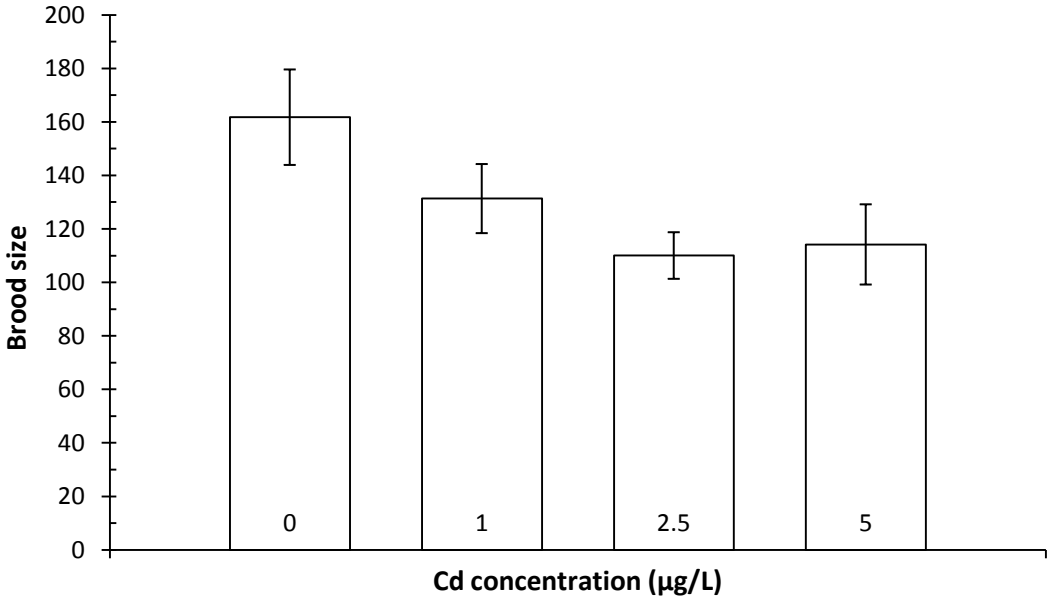

Figure 3.4.6 The average brood size per female during a 21-day exposure to 0 (control), 1, 2.5 and 5 µg/L of water-borne Cd. Data are presented as mean ±SE (n=10-15).

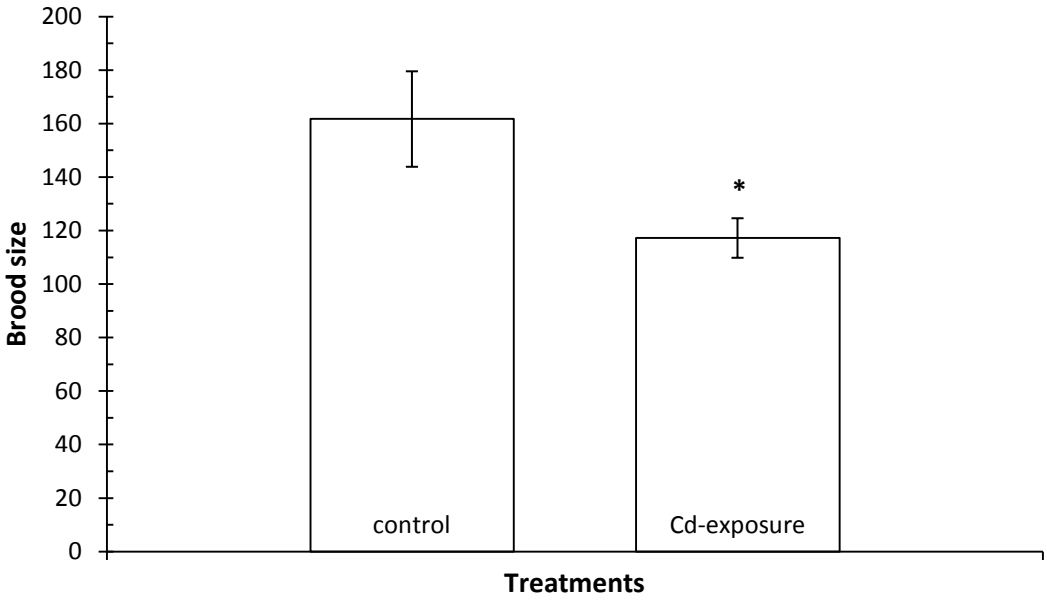

Figure 3.4.7 The average brood size per female of the control and all three Cd-exposure treatments combined during a 21-day exposure phase. Data are presented as mean ±SE (n=13, 38). Asterisk denotes significant difference determined by one-way ANOVA ($P<0.01$).

For cumulative egg production and cumulative number of breeding events, Kolmogorov-Smirnov tests (n=21) showed that there was no significant difference among four treatments (all $P>0.05$), even though cumulative egg production tended to increase faster in the control relative to each of the Cd exposure treatment (Figure 3.4.8. & 3.4.9.).

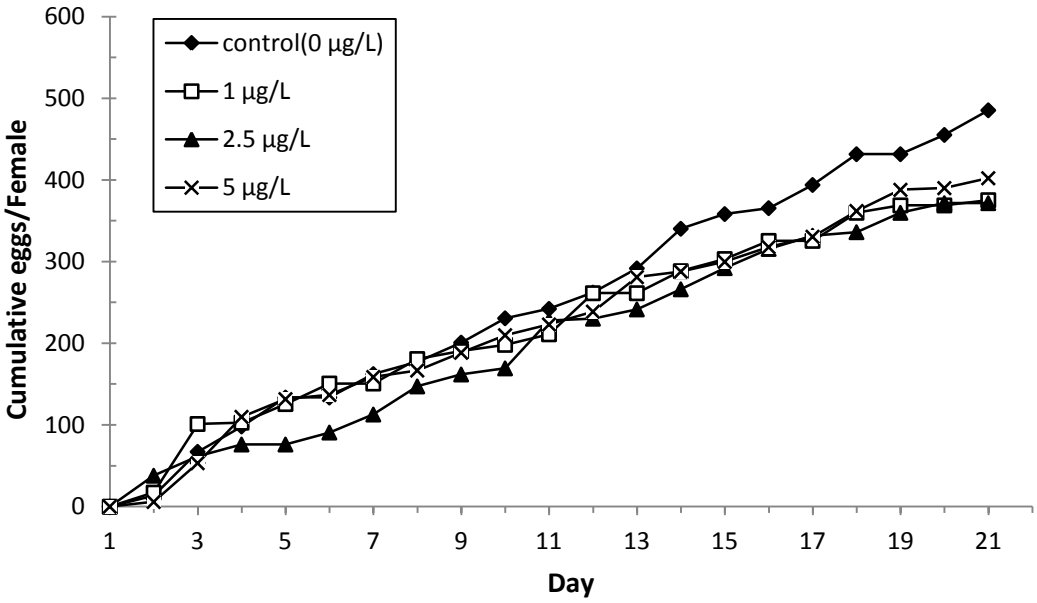

Figure 3.4.8 Cumulative eggs per female over a 21-day exposure to 0 (control), 1, 2.5 and 5 µg/L of water-borne Cd (n=21).

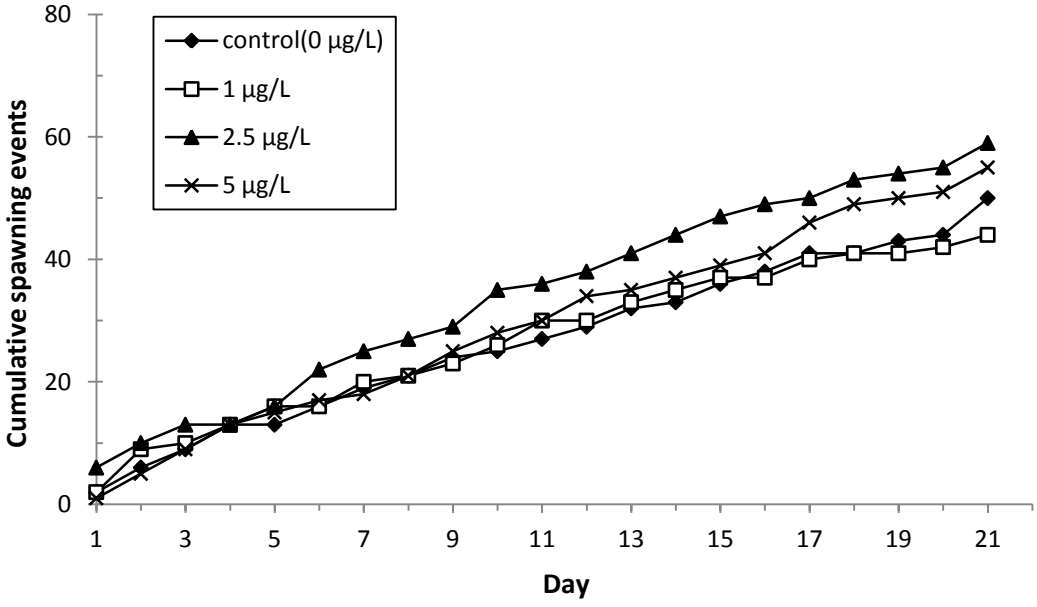

Figure 3.4.9 Cumulative number of spawning attempts of fathead minnows during a 21-day exposure to 0 (control), 1, 2.5 and 5 µg/L of water-borne Cd (n=21).

Egg size after one-day incubation was measured from all broods produced by fathead minnows over the exposure period and analyzed by averaging measurements by tank (n=14 for

control and 2.5 μg Cd/L, n=13 for 1 μg Cd/L and n=15 for 5 μg Cd/L; samples that cannot be measured accurately was excluded). A one-way ANOVA showed there was no significant difference in egg size among treatments ($F_{(3, 52)}$=0.1801, P>0.9, Figure 3.4.10.).

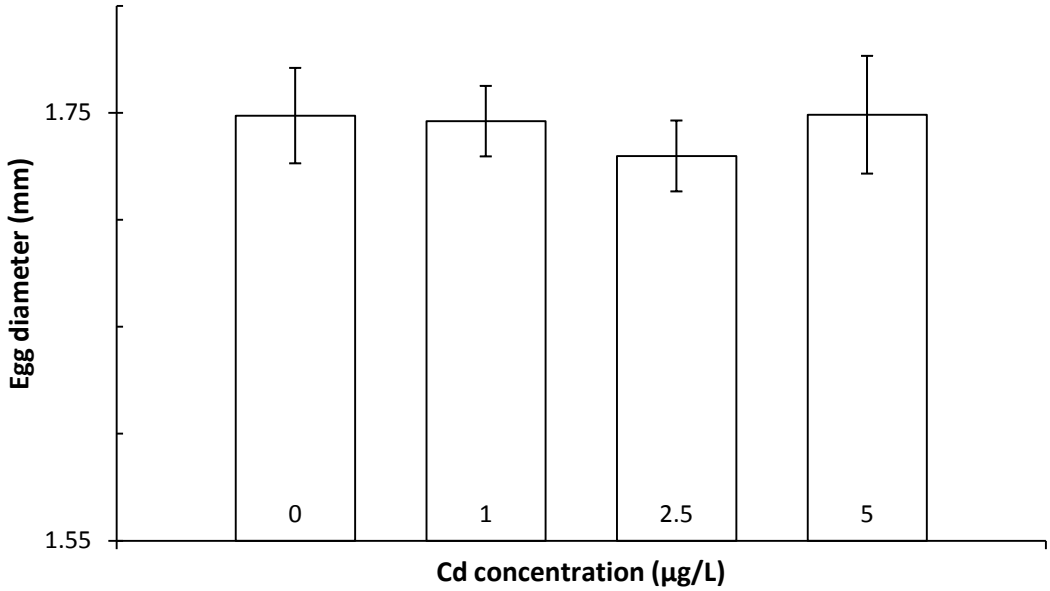

Figure 3.4.10 The average diameter of eggs laid by fathead minnows during a 21-day exposure to 0 (control), 1, 2.5 and 5 μg/L of water-borne Cd. Data are presented as mean±SE (n=13-15).

3.5 Egg Hatching

The length of hatching period and the hatching success were assessed from the broods which were randomly chosen to incubate (n=12 for control and 5 μg Cd/L, n=9 for 1 μg Cd/L and n=13 for 2.5 μg Cd/L). There was no significant difference in the number of days required by each brood to hatch among treatments (One-way ANOVA: $F_{(3, 42)}$=0.4874, P>0.7, Figure 3.5.1.). However, a significant difference was detected when assessing the period from the day a brood was produced to the first egg hatched in this brood (one-way ANOVA: $F_{(3, 42)}$=2.8242, P<0.05), and Tukey post-hoc test revealed that the time required by the eggs of 2.5 μg Cd/L treatment was significantly longer than the control (P<0.04, Figure 3.5.2.). Subsequently, in the

Cd-exposure groups, the time required by the rest of eggs to hatch in the brood were shorter than the control (one-way ANOVA: $F_{(3, 42)}$=3.2706, P<0.04; Tukey post hoc test: control vs. 2.5 µg Cd/L: P<0.04, Figure 3.5.3.).

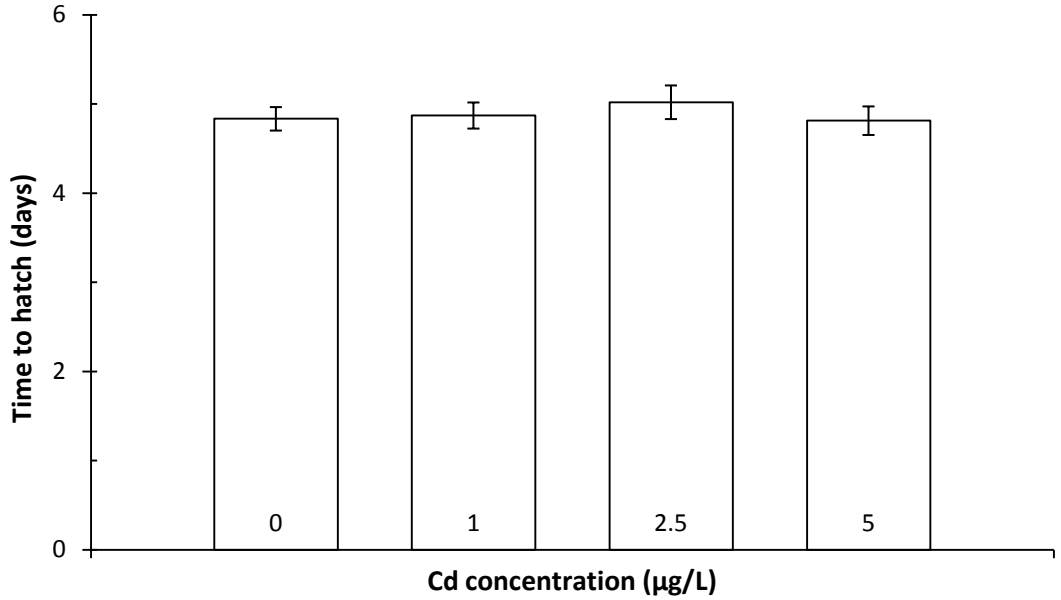

Figure 3.5.1 The number of days required for the eggs to hatch during a 21-day exposure to 0 (control), 1, 2.5 and 5 µg/L of water-borne Cd. Data are presented as mean ±SE (n=9-13).

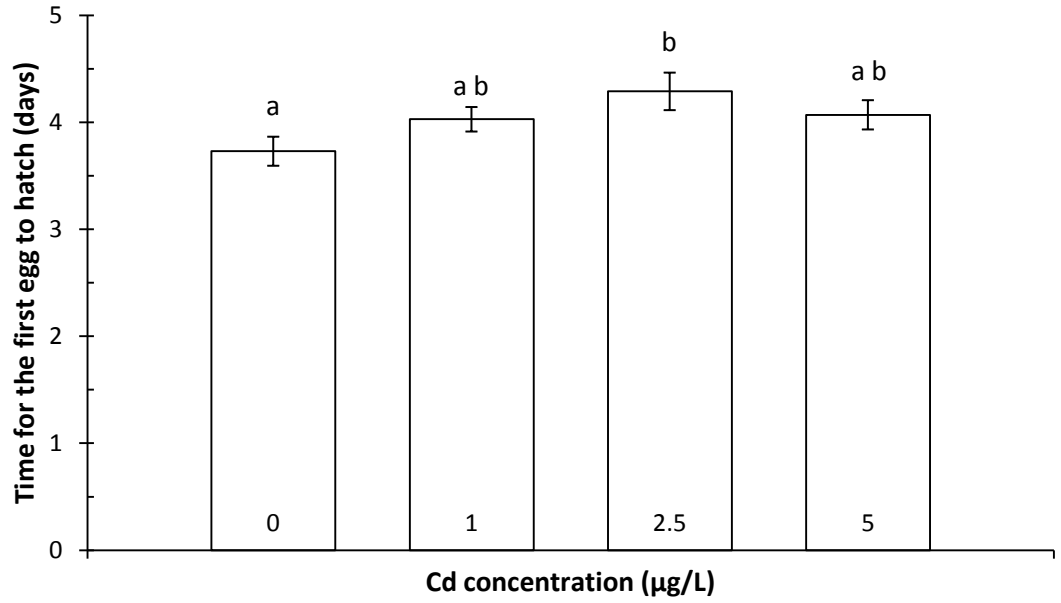

Figure 3.5.2 The number of days required for the first egg of each brood to hatch over a

21-day exposure to 0 (control), 1, 2.5 and 5 μg/L of water-borne Cd. Data are presented as mean±SE (n=9-13). Different letters denote significant differences among treatments, determined by one-way ANOVA followed by Tukey post-hoc test (P<0.04).

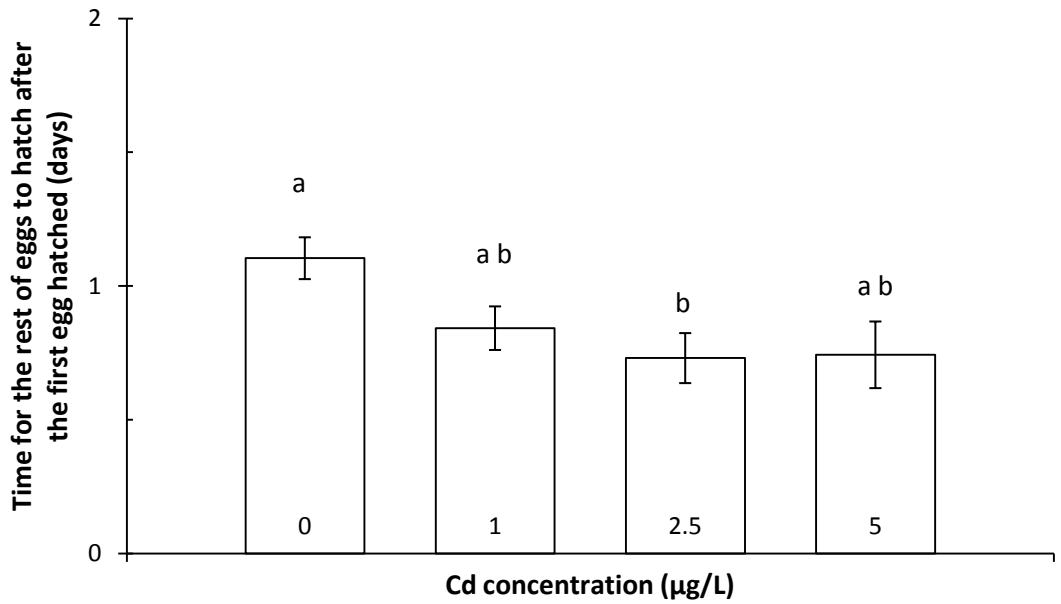

Figure 3.5.3 The number of days required by the rest of eggs in the brood to hatch (after the first egg hatched) over a 21-day exposure to 0 (control), 1, 2.5 and 5 μg/L of water-borne Cd. Data are presented as mean±SE (n=9-13). Different letters denote a significant difference among treatments determined by one-way ANOVA followed by Tukey post-hoc test (P<0.04).

Hatching success of four treatments did not significantly differ from each other (one-way ANOVA: $F_{(3, 42)}$=1.4656, P>0.2). However, hatching success slight decreased when the concentration of Cd increased in the water, indicating that water-borne Cd had some effects on the eggs. (Figure 3.5.4.). A one-way ANOVA further showed that the hatching success of all the Cd-exposed fish combined (n=34) was significantly lower than that of the control (n=12, $F_{(1, 44)}$=4.408, P<0.04, Figure 3.5.5.).

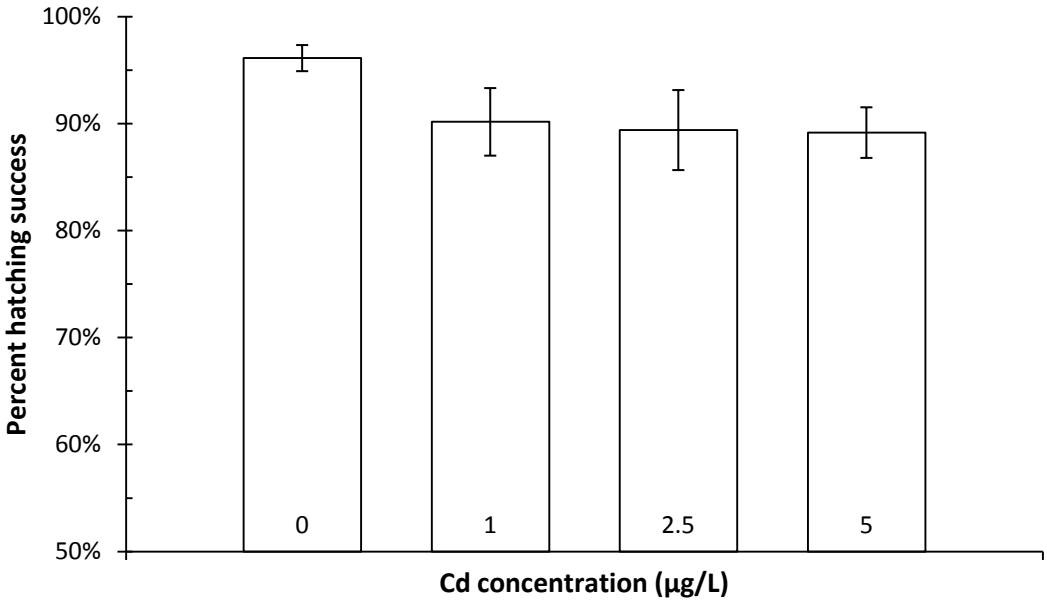

Figure 3.5.4 Percent hatching success of eggs produced by fathead minnows over a 21-day exposure to 0 (control), 1, 2.5 and 5 µg/L of water-borne Cd. Data are presented as mean ±SE (n=9-13).

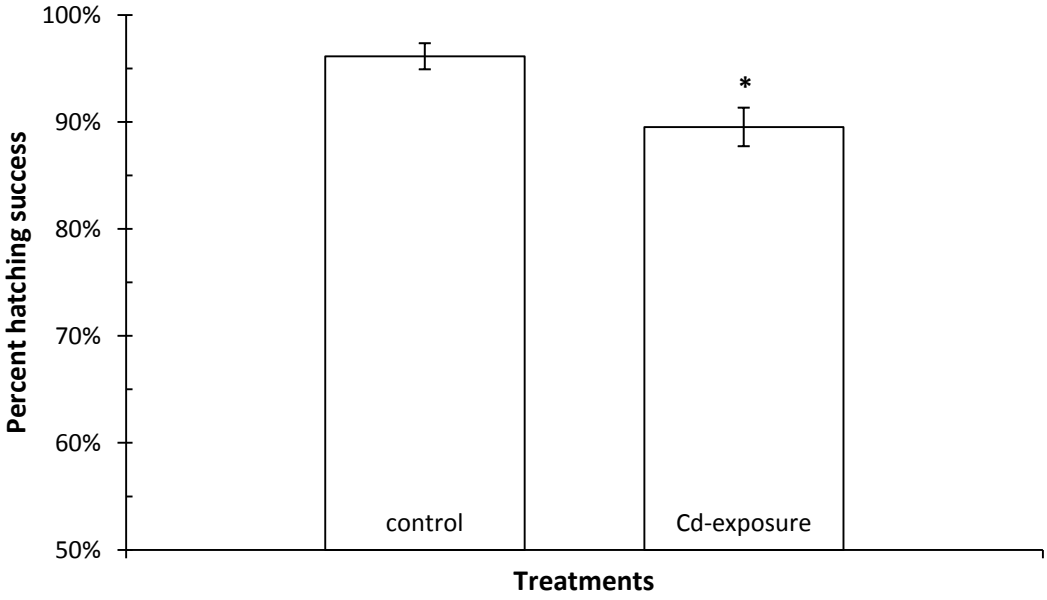

Figure 3.5.5 Percent hatching success of eggs produced by fathead minnows over a 21-day exposure to the control and all three Cd-exposure treatments combined. Data are presented as mean ±SE (n=12, 34). Asterisk denotes significant difference determined by one-way ANOVA ($P<0.04$).

3.6 Larval Deformities

Larval deformities were analyzed by different deformity categories and severity degrees from the broods in which eggs successfully hatched (n=12 for control and 5 μg Cd/L, n=9 for 1 μg Cd/L and n=13 for 2.5 μg Cd/L). When larvae were evaluated by 4 deformity categories separately, including spinal curvature (kyphosis, lordosis and scoliosis), craniofacial malformation, pericardial and yolk sac edema, no significant difference was detected among treatments (one-way ANOVA: spinal curvature: $F_{(3, 42)}$=1.3874, P>0.2; craniofacial malformation: $F_{(3, 42)}$=1.1903, P>0.3; pericardial edema: $F_{(3, 42)}$=0.3143, P>0.8; yolk sac edema: $F_{(3, 42)}$=0.3463; P>0.7, Figure 3.6.1. & Figure 3.6.3.). Similarly, larval deformities assessed based on three severity degrees (negligible, moderate and severe) did not show significant difference among treatments either (one-way ANOVA: negligible: $F_{(3, 42)}$=1.0536, P>0.3; moderate: $F_{(3, 42)}$=0.3501, P>0.7; severe: $F_{(3, 42)}$=0.2066, P>0.8, Figure 3.6.2. &Figure 3.6.3.).

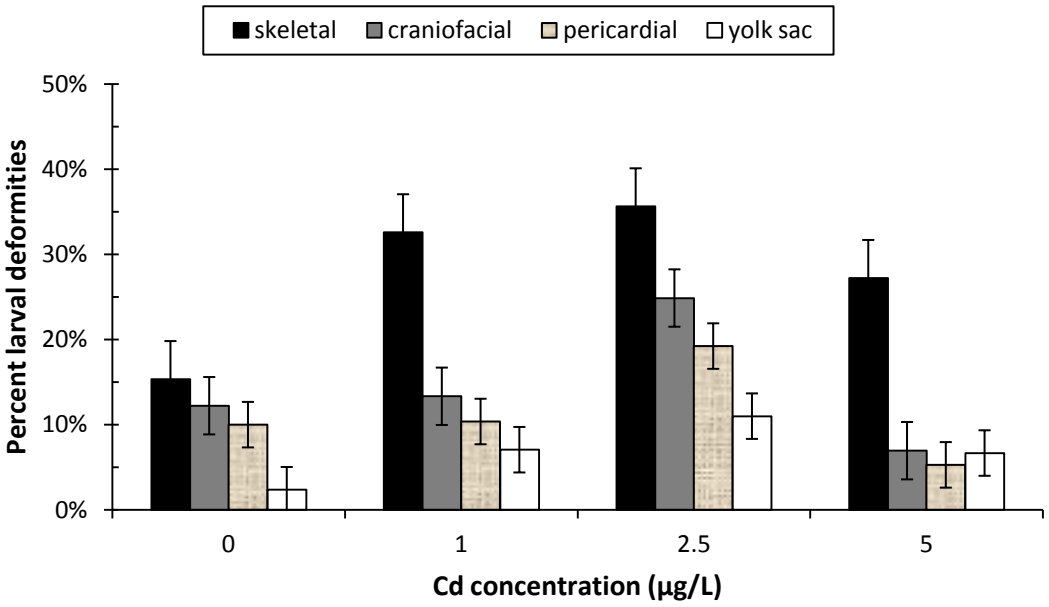

Figure 3.6.1 Percent larval deformities in different categories over a 21-day exposure to 0 (control), 1, 2.5 and 5 μg/L of water-borne Cd. Data are presented as mean ±SE (n=9-12).

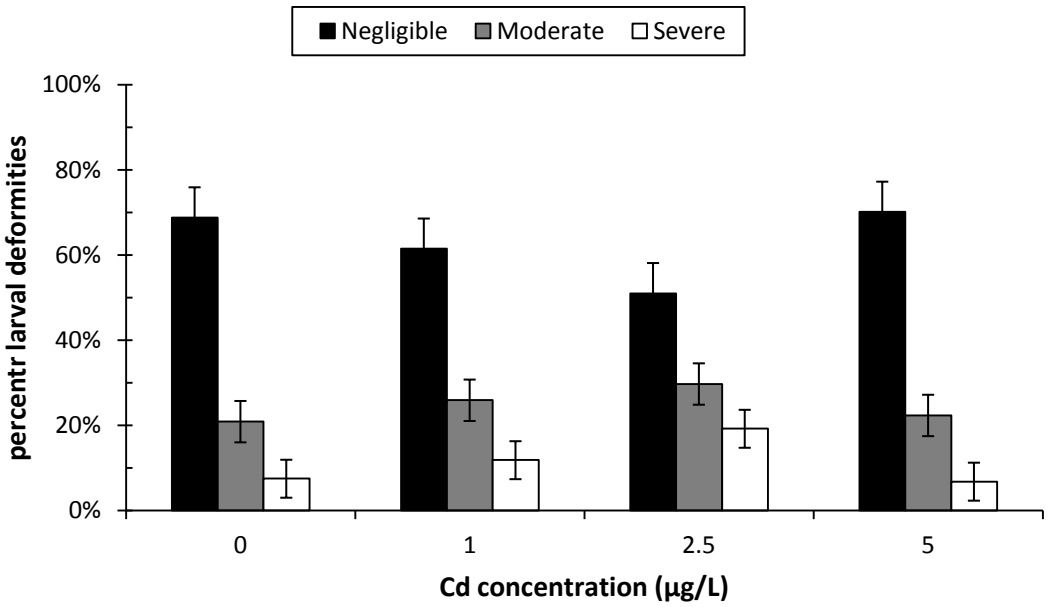

Figure 3.6.2 Percent larval deformities in different severity degrees over a 21-day exposure to 0 (control), 1, 2.5 and 5 µg/L of water-borne Cd. Data are presented as mean ±SE (n=9-13).

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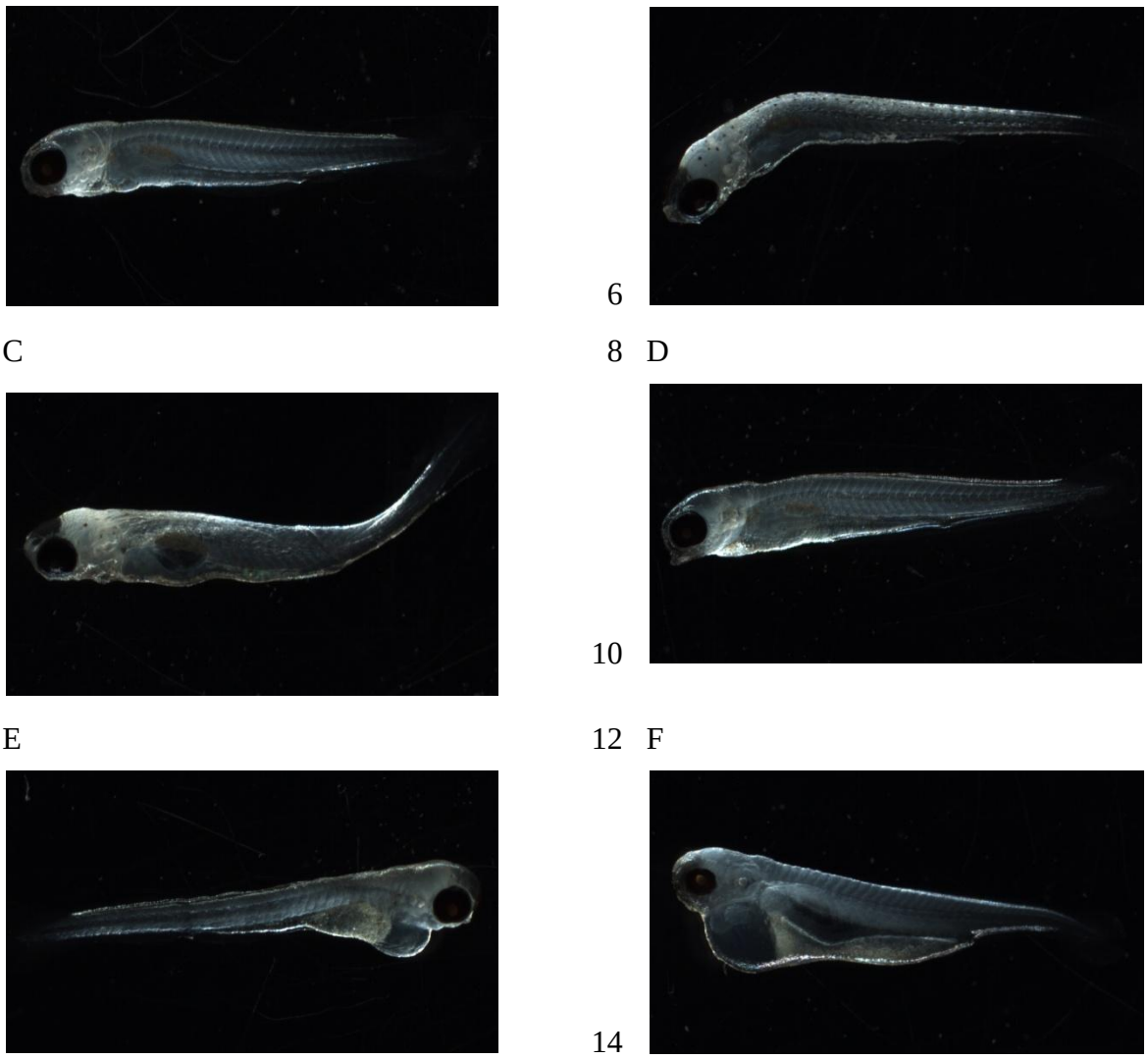

Figure 3.6.3 Deformities observed in fathead minnow larvae.
A: Normal fathead minnow larva
B: Kyphosis
C: Lordosis
D: Jaw malformation
E: Pericardial edema
F: Pericardial and yolk sac edema
G:Kyphosis, jaw malformation and pericardial edema (severe deformity)

3.7 Reproductive Behaviour

The reproductive behavioural analyses included the number of spawning activities, the number of times the male courted the female in or outside the nest, the percent time spent in the nest by female or male and the percent time spent on maintaining the nest by male (n=13 for control, n=12 for 1 μg Cd/L, n=15 for 2.5 μg Cd/L and 5 μg Cd/L). No significant difference was detected by one-way ANOVA among treatments for any of these six behavioural endpoints (spawning: $F_{(3, 51)}$=1.7039, P>0.1; courting in the nest: $F_{(3, 51)}$=0.9292, P>0.4; courting outside the nest: $F_{(3, 51)}$=0.5375, P>0.6; female in the nest: $F_{(3, 51)}$=0.7454, P>0.5; male in the nest: $F_{(3, 51)}$=0.8827, P>0.4; male cleaning the nest: $F_{(3, 51)}$=0.4553, P>0.7). However, the data indicated that in Cd exposure treatment groups, the number of spawning activity and the percent time female spend in the nest both decreased relative to the control. On the other hand, Cd-exposed male courted the female more frequently outside the nest (Figure 3.7.1. & 3.7.2.).

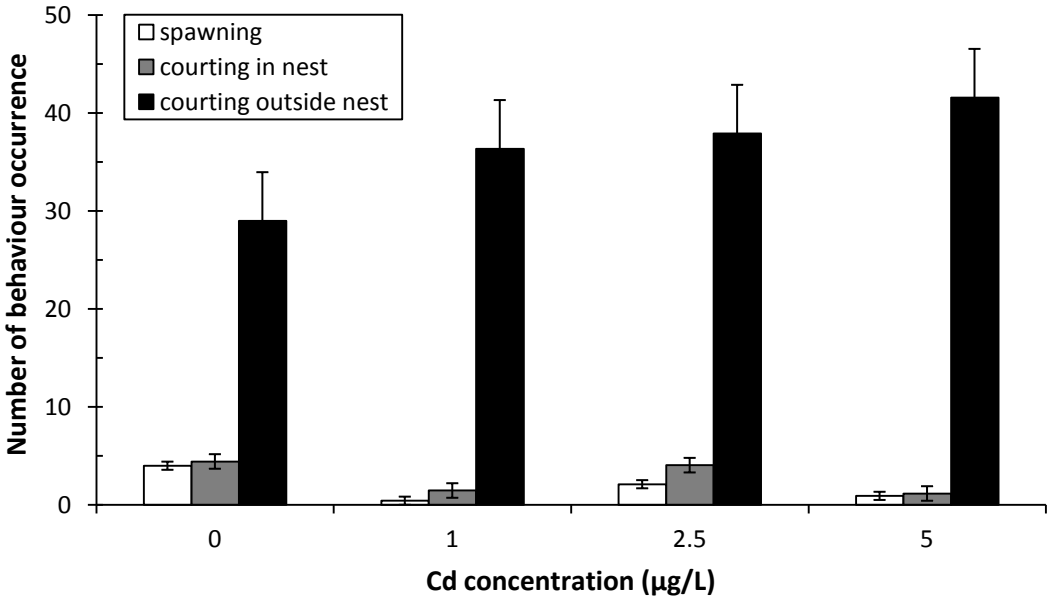

Figure 3.7.1 The average number of the occurrence of reproductive behaviour in a 20 min observation each day during a 21-day exposure to 0 (control), 1, 2.5 and 5 μg/L of water-borne Cd. Data are presented as mean±SE (n=12-15).

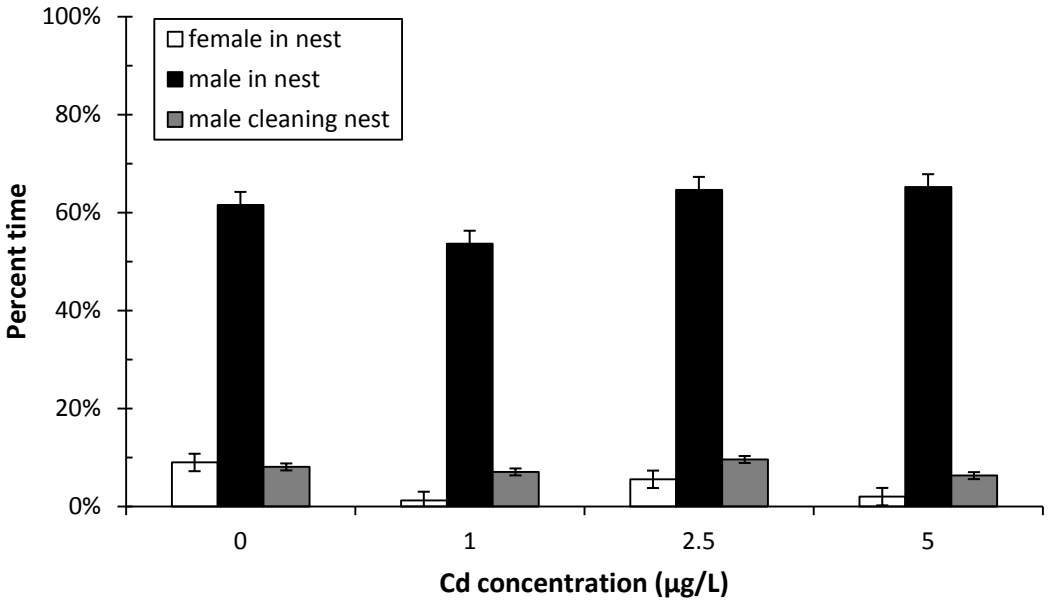

Figure 3.7.2 Percent time spent on reproductive behaviour over a 21-day exposure to 0 (control), 1, 2.5 and 5 µg/L of water-borne Cd. Data are presented as mean ±SE (n=12-15).

3.8 Blood Plasma β-Estradiol Level

Due to the small volume of blood collected from each female fathead minnow, E2 level was analyzed from pooled samples which combined 3-4 fish blood samples within a same treatment (n=3 for control and 2.5 µg Cd/L, n=5 for 1 µg Cd/L, and n=4 for 5 µg Cd/L). No significant difference was found among the four treatments (one-way ANOVA: $F_{(3, 11)}$=1.4051, P>0.2, Figure 3.8.1.).

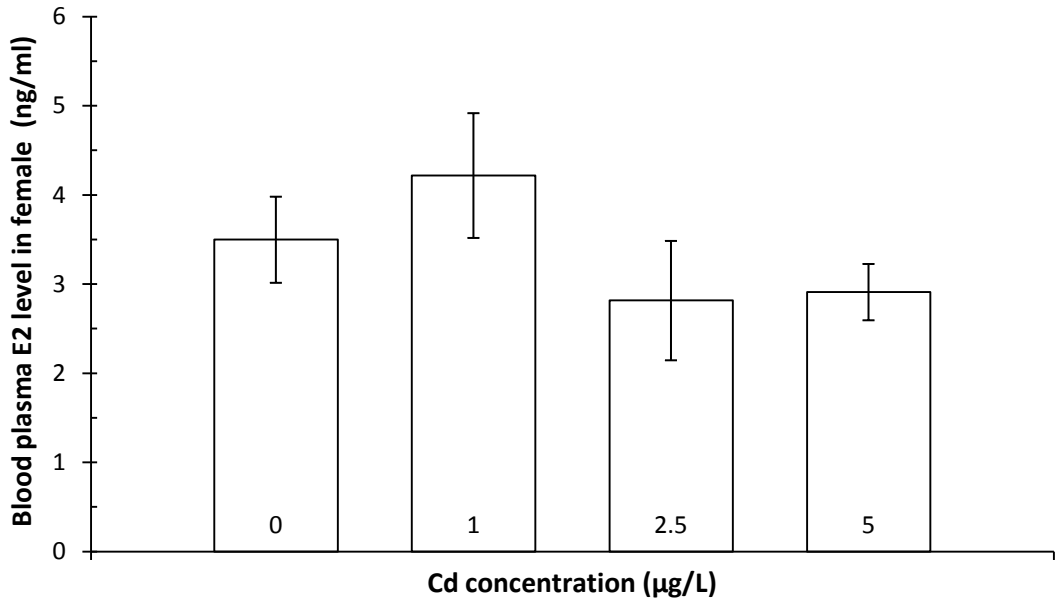

Figure 3.8.1 E2 levels in blood plasma of female fathead minnows after a 21-day exposure to 0 (control), 1, 2.5 and 5 µg/L of water-borne Cd. Data are presented as mean ±SE (n=3-5).

CHAPTER 4. DISCUSSION

4.1 Water Quality

In this experiment, significant differences in water-borne Cd concentration, water temperature and conductivity were detected. However, water temperature was found to have no effect on fathead minnow reproduction in this experiment by stepwise deletion of saturated general linear models. Water conductivity was a significant explanatory variable in water quality for the significant changes in reproductive performance (brood size) in the general linear model. However, the largest difference in water conductivity among treatments was 14.5 μS/cm. It was actually a very small magnitude of change comparing to the control water conductivity (476 μS/cm). Furthermore, the highest water conductivity recorded in this test was 528 μS/cm. It was unlikely that such a low level of water conductivity would have any biological effect on fish reproduction. Additionally, no difference was detected in DO and pH between treatments during the exposure period, while the water-borne Cd concentration of each treatment significantly differed from others. The results of water quality demonstrate that water-borne Cd was the main factor which probably affected the reproduction of fathead minnows in this experiment.

4.2 Reproductive Performance

In toxicology studies, reproductive outputs are important criteria to assess the effects of toxicants on fish population, and are useful for regulatory purposes and risk assessments (Ankley *et al*., 2001). Therefore, reproductive performance endpoints, such as mean egg production, the number of breeding attempts, brood size, fertilization success and egg size were the primary concern in this experiment in order to evaluate potential reproductive effects of Cd on fathead minnows.

While there was no statistically significant difference in mean egg production (eggs per female per day) among four treatments, the data showed that exposed fish appeared to produced fewer eggs than the control on average, suggesting biologically relevant effects (Figure 3.3.1.). The significant difference in mean egg production between the control and

Cd-exposure group (1, 2.5 and 5 μg Cd/L treatment groups combined) further indicated that Cd had adverse effects on fish reproductive performance. Therefore, it would be misleading to conclude that Cd at a concentration ≤ 5 μg/L does not affect the reproductive capacity of fathead minnow based only on the analysis of mean egg production for four treatments separately. The lack of significance may be a simple statistical power issue associated with a small sample size. However, it is to be noted that the sample size was 15 per treatment group, which far exceeds the sample size of 4 recommended by the Organisation for Economic Co-operation and Development (OECD) guidelines for fish reproductive bioassays (OECD, 2009). Low concentrations of Cd used in this experiment might be a potential reason for no significant difference detected among difference Cd levels. However, the comparisons for mean egg production between the pre-exposure and exposure phases showed that significant differences existed within each treatment (Figure 3.3.3.). The eggs produced by the control fish in the exposure period were significantly more than that in the pre-exposure period. In contrast, in the three Cd-exposure treatment groups, egg production in the exposure period significantly declined relative to that in the pre-exposure period. Since each group had identical conditions in either phase except the Cd concentrations in the water, the decreasing trend of egg production in Cd-exposure groups is evidence that even exposing to Cd at 0.64 μg/L can result in reproductive impairment in fish. Caution is therefore needed in interpreting other experiments that have failed to find effects of particular pollutants, since usually, assessments based on short-term reproduction assay would not include the differences in the baseline levels of performance between the pre- and post-exposure phase for individual treatment group while evaluating reproductive effects on fish. That may result in underestimating toxic effects of these pollutants.

No statistical difference was detected in the number of breeding attempts, brood size, egg size or fertilization success. Once again, there was a decreasing trend in brood size of Cd-exposed fish, and a significant difference existed between the control and the three Cd-exposure groups combined. This result indicates that Cd at a concentration ≤ 5 μg/L is able to reduce the brood size of fathead minnow. Given that the number of breeding attempts was

similar in each treatment during the exposure period, lower brood size was likely the primary cause of smaller egg productions in Cd-exposure treatments relative to the control.

Previous studies indicated that female fish inhabiting adverse environments, like water contaminated by Cd, would make a trade-off between the egg size and fecundity to maximize their fitness. Mothers would produce fewer but larger eggs to increase their offspring survival rate since larger eggs would result in larger offspring, and most often, larger young have higher burst swimming speed and survival rate (Hutchings, 1991; Morrongiello *et al*., 2012; Segers & Taborsky, 2011; Segers & Taborsky, 2012). Female fish may also lay more eggs that are varied in size to spread the risk and ensure that at least some offspring can survive (Morrongiello *et al*., 2012). In this experiment, however, fecundity declined in Cd-exposed fish and their egg size did not increase relative to the control. No obvious difference in variance of egg size between treatments was found. These results indicate that in a Cd contaminated environment, female fathead minnows might be affected by compromised quantity and quality of eggs as a result of reproductive impairment. Therefore, Cd exposure probably leads to lower fitness of adult female fish and their offspring.

Mean fertility rates of all the treatments were around 95%, which is the normal fertility level in fathead minnow according to previous experiments (Ankley *et al*., 2001). This result indicates that water-borne Cd exposure at such low concentrations might not affect sperm quantity, quality or spawning behaviour between male and female, which are important factors that influencing fish fertilization success.

The Kolmogorov-Smirnov tests showed that there was no statistically significant result in the cumulative egg production and the cumulative number of breeding attempts when the adult mortality during the exposure period was taken into account. However, differences in cumulative egg production between the control and Cd-exposure treatment groups are apparent (Figure 3.3.8.). It is likely that if the exposure period had been extended, the effects of Cd on fish would have been more obvious. Since Cd tends to accumulate in fish tissues including ovary (Allen, 1995; Pelgrom *et al*., 1995), it is quite reasonable to predict that the longer the exposure to Cd, the more severely fish reproductive performance is impaired, even at

environmentally relevant exposure levels.

4.3 Morpho-metrics and β-Estradiol Level

In this experiment, Cd accumulated in the liver and ovary of exposed female fish in a dose-dependent manner. In the same treatment, the Cd accumulation in the liver was higher than that in the ovary. These results agree with previous reports that Cd had a tendency to bioaccumulate in specific tissues including liver and gonad (Allen, 1995; Pelgrom *et al*., 1995; Rani, 2000).

However, statistical tests of GSI and LSI for both male and female fish did not show any difference among these treatments. While some previous studies focusing on fish reproduction in Cd contaminated environments reported similar results (Pelgrom *et al*., 1995; Sellin & Kolok 2006), there is also evidence that Cd can reduce GSI of both male and female fish (Sellin & Kolok 2006). It is likely that the conflicting results of these studies are related to differences in exposure dose, duration, timing and experimental species. Our experiment indicates that with low exposure levels and short exposure time, Cd probably does not influence the conditions of liver and gonad (ovary and testis) in fathead minnow.

The result of female blood plasma E2 level test in this experiment was less reliable, as the sample size of blood plasma was extremely small. Moreover, previous studies showed that the level of E2 in female blood varied cyclically depending on the spawning cycle, with the highest concentration arising one day after spawning followed by a gradual decline (Jensen *et al*., 2001). Since all the female fish in this experiment were sacrificed within three days after the Cd exposure phase ended, they were likely in different stages of their spawning cycles. Large variances in E2 levels therefore came along with the small sample sizes, which made it more difficult to determine whether Cd altered E2 levels.

Reproduction in fathead minnow is mediated by a series of endocrine factors. Luteinizing hormone (LH), maturation inducing hormone (MIH), maturation promoting factor (MPF) and E2 regulate final oocyte maturation (Clelland & Peng 2009). Ovulation is stimulated by prostaglandin and also influenced by E2 (Clelland & Peng 2009; Young *et al*. 2005). Vtg is

responsible for yolk, lipid droplets and other required substances accumulating in the egg, therefore it influences the size and quality of egg (Young *et al*. 2005). Any factor that affects the functions of reproductive hormones will probably disrupt the normal reproduction of fathead minnow.

Previous studies indicated that Cd affected fish endocrine receptors, Vtg and sex steroids concentrations in fish blood even at sub-lethal levels (Sellin & Kolok 2006; Thomas 1989; Tilton *et al*., 2003). Elevated plasma E2 levels in Cd-exposed fish were observed in several experiments (Foran *et al*., 2002; Thomas 1989). Tilton *et al*. (2003) reported similar results except that E2 level decreased in highest Cd-exposure group (10 μg Cd/L). However, contrary to the *in vivo* results, Cd significantly reduced gonad sex steroids release in *in vitro* tests (Tilton *et al*., 2003). Two possible explanations given by the authors were 1) during *in vivo* exposure, Cd stimulated singling pathways to increase the production of E2 rather than influencing the gonad directly, and 2) the adverse effects of Cd on liver inhibited the metabolism of E2 and resulted in high E2 concentrations left in blood plasma (Foran *et al*., 2002; Tilton *et al*., 2003).

Even though in the present experiment, no difference was detected in blood plasma E2 levels among treatments, there is still a possibility that Cd has effects on E2 or other hormones. Given that Cd exposure reduced the egg production and brood size in this experiment, it is possible that Cd disrupted hormones which involved in the maturation of eggs, then resulted in fewer eggs produced in Cd-exposure groups. Additionally, high Cd concentrations in liver and gonad also indicated that Cd has the potential to influence hormones since these two organs are both important glands of endocrine system.

4.4 Reproductive Behaviour

There is a general consensus that behaviour is a sensitive indicator for toxicology tests (Atchison *et al*., 1987; Faucher *et al*., 2008; Kusch *et al*., 2008; Scott *et al*., 2003; Scott & Sloman 2004). Fish in sub-lethal contaminated environments probably do not show obvious morphological changes or have high mortality, but the fitness of fish are impaired if they cannot respond to stimuli with appropriate behaviour. For example, anti-predator behaviour like

escaping, hiding and shoaling are critical for the survival of fish. An inability to react to alarm cue immediately with appropriate avoidance behaviour inevitably lowers the chance of survival for fish. Specific reproductive behaviour such as courtship and spawning activities of each fish species directly influence their reproductive success, and alternations in timing and occurrence of appropriate reproductive behaviour can disrupt mate selection, reduce fertilization success and the chance of offspring survival in nature. Consequently, evaluating the alternations in fish behaviour is helpful in examining the chronic impacts of contaminants on fish populations. Standard fish behavioural assessments should be included in regulations relevant to species conservation, environment protection or contaminants release (Atchison *et al*., 1987; Scott & Sloman 2004).

Cd was found to disturb fish behaviour mediated by olfactory or lateral system (Blechinger, 2007; Faucher *et al*., 2008; Kusch *et al*., 2008; Scott *et al*., 2003). It is therefore reasonable to hypothesize that Cd also has effects on reproductive behaviour (Sellin & Kolok 2006). Although, the data from this experiment did not seem to support that the reproductive behaviour of fathead minnow could be altered by water-borne Cd exposure at such low concentrations, the number of times the male courting the female outside the nest showed a minor difference between Cd exposure groups and the control (Figure 3.6.1.). Cd exposed male fathead minnows courted their partners more frequently than the unexposed ones. Furthermore, the average times of the outside nest courting in the 5 μg Cd/L treatment group was much more than that in the lower Cd treatment groups. Some studies reported that male fish (like bluegill and brook trout) in Cd contaminated water became hyperactive at the onset of courtship (Benoit *et al*., 1976; Eaton, 1974). Courting female more frequently seems to be an expression of becoming hyperactive for breeding male fathead minnows, and thus these findings are in agreement with the previous studies.

On the other hand, the percent of time exposed female fish spend in the nest was less than the control, followed by a decline in the number of spawning activities between the male and female fish, which was a potential cause of reduced fecundity in Cd exposure treatments (Figure 3.6.1. & 3.6.2.). These results reflect the fact that Cd-exposed female fish may not be willing to

spawn, or not in the optimal breeding conditions. Male fish in Cd treatment groups therefore had to increase their courtship frequency since they needed to spend more effort to convince the female to spawn.

From the regulatory perspective, the assessment of behavioural endpoints could be preferable to other endpoints, particularly traditional physiological endpoints, which are more expensive and tedious. However, the results of reproductive performance and behaviour analyses in this experiment indicate that behavioural endpoints are not as sensitive as traditional endpoints such as egg production and brood size. It may demonstrate that behaviour is not an appropriate indicator in the toxicity assessment when the concentration of a toxicant is low. On the other hand, no significant result in the behavioural analyses in this experiment may also be due to the limit data set of reproductive behaviour. The length of observation time (20 min per tank per day) might not be enough for reproductive behaviour analysis. Even though there was no significant result in the current experiment, behavioural analysis still has potential uses in toxicity assessments. Future studies should extend the time period for behavioural observation to an hour, or have additional cameras available for recording behaviour in each tank every day.

4.5 Egg Hatching

The number of days required by a brood of eggs (15) to hatch was not significantly different among treatments. Most of the eggs hatched within 5 days, which was similar to the results of previous studies (Ankley *et al*., 2001; Jensen *et al*., 2001; U.S. EPA, 2002). However, when the whole hatching period was divided into two portions and analyzed separately, statistically significant differences was revealed. The first portion of the hatching period was the time required by the first egg to hatch in each brood. In this portion, Cd-exposed eggs required longer time than the control, and significant difference occurred between the 2.5 μg Cd/L and the control groups (Figure 3.4.2.). The second portion of the hatching period was the time required by the rest of the brood to hatch after the first egg hatched. The second portion of Cd-exposed eggs was all shorter than the control and again, significant difference was detected between the

2.5 μg Cd/L and the control groups (Figure 3.4.3.).

Multiple studies focused on the effects of Cd on fish egg hatching had conflicting results. Some of them found that Cd extended the development time of fish embryos resulting in delayed hatching (Witeska *et al*., 1995), whereas others reported opposite results that Cd promoted premature hatching of eggs (Jezierska *et al*., 2009; Woodworth & Pascoe 1982). The hatching process is the combination of a biochemical and a behavioral phenomenon. The inhibition of embryo development, chorionase activity and embryo movement could be the causes of delayed hatching in fish eggs (Fraysse *et al*., 2006). On the other hand, stimulation of chorionase activity and abnormal distribution of chorionase which results in the enzyme concentrating in one region of the shell could lead to premature hatching (Lizardo-Daudt & Kennedy, 2008; Woodworth & Pascoe, 1982). In this experiment, these results indicate that Cd exposure may have a dual effect on the hatching of fathead minnow eggs. Cd seemed to delay the hatching of the first egg, resulting in Cd-exposed eggs started to hatch later than the control. Subsequently, Cd promoted the rest of eggs in the brood to hatch more rapidly. Therefore, the whole hatching period of each treatment group had almost the same length. Similar results were found in rainbow trout and common carp early life trials (Lizardo-Daudt & Kennedy, 2008; Witeska *et al*., 1995). Lizardo-Daudt *et al*. (2008) reported that 2.5 μg Cd/L exposure delayed eggs hatching with > 90% hatching occurring on the last day of the hatching period. In the end, the Cd-exposed eggs had the same length of hatching period as the control (Lizardo-Daudt & Kennedy, 2008). They also found premature hatching in rainbow trout eggs at lower Cd concentrations (0.05 and 0.25 μg/L). They concluded that Cd might affect the activity of chorionase as other divalent cations (Ca^{2+} and Mg^{2+}) which inhibit chorionase activity at high concentrations while slightly stimulate it at low concentrations (Lizardo-Daudt & Kennedy, 2008).

No premature hatching was observed in lower Cd exposure group (1 μg/L) in this experiment because 1 μg Cd/L might not be low enough to promote eggs to hatch. The different effects of Cd on the egg hatching before and after the first egg hatched seemed a result of the combination of Cd and certain cues released in the hatching process, which led to promote the

synchronous hatching of fathead minnow eggs in the same brood. Synchronous hatching refers to a situation where all the eggs from the same clutch or even the same population hatch at (more or less) the same time (Weaver, 2010). Synchronous hatching has been observed in many oviparous taxa, including invertebrates (Frechette & Coderre 2000), fishes (Bradbury *et al*. 2005), amphibians (Sih & Moore 1993; Warkentin 1995), turtles (Colbert *et al*., 2010; Doody *et al*., 2001) and birds (Davies & Cooke 1983). Synchrony in the timing of births is thought to have evolved as a general predator avoidance strategy, limiting predation through swamping predators or diluting an individual's risk of predation (Colbert *et al*., 2010; Doody, 2011). Different species rely on different cues to manipulate the hatching period of their eggs in order to achieve synchronous hatching, like sounds, temperature, moisture, light/night alternation, chemical cue released from a broken egg shell or from predators (Bradbury *et al*. 2005; Colbert *et al*., 2010; Doody, 2011). Although the mechanism of synchronous hatching in fathead minnows eggs is still unclear, this experiment demonstrated that Cd might accelerate this process. Considering that the delayed hatching of Cd-exposed eggs might result from inhibiting embryo development (Romhough & Garside, 1982), promoting synchronous hatching could lead to the release of undeveloped larvae, causing increased mortality.

Hatching success had an obvious decreasing trend when the Cd concentrations increased in the water and the statistical test showed a significant difference between the control and the combined Cd-exposure treatments. This result indicated that Cd reduced the hatching success rate of fathead minnow eggs. Multiple studies indicated that the hatching success of Cd exposed eggs significantly decreased (Pelgrom *et al*., 1995; Romhough & Garside, 1982; Williams & Holdway, 2000), even resulting in 100% mortality of eggs in relatively high Cd concentration (e.g. 0.1 mg/L for Australian crimson spotted rainbow fish, see Williams & Holdway, 2000). The death of the embryo, the diminished activity of the embryo with abnormal distribution of chorionase, and the deactivation of proteolytic enzyme could be the causes of hatching failure (Fraysse *et al*., 2006; Hallare *et al*., 2005). According to previous studies, Cd could affect eggs through accumulating in female ovary, and then being deposited into the eggs (Pelgrom *et al*., 1995) or being absorbed by eggs when they were incubated in

Cd-contaminated habitats (Williams & Holdway, 2000). In this experiment, since fertilized eggs were incubated in the identical environmental conditions as their parents, it was not possible to separately evaluate the Cd effects for breeding fish and embryos. Further studies should focus on differentiating the effects of Cd on breeding fish and embryos.

4.6 Larval Deformities

Larval deformities which were evaluated by deformity categories and by severity degrees did not show any significant difference among treatments. Our data also showed no obvious trend except that the percentage of skeletal deformities and yolk sac edema in Cd-exposed larvae were both higher than the control. Cd exposure can cause many types of deformities in different taxa (Nguyen & Janssen, 2002), and spinal deformities are common at low concentrations exposure (Witeska *et al*., 1995; Williams & Holdway, 2000). Jezierska *et al*. (2009) argued that these deformities in new hatchlings probably resulted from the reduction of egg swelling. Smaller egg means less space for the embryo to move. Witeska *et al*. (1995) observed that the swelling of common carp eggs was highest in the control (40.0%), and decreased with Cd exposure. On the other hand, abnormal cell development of future vertebral or muscle tissue could result in spinal deformities as well. Cd might induce larval deformities through disturbing ectopic expression of developmental regulatory genes (Cheng *et al*., 2000; Williams *et al*., 2000).

Although larvae with slight deformities like abnormal spinal curvatures could continue to survive in the laboratory, their fitness was indeed impaired. The predation risk for deformed larvae would increase and they would have decreased competitive ability relative to their siblings. In the wild, deformed larvae would be not likely to survive and reproduce (Williams & Holdway, 2000).

4.7 Breeding Fish Survival

Even though there was no statistical difference, the results of adult (mainly female) fish survival were enigmatic, as more female death occurred in lowest Cd-exposure treatment group (1 μg Cd/L) rather than in the highest one (5 μg Cd/L). During the first half of the exposure period

(1^{st}-11^{th} day), two females from the control and one from the 1 μg Cd/L group died with swelled abdomen, which had this symptom and ceased spawning for 4-5 days on average before their death. Since most of the dead females were from the control group and the similar symptom was observed in the pre-exposure period, it is likely that Cd was not the primary cause of female death in the first half of the exposure period.

In the latter half of the exposure period (12^{th}-21^{st} day), three females in the 1 μg Cd/L and two in the 2.5 μg Cd/L treatment groups died with totally different symptoms compared to that mentioned above. The fins and tails of these female had undergone gradual rotting, females therefore lost their balance gradually and were unable to swim in the end. These symptoms were all found after one day of spawning, and these female died within 1-2 days when they had the symptoms. These symptoms seemed a result of bacterial or fungal infections in fish. Other studies have indicated that 2 μg Cd/L could result in a reduced macrophage-mediated immune function in fish which would translate into a reduced ability to fight off bacterial or fungal infections as well as other diseases (Kusch *et al*., 2008). Therefore higher female mortality occurred in the low (1 μg Cd/L) and medium (2.5 μg Cd/L) Cd-exposure treatment groups than the control possibly resulted from Cd reducing the resistance of fish to diseases. However, in the 5 μg Cd/L treatment group, high Cd concentration might also inhibit the growth of pathogens, therefore, female mortality declined in this treatment.

4.8 Conclusions

Table 4.8.1 The summary of the reproductive endpoints measured in this experiment.

Reproductive endpoint	Significant change	No significant change
Mean egg production[a]	√	
Brood size[a]	√	
No. of breeding attempts		√
Fertilization success		√
Egg size		√
Reproductive behaviour		√
Hatching success[a]	√	
Hatching period (whole)		√
Hatching period (first egg)	√	
Hatching period (rest of eggs)	√	
Larval deformities		√
Cd accumulation in liver/ovary	√	
LSI/GSI		√
Female blood plasma E2 level		√
Survival of breeding fish		√

[a] The significant effect of Cd on the endpoint was detected when comparing the control with three Cd treatments combined.

This research demonstrates that chronic exposure to water-borne Cd at environmentally relevant levels (≥ 0.64 μg/L) can impair reproduction of fathead minnow through reducing brood size and fecundity, which probably results from Cd significantly accumulating in female ovary and liver. Moreover, Cd has complex effects on eggs hatching: it delays egg hatching at the beginning while promotes synchronous hatching afterwards. Meanwhile, hatching success decline in the exposed eggs indicates that Cd also influences the quality of eggs or embryo development or both. No significant result was found in reproductive behaviour analyses, which provides evidence that traditional endpoints used in standard testing (e.g. egg production, brood size or hatching success) were more sensitive to Cd exposure than the behavioural endpoints examined in this study. Behavioural analyses, however, are still potentially useful in toxicants assessments.

This is the first study to conduct a comprehensive assessment of chronic Cd exposure on

both reproductive capacity as well as behaviour in fish. The findings of this study shows the adverse effects of Cd on reproductive performance in fish at environmentally relevant concentrations and that the traditional protocols used to identify effects of toxicants may underestimate their adverse effects. They thus have important implication for protecting fish populations in Cd or other toxicants contaminated aquatic systems.

Due to the limitations in this experiment, some effects of Cd on fish reproduction were not statistically significant, nevertheless, they could be biologically relevant. Additionally, difficulty will arise when utilizing laboratory results to predict the effects in a natural environment, as relatively ideal conditions were used in this experiment. Future work in this area should focus on adjusting experimental conditions in order to simulate natural condition as well as extending the exposure period and sample size. The mechanisms by which Cd impairs eggs production or inhibits embryo development also require further studies.

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