The Effects of Various Levels of Dissolved Oxygen on Fish Reproduction

A Thesis Submitted to the College of

Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree Masters Of Science

in the Department of Biology

University of Saskatchewan

Saskatoon

By Shelly E. Fisher

© Copyright Shelly E. Fisher December 2009. All Rights Reserved.

Permission to use

In presenting this thesis in partial fulfillment of the requirements for a postgraduate degree from the University of Saskatchewan, I agree that the libraries of this university may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Request for permission to copy or make other use of material in this thesis in whole or in part should be addressed to:

Head of the Department of Biology
University of Saskatchewan
112 Science Place, Saskatoon
Saskatchewan S7N 5E2

Abstract:

Adequate levels of dissolved oxygen (DO) are essential to the health of most aquatic organisms. While diel fluctuations in DO concentration are a normal occurrence in aquatic ecosystems, anthropogenically-produced periods of prolonged hypoxia have the potential to cause changes in growth, reproduction and behaviour in animals. My thesis examined reproductive behaviour and physiology of fathead minnows (*Pimephales promelas*) following exposure to several concentrations of dissolved oxygen. Using a custom-built system that was able to maintain DO concentrations at precise levels, reproductive performance was analyzed under 3.5 mg/l, 4.5 mg/l, 5.5 mg/l and a control of 7.5 mg/l of DO. A second experiment evaluated reproductive performance at 5.0, 5.7, 6.5 and 7.5(control) mg/L.

Breeding attempts ceased altogether at 4.5 mg/l and lower. At higher concentrations, the effects of DO on reproductive output were contradictory between experiments. When DO was maintained at 5.5 mg/L in the first experiment, egg production was lower than in the controls. When DO levels of 5.7 mg/L were used in the second experiment, egg production was higher than in the controls. Courtship behaviour decreased significantly compared to the control at DO levels of 4.5 mg/L and lower. No significant differences were observed between treatments in morphometrics, survival, larval deformities, sex steroid levels, vitellogenin levels, hatching success, egg size, fertility, or gonad histology.

The results of this study demonstrate that reproductive behaviour may represent a sensitive early marker of reproductive impairment in fathead minnows. Inconsistencies between the two experiments suggest a possible hormetic effect in response to depressed

DO in fathead minnows. My results have important implications with respect to Canadian water quality guidelines and applications in the restoration of aquatic systems with lowered DO due to human activities.

Acknowledgements

I would like to thank my supervisor, Dr. Doug Chivers for all the help and support he provided throughout this project. I would also like to thank the members of my advisory committee, Drs. Lynn Weber and Som Niyogi for their excellent advice and ideas. Thank you to my lab mates, Allison Squires, Lisa Ramillo, Melissa Dreissnack, Dennis Duro and Aditya Manek for all their input and support. Thanks to Maud Ferrari for her guidance with statistics and for all the editing. I would additionally like to thank Mike and Robyn Pollock, who provided me with much guidance throughout this whole process. This study was initiated by Monique Dube in association with Domtar Pulp and Paper.

Funding for this research and personal stipend was provided by an NSERC CRD grant, an NSERC Discovery grant, an NSERC IPS scholarship, and through the Department of Biology.

Table of Contents:

Permission to use…………………………………………………………………….……..…i
Abstract………………………………………………………………………….......ii
Acknowledgements………………………………………………………………………iv
Table of contents………………………………………………………………………..v
List of tables…………………………………………………………………..…vii
List of figures………………………………………………………………….....vii
List of abbreviations……………………………………………………………...xi

Chapter 1: Introduction…………………………………………………...……..…1
1.1 General information ……………………………………………….…....1
1.2 Known effects of low DO on fishes…………………………….….……2
1.3 Strategies and species differences in low DO environments…………….......3
1.4 Test species: Fathead minnow life history………………………….………..4
1.5 Normal gamete and hormone development in the fathead minnow……..…...7
1.6 Research objectives………………………………………………………..10

Chapter 2:
Methods……………………………………………………………………...…..11
2.1 Fish maintenance ……………………………………………………11
2.2 Pre-exposure period……………………………………………..…...11
2.3 Criteria for selection of test fish…………………………………….......12
2.4 Experimental apparatus……………………………………………………...13
2.5 Exposure conditions and procedures……………………………………...15
2.6 Analysis of reproductive potential………………………………………...16
2.7 Analysis of survival and morphometrics…………………………..…...17
2.8 Analysis of reproductive behaviour……………………………….…...18
2.9 Histology and hormone analysis……………………………………......19
2.10 Experiment 1: The effects of various levels of dissolved oxygen on fathead minnows.……………………………… ………….……...21
2.11 Experiment 2: The threshold for reproductive impairment in fathead minnows exposed to several concentrations of dissolved oxygen………………………………………………………….…....21
2.12 Statistical analysis……………………………………...………..23

Chapter 3: Results…………………………………………....……………………24
Experiment 1 The effects of various levels of dissolved oxygen on fathead minnows. ……………………………….……...….………24
3.1 Water quality…………………………………………………..……...24
3.2 Reproductive endpoints……………………………………….….……27
3.3 Survival and morphometrics……………………………...…...……...33
3.4 Histology ……………………………………………...…...…...…..36
3.5 Hormone and vitellogenin analysis……………………………….…...44
3.6 Behavioural data…………………………………………….……….…...48

Experiment 2: The threshold for reproductive impairment in fathead minnows exposed to several concentrations of dissolved oxygen……………………………………………………………….…….50
3.7 Water quality………………………………………………………......50
3.8 Reproductive endpoints…………………………………….…………53
3.9 Survival and morphometrics……………………………………..……58
3.10 Behavioural data……………………………………………………..…60

Chapter 4: Discussion………………………………………….……….………63
4.1 Water Quality……………………………………….…………………63
4.2 Reproductive endpoints……………………………..…...…………...63
4.3 Survival and Metrics…………………………………….….……………68
4.4 Histology…………………………………………………………………70
4.5 Hormone and vitellogenin analysis………………………………………72
4.6 Behaviour………………………………………………………………..74
4.7 Conclusions ………………………………………………………...77

Literature cited…………………………………………………………………..…79

List of Tables

Table 3.1.1. Water quality parameters measured daily over an 11-day exposure to 3.5, 4.5, 5.5, and 7.5 (control) mg/L of dissolved oxygen………………….26

Table 3.3.1. Morphometric endpoints from fathead minnows after an 11-day exposure to 3.5, 4.5, 5.5 and 7.5(control) mg/L dissolved oxygen………………………35

Table 3.7.1. Water quality parameters measured daily over an 21-day exposure to 5.0, 5.7, 6.4, and 7.5 (control) mg/L of dissolved oxygen………………….52

Table 3.9.1. Morphometric endpoints from fathead minnows after a 21-day exposure to 5.0, 5.7, 6.4, and 7.5 (control) mg/L dissolved oxygen……………………..60

List of Figures

Figure 2.1. Experimental apparatus……………………………………………...………14

Figure 3.1.1. Mean (± standard error) dissolved oxygen concentration measured from four treatment groups during an 11-day exposure period……………….25

Figure 3.1.2. Mean (± standard error) water temperature over an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5 (control) mg/L of dissolved oxygen…………..26

Figure 3.2.1. Percent fertilization success (± standard error) for eggs laid by fathead minnows over an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5 mg/L of dissolved oxygen……………………………………………...…. 28

Figure 3.2.2. Mean number of days required to hatch (± standard error) for eggs laid by fathead minnows over an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen……………………………...…..29

Figure 3.2.3. Percent hatching success (± standard error) for eggs laid by fathead minnows over an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen………………………………………...……29

Figure 3.2.4. Percent larval deformities (± standard error) for newly-hatched offspring produced by fathead minnows over an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen…………………………………….30

Figure 3.2.5 Deformities observed in fathead minnow larvae…………………………...31

Figure 3.2.6. Cumulative eggs/female produced by fathead minnows over an 11- day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen……………………………………………………………………………32

Figure 3.2.7. Mean (+ SE) of the average number of eggs produced per female per day over an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen…………………………………………………………………...….32

Figure 3.2.8. Mean (+ SE) of the total number of eggs produced per female per day over an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen…………………………………………………………..33

Figure 3.3.1. Percent survival (+ standard error) of fathead minnows over an 11-day exposure to 3.5, 4.5, 5.5 and 7.5(control) mg/L dissolved oxygen………...…34

Figure 3.4.1. Representative cross sections of fathead minnow testes after exposure to 3.5 (A), 4.5 (B), 5.5 (C) and 7.5 (D) mg/L DO……………………………. 37

Figure 3.4.2. Percentage of spermatogonia (+ standard error) present within cross sections of fathead minnow testis after an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen………………………………..39

Figure 3.4.3. Percentage of spermatocytes (+ standard error) present within cross sections of fathead minnow testis after an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen………………………………..39

Figure 3.4.4. Percentage of mature sperm (+ standard error) present within cross sections of fathead minnow testis after an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen………………………...……40

Figure 3.4.5. Representative cross sections of fathead minnow ovaries after exposure to 3.5 (A), 4.5 (B), 5.5 (C) and 7.5 (D) mg/L DO……………………...……41

Figure 3.4.6. Percentage of early stage(1-3) oocytes (+ standard error) within cross sections of fathead minnow ovaries after an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen…………………….. 43

Figure 3.4.7. Percentage of late stage(4-5) oocytes (+ standard error) within cross sections of fathead minnow ovaries after an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen……………….……..43

Figure 3.4.8. Percentage of atreitic oocytes (+ standard error) within cross sections of fathead minnow ovaries after an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen……………………………...…..44

Figure 3.5.1. Mean estradiol level (± standard error) in the gonad tissue of female fathead minnows after an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen……………………………………….45

Figure 3.5.2. Mean testosterone level (± standard error) in the gonad tissue of female fathead minnows after an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen…………………………..……….46

Figure 3.5.3. Mean estradiol level (± standard error) in the gonad tissue of male fathead minnows after an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control)…………………………………………………………..……46

Figure 3.5.4. Mean testosterone level (± standard error) in the gonad tissue of male fathead minnows after an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen………………………………….…..47

Figure 3.5.5. Mean vitellogenin level (± standard error) in the liver tissue of male fathead minnows after an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen……………………………………..……47

Figure 3.6.1. Percent time spent in the nest (± standard error) by male fathead minnows over an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5 (control) mg/L dissolved oxygen………………………………………………………..49

Figure 3.6.2. Percent time spent on nest maintenance (± standard error) by male fathead minnows over an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5 (control) mg/L dissolved oxygen……………………………………….…….49

Figure 3.6.3. Time spent in courtship (± standard error) by male fathead minnows over an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5 (control) mg/L dissolved oxygen…………………………………………………..……50

Figure 3.7.1. Mean (± standard error) dissolved oxygen concentration measured from four treatment groups during a 21-day exposure period…………….…….51

Figure 3.7.2. Mean (± standard error) water temperature over a 21-day exposure period to 5.0, 5.7, 6.4, and 7.5 (control) mg/L of dissolved oxygen…………..52

Figure 3.8.1. Percent fertilization success (± standard error) for eggs laid by fathead minnows over a 21-day exposure period to 5.0, 5.7, 6.4, and 7.5 mg/L of dissolved oxygen…………………………………………………...….54

Figure 3.8.2. Percent hatching success (+ standard error) for eggs laid by fathead minnows over a 21-day exposure period 5.0, 5.7, 6.4, and 7.5(control) mg/L of dissolved oxygen……………………………………………..……55

Figure 3.8.3. Mean number of days required to hatch (+ standard error) for eggs laid by fathead minnows over a 21-day exposure period to 5.0, 5.7, 6.4, and 7.5(control) mg/L of dissolved oxygen…………………………………..…55

Figure 3.8.4. Percent larval deformities (+ standard error) for newly-hatched offspring produced by fathead minnows over a 21-day exposure period to .0, 5.7, 6.4, and 7.5(control) mg/L of dissolved oxygen…………………………………56

Figure 3.8.5. Mean diameter (+ SE) of newly-laid fathead minnow eggs during a 21-day exposure period to 5.0, 5.7, 6.4, and 7.5(control) mg/L of dissolved oxygen. Eggs were measured within several hours of laying………...……56

Figure 3.8.6. Cumulative eggs/female produced by fathead minnows over a 21- day exposure period to 5.0, 5.7, 6.4, and 7.5(control) mg/L of dissolved oxygen………………………………………………………………………57

Figure 3.8.7. Mean (+ SE) of the average number of eggs produced per female per day over a 21-day exposure period to 5.0, 5.7, 6.4, and 7.5(control) mg/L of dissolved oxygen…………………………………...……….. 57

Figure 3.8.8. Mean (+ SE) of the total number of eggs produced per female per day over a 21-day exposure period to 5.0, 5.7, 6.4, and 7.5(control) mg/L of dissolved oxygen…………………………………………………………...……58

Figure 3.9.1. Mean survival (+ standard error) of fathead minnows over an 11-day exposure to 5.0, 5.7, 6.4, and 7.5(control)mg/L dissolved oxygen……...……59

Figure 3.10.1. Percent time spent on courtship (+ standard error) by male fathead minnows over a 21-day exposure period to 5.9, 5.7, 6.4, and 7.5 (control) mg/L dissolved oxygen……………………………………………….…61

Figure 3.10.2. Percent time spent in the nest (+ standard error) by male fathead minnows over a 21-day exposure period to 5.9, 5.7, 6.4, and 7.5 (control) mg/L dissolved oxygen………………………………………………..……62

Figure 3.10.3. Percent time spent on nest maintenance (+ standard error) by male fathead minnows over a 21-day exposure period to 5.9, 5.7, 6.4, and 7.5 (control) mg/L dissolved oxygen………………………………………………..……62

List of Abbreviations

ANOVA = analysis of variance

ANCOVA = analysis of covariance

ºC = degrees celsius

CF = condition factor

cm = centimeter

DO = dissolved oxygen

ELISA = enzyme linked immunosorbent assay

g = gram

g/cm^3 = gram per cubic centimeter

GSI = gonadosomatic index

L = liter

L:D = light:dark

LSI = liversomatic index

mg = miligram

mg/L = milligrams per litre

mm = millimeter

MS222 = Tricaine Methanesulfonate

ng/g = nanograms per gram

PVC = Polyvinyl chloride

µS/cm = microsiemens per centimeter

Chapter 1: Introduction

1.1 General information

Good water quality is essential to the survival and reproduction of fishes and other aquatic organisms. Changes in water quality parameters, such as temperature, dissolved oxygen, pH, turbidity, and flow rate, as well as the presence of toxicants may result in immediate mortality or provoke long-term changes in community structure through impairment of reproduction, recruitment or other essential ecological processes (Karr & Dudley, 1981; Lloyd, 1987; McCormick et al., 1989). While many natural events alter water quality, there is great potential for anthropogenic influences to degrade habitat quality such that populations are negatively affected.

In aquatic environments, dissolved oxygen concentration can represent a limiting factor, as oxygen availability is lower than in terrestrial environments and the energetic costs of oxygen extraction are higher (Kramer 1987). DO naturally fluctuates seasonally and annually in aquatic systems and can be affected by changes in temperature, surface mixing, and chemical and biological oxygen demand (Breitberg, 2002). While depressed DO was once associated primarily with natural phenomena, more attention has recently been directed towards changes in DO in response to human activity (Rosenberg et al. 1991, Eby & Crowder, 2002; Landry et al, 2007). Numerous anthropogenic influences can also result in lowered DO, due to both increased chemical oxygen demand and eutrophication (Breitberg, 2002). DO may be lowered by industrial discharges, municipal waste water, and a variety of other pollutants and has caused extensive dead zones in many aquatic systems worldwide.

Canadian water quality guidelines for warm water ecosystems recommend DO

levels of 5 mg/L. It is known that hypoxia, defined as a concentration of DO below 2 mg/L (Thomas et al., 2006; Pihl et al., 1991), can directly result in fish mortality and can occasionally cause massive fish kills. Moderate depressions in DO may affect fish from an ecological perspective, limiting growth, locomotion, or reproductive capacity (Kramer 1987). Therefore, depressed DO has the potential to disrupt fish populations at concentrations exceeding those which cause immediate mortality.

1.2 Known effects of low DO on fishes

As certain levels of DO are essential to fish survival and reproduction, prolonged decreases in DO have the potential to cause severe effects on fish populations. For example, it has been demonstrated that with declining oxygen concentrations, the abundance and diversity of demersal fish declines (Pihl, 1991). Hypoxia has also been documented to induce changes in species composition in aquatic environments (Thomas et al., 2007).

Studies performed on a wide variety of species have shown a range of effects in response to hypoxia including reduced growth, reduced fertility and gamete production, altered sex steroid levels, lowered GSI, and lowered hatching success (Landry et al., 2007; Wu et al., 2003; Thomas et al., 2006; Thomas et al., 2007; Wang et al, 2008).

Given that these effects have been demonstrated in several different species, and that organic pollution and eutrophication have caused hypoxic conditions in numerous ecosystems worldwide, hypoxia may represent a more serious ecological risk than the majority of anthropogenic chemicals (Wu et al., 2003). It is also possible that artificially

lowered DO may render an ecosystem more susceptible to other forms of stress (Breitburg, 2002; Hlohowskyj & Chagnon, 1991). Furthermore, there is a lack of research focusing on levels of DO above what is considered true hypoxia (2 mg/L). Research regarding environmentally relevant concentrations of DO is therefore critical to the maintenance of fish populations inhabiting environments with depressed DO as a result of human activities..

1.3 Strategies and species differences in low DO environments

Fishes may use a variety of strategies in coping with lowered DO. In aquatic environments, oxygen may constitute a limiting resource and can often vary seasonally or daily (Kramer, 1987). Therefore, a wide variety of behavioural and physiological coping mechanisms are utilized by fishes, including avoidance, increasing oxygen uptake, changes in behaviour, and metabolic changes (Kramer, 1987; Dean & Richardson, 1999). Tolerance of fishes to low DO can vary considerably between individuals and species (Kramer 1987; Robb & Adams, 2003). Species inhabiting environments where hypoxic events are common tend to have evolved coping mechanisms for low levels of DO, whereas those inhabiting environments where hypoxia is rare may be more vulnerable when DO is artificially reduced (Breitburg, 2002).

Avoidance of low DO areas may sometimes be accomplished by vertical or horizontal migrations (Kramer 1987). Migrations in response to limited DO have been recorded in a variety of fishes, however the effectiveness of this strategy is limited by the extent and severity of the hypoxic zone, the danger associated with changing habitat, and

the mobility, feeding habits, and tolerance of individual species (Pihl et al., 1991; Eby & Crowder, 2002).

A second coping strategy is to increase oxygen uptake. This may be accomplished in a variety of ways, such as air-breathing, increasing ventilation rate, or aquatic surface respiration (Kramer 1987). The optimal mode of respiration may vary with the amount of available DO and the species of fish. For example, only a minority of fish species are capable of air breathing, and fishes with upturned mouthparts more easily perform aquatic surface respiration (Kramer, 1987). One must also note that there is often a cost associated with these behaviours in terms of energy, time, and risk of predation (Kramer, 1987; Dean & Richardson, 1999).

Additional strategies used by some fishes include decreasing activity levels to reduce oxygen demand, or changes in metabolic processes (Kramer 1987). Metabolic responses to hypoxia occur in some species and in some cases, anaerobic pathways are relied upon more heavily (Dalla Via et al, 1998). Fishes such as Atlantic sole (*Solea solea*) or Crucian carp (*Carassius carassius*) can upregulate glycolosis to alleviate energetic demands (Dalla Via et al, 1998)**.** Some cyprinids have been shown to shift entirely to anaerobic pathways in the absence of available oxygen (Klinger et al., 1982).

1.4 Test species: Fathead minnow life history

The fathead minnow is a fairly ubiquitous North American species, its range extending through most of central North America (Scott & Crossman, 1998). Habitat preference varies widely throughout its range, however they prefer still water above

running water and may be found in reservoirs, muddy brooks, beaver ponds, and alkaline lakes (Scott & Crossman, 1998). Wild fish feed on algae, insect larvae, and zooplankton (Scott & Crossman, 1998), but they will take a variety of food in captivity.

Under natural conditions, spawning occurs in summer when water temperatures reach approximately 18ºC (usually in June) (Scott & Crossman, 1998). Spawning activity may persist into August in some parts of its range (Scott & Crossman, 1998). Mature fish in breeding condition are sexually dimorphic and develop secondary sex characteristics (Ankley et al., 2001). Nuptial tubercles develop on males in breeding condition, appearing on the snout, and body coloration becomes dark with light vertical bands (Ankley et al, 2001) A spongy dorsal pad also develops, extending from the nape to the origin of the dorsal fin. Females develop an ovipositor, but otherwise do not change in morphology or coloration (Ankley et al, 2001). .

A spawning site is chosen by the male on the underside of rocks or submerged logs and vegetation. Under laboratory conditions, a small shelter made out of PVC pipe is sufficient. Females approaching the nest are courted by the males; they are pursued or nudged into the nest, where egg laying occurs. Males remain at the nest location to guard and clean the eggs until they hatch (Scott & Crossman, 1998). In the wild, or when laboratory housing allows, males may receive eggs from multiple females, and females may lay eggs in more than one nest (Scott & Crossman, 1998). The number of eggs produced per spawn is highly variable, and under ideal lab conditions often ranges between 50 and 100 eggs (Ankley et al., 2001) Fish typically spawn every 3-4 days. Larvae usually hatch at 4 to 5 days when maintained at 25ºC, and can derive nutrition from their yolk sac for about 2 days post-hatch (Ankley et al., 2001). In the wild,

maturation times vary greatly with temperature and food availability, but under ideal lab conditions larvae may reach reproductive maturity in four months (Ankley et al., 2001). Wild fathead minnows are reported to rarely live beyond two or three years. High mortality in wild male fish has been reported after spawning (Scott & Crossman, 1998). This is presumably due to exhaustion and low food intake, as this is not generally witnessed in the lab (Ankley et al., 2001).

In terms of biological or toxicological testing, fathead minnows are among the most extensively used fishes and much is known about their culture in the lab (Ankley et al., 2001). They are members of the Cyprinidae, a very large and ecologically important family (Jenson et al, 2001). They have a rapid life cycle, are easy to culture and maintain, and spawning can be stimulated by manipulating photoperiod, temperature and diet (Ankley et al., 2001). For these reasons, they represent a nearly ideal test species for a wide variety of experimentation.

Fathead minnows utilize several strategies when facing decreases in available oxygen, and are fairly tolerant to low DO (Klinger et al., 1982). Their initial response when confronted with low DO is to increase ventilation frequency (Robb & Abrams, 2003; Klinger et al., 1982). Both lowered activity levels, and migrations have been reported in fathead minnows living under the ice in winterkill lakes (Magnuson et al, 1985). These migrations occurred both horizontally and vertically to the water-ice interface.

While fathead minnows are unable to shift entirely to anaerobic pathways as in some other cyprinids, they can rely on them more heavily with decreasing DO (Klinger et al., 1982). They may also increase haematocrit and hemoglobin concentrations in the

blood to allow for more efficient oxygen transport (Robb & Adams, 2003). Tolerance to low DO has been shown to vary between individuals, and there is some evidence that smaller individuals are more tolerant (Robb & Adams, 2003).

1.5 Normal gamete and hormone development in the fathead minnow

In fathead minnows, ovaries are large, paired organs occupying a large proportion of the abdominal cavity (Leino et al., 2005) Oocytes develop within the ovaries, originating as mitotically proliferating oogonia. These oogonia enter into meiosis but are arrested at prophase I (Suwa & Yamashita, 2007). At this stage in their development, they accumulate yolk, lipid droplets and other substances required for embryonic development, and attain their full size (Suwa & Yamashita, 2007; Leino et al., 2005). Stimulation by hormones then causes the oocytes to resume meiosis until metaphase II, where meiosis is arrested once more (Suwa & Yamashita, 2007). The period between prophase I and metaphase II is called final maturation, and after this occurs oocytes are mature and ready to be fertilized (Suwa & Yamashita, 2007). Final oocyte maturation is regulated by luteinizing hormone (LH), which stimulates the production of maturation inducing hormone (MIH). MIH activates maturation promoting factor (MPF), which ceases the arrest at prophase I (Clelland & Peng, 2009). Final maturation is a rapid process, which generally occurs within 24 hours (Clelland & Peng, 2009).

During final maturation, chromosome condensation occurs and the germinal vessel (oocyte nucleus) migrates from the center of the cell to the animal pole where germinal vessel breakdown occurs (Suwa & Yamashita, 2007; Leino et al., 2005).

Ovulation is stimulated by prostaglandin and occurs when the follicles ruptures, and the eggs are released (Clelland & Peng, 2009).

Fathead minnow testes are paired organs, located in the dorsal body cavity (Leino et al., 2005). Sperm originate from germ cells in the seminiferous tubules within the testes (Grier, 1981). Germ cells give rise to primary spermatogonia, which divide several times via mitosis to form secondary spermatogonia (Leino et al., 2005). These stages can be recognized by their lightly staining, or slightly basophilic nuclei (Leino et al., 2005) Another round of mitotic divisions form primary spermatocytes, which undergo meiosis I to produce secondary spermatocytes. Spermatocytes are smaller than spermatogonia, with basophilic nuclei (Leino et al., 2005). These cells undergo meiosis II to form spermatids which mature into spermatozoa. This maturation process is called spermiogenesis, and is characterized by the development of a flagellum, and the compaction of DNA into a small nucleus (Schulz et al., 2010). The entire process of spermatogenesis occurs within cysts formed by Sertoli cells, and all developing germ cells within a single cyst are derived from a single primary spermatogonium (Grier, 1981; Leino, 2005). Once cells within a cyst have matured into spermatozoa, the cyst ruptures, releasing spermatids into the lumen where final maturation into spermatozoa occurs (Grier, 1981).

Sex steroids are fundamental to the regulation of reproduction in fishes. They are produced in specialized cells within gonadal tissues; within follicular cells in females, and in Leydig cells in males (Young et al., 2005). Production of sex steroids is stimulated by the pituitary gonadotropins LH and follicle stimulating hormone (FSH) (Schulz, 2010). Testosterone is produced through several enzymatic conversions, using cholesterol as a precursor (Clelland & Peng, 2009). It can then be converted to estradiol by

aromatase in both males and females (Clelland & Peng, 2009). Sex steroids have a variety of functions, including the regulation of development, sexual cycles, and reproductive behaviour (Young et al., 2005). Two sex steroids which are commonly measured in fathead minnows are estradiol and testosterone. Estradiol plays an important role in vitellogenesis, oocyte proliferation, ovulation, and final oocytes maturation in females, and regulates germ cell proliferation in males (Young et al., 2005). Testosterone is a precursor to other sex steroids, including estradiol and 11-ketotestosterone, an androgen important in the development of secondary sex characteristics and sperm maturation in males (Gazola & Borella, 1997; Young et al., 2005). While present in both males and females, testosterone levels are higher in male fathead minnows, where they play roles in spermatogenesis (Gazola & Borella, 1997).

Vitellogenin, a precursor to egg protein, is also commonly measured in fathead minnows. It is produced in the hepatocytes of female fathead minnows and is moved via the blood to the ovaries, where it is incorporated into developing oocytes (Young et al., 2005). Vitellogenesis is regulated by estradiol, which binds to nuclear receptors in hepatocytes and initiates vitellogenin synthesis by activating the vitellogenin genes (Clelland & Peng, 2009). The genetic machinery necessary for the production of vitellogenin is present in male fathead minnows but is not normally expressed, except in the presence of estrogen-mimicking compounds (Folmer et al, 1996; Leino et al., 2005; Ankley et al., 2001). Measurable quantities of vitellogenin in male fathead minnows are therefore indicative of endocrine disruption.

Estradiol and testosterone levels were measured after exposure to depressed DO because previous authors have found lowered sex steroid hormones in fish exposed to

long-term hypoxia (Landry et al. 2007; Wu et al., 2003). Vitellogenin levels in male fish were measured for future comparison to other experiments performed in the same laboratory.

1.6 Research objectives

The focus of this research was to determine the effects of depressed DO on fathead minnow reproductive behaviour and physiology. Levels of DO that were tested in my experiments were above those associated with true hypoxic conditions (>2 mg/L). These ranged from 3.5 to 6.4 mg/L. These levels were chosen based on real DO concentrations from a Canadian river system exposed to multiple point discharges from a bleached kraft pulp mill and municipal waste water. This research is the first to test the effects of depressed DO on reproductive processes and behaviour in fathead minnows. It is also the first to encompass such a comprehensive suite of endpoints in response to low DO, including reproductive behaviour, egg production, hatching success, fertility rate, larval deformities, hormone and vitellogenin levels, morphometrics, survival, and gonad histology.

Chapter 2: Methods

2.1 Fish maintenance

This research was conducted in an aquatic laboratory in the Animal Care Unit of the Veterinary Medical Sciences building at the University of Saskatchewan. Minnows used in the study were purchased from a commercial supplier (Thomas Fish Supply, California, USA). Fish were maintained in the laboratory in 529L holding tanks with a flow-through rate of four turnovers per day of filtered, de-chlorinated tap water set at 24° C. Light was adjusted to a 16L:8D cycle and all fish were fed 10% of their body weight with brine shrimp (*Artemia* spp.) and bloodworms (*Chironomus tentans*) twice daily.

2.2 Pre-exposure period

Experiments were divided into two portions, the pre-exposure (normoxic conditions) and exposure (experimental conditions). The purpose of dividing the study into two components was to use the pre-exposure phase to establish successful breeding trios which were then used in the exposure phase. Pre-exposure periods were a minimum of one week in duration, plus any subsequent time required to attain sufficient breeding trios for the exposure phase.

In the pre-exposure stage, 61 trios (two females: one male) were randomly placed into a series of 10L experimental tanks containing a nesting object (10 cm x 10 cm PVC pipe cut lengthwise). Breeding trios were used because the tanks could not house more than three fish, and using two females rather than one increased the likelihood that egg production would occur in any given replicate tank (Pollock et al., 2008). These tanks were held at 25° C with 16L:8D light cycle and had a flow-through rate of four

turnovers/day of filtered, de-chlorinated tap water. Trios of fish were fed twice daily, 1.0 g of brine shrimp at 09:00 and 1.0 g of chironomids at 16:00.

Prior to the morning feeding, water samples were collected from a representative number of aquaria (one tank per row in the housing systems, with a different tank being chosen each day). These samples were tested for ammonia, pH, conductivity, hardness and chlorine. Following feeding, trios were left for one hour, after which all nesting objects were checked for eggs. If eggs were present they were gently rolled into a Petri dish, counted, and scored for fertility (number of fertilized eggs/total number of eggs). Unfertilized eggs were opaque and were easily differentiated from those that were viable.

2.3 Criteria for selection of test fish

Of the 61 trios that started the pre-exposure phase, 36 trios were selected for use in the exposure phase. Trios were chosen based on three criteria: 1) Survival of all three adults, 2) At least one breeding attempt, and 3) Fertility rates greater than 80 % (derived from Rickwood et al. 2006a and b). As in Rickwood et al. (2006a and b) and similar studies (Ankley et al. 2001), statistics were performed once trios were assigned to treatment groups to ensure there was no significant difference between trios with regard egg production (one-way ANOVA) and fertility rate (one-way ANOVA) before experimental conditions were applied. At the end of the pre-exposure, these trios were relocated to the experimental apparatus and DO (dissolved oxygen) levels were gradually adjusted to experimental levels over the course of several hours.

2.4 Experimental apparatus

Levels of DO used in the exposure phase were controlled by a custom-built system (Figure 2.1) designed for the specific purpose of controlling oxygen and temperature in experiments over extended periods of time (several weeks). This apparatus was a partial flow-through system, operating on approximately four turn-overs a day. A vacuum pump removed DO from treated water supplied by a head tank, then this deoxygenated water was fed into four mixing tanks, one for each treatment group. Each mixing tank was air-tight, and equipped with a valve that supplied oxygen back into the water in small quantities. This valve regulated DO concentrations based on a feedback system, using an in-situ DO probe submerged inside each mixing tank. Temperature was regulated in a similar fashion, each mixing tank being supplied with an in-situ heater and temperature probe. These probes automatically recorded temperature and DO at 22-minute intervals and sent these data wirelessly to a dedicated computer. All four treatment groups could therefore self-regulate temperature and DO levels according to experimental requirements.

Figure 2.1. Experimental apparatus. Deoxygenated water is pumped into air-tight mixing tanks (A). Air is added in short bursts through a valve located inside each mixing tank, and is regulated by an in-situ DO probe (not shown) and monitron system (B). This water is then pumped into the row of 10 liter tanks adjacent to the mixing tank. One tank in each treatment group (C) is modified to hold eggs under the same conditions as parents. Camera units (D) were set up and moved daily to record reproductive behaviour.

2.5 Exposure conditions and procedures

Aside from adjustments in DO levels, fish maintenance and water quality testing were identical to the pre-exposure phase. The only shift in the daily protocol that occurred at the outset of the exposure period was the recording of breeding behaviour beginning at 09:00, followed by feeding at 9:20.

Additional data pertaining to reproductive output and success were collected throughout the exposure phase of both studies. After eggs were counted and scored for fertility, they were subsequently placed into egg cups (10 cm x 2 cm plastic with a screened bottom and top) which were placed into tanks with conditions identical to those of the parents. When eggs hatched, larvae were maintained in the egg cup for one day to ensure full brood hatch, after which larvae where counted to determine percent hatching success (number of eggs that hatched/number of fertilized eggs x 100) and scored for deformities. Such deformities included pericardial and yolk sac edema, craniofacial abnormalities, hemorrhaging, lordosis, and scoliosis (Jezierska et al., 2000). These data were also expressed as a percentage (number of deformed larvae/total number of larvae x 100). The number of days required for eggs to hatch was also recorded.

At the end of the exposure period, all fish were sacrificed using a MS222 solution, and were subsequently dissected. Total body weight, fork length, and liver and gonad weights were recorded.

2.6 Analysis of reproductive potential

Egg production was measured in several different ways, and was assessed per replicate tank or for entire treatment groups over time. Egg production could not be tied to individual females in a trio. Instead, each tank was used as the level of replication.

Egg production was first calculated as a grand mean, by analyzing the average brood size per replicate tank. Each brood was divided by the number of females present in the tank (two) and by the duration of the experiment so that egg production was measured in eggs/female/day. The purpose of this was to allow for comparison to other studies which either had exposure periods of different lengths, or used pairs of fish rather than trios. This endpoint provides an estimation of the average reproductive output of a breeding group.

Mean egg production was calculated in a similar fashion, however, using the *total* number of eggs produced per replicate tank rather than the average brood size. This endpoint was also expressed in terms of eggs/female/day so that comparisons are possible between experiments. Mean egg production represents an expression of the overall reproductive potential of breeding groups over time.

A third measure of egg production, cumulative eggs/female/day, was used to estimate reproductive ability while compensating for mortality. A cumulative record of egg production in each treatment group was constructed with the experimental day as the replicate. The cumulative egg count for each day was divided by the number of females capable of reproduction present in the treatment group at the time. The number of breeding females at the outset of experimentation was 18 for each group, but was reduced throughout the experiment with both male and female mortality. (If a male in a breeding

group died, both females in the trio were considered incapable of reproduction). This measurement lends an estimate of the breeding potential of each treatment group while compensating for adult mortality.

Cumulative number of spawns, the number of replicate tanks that bred, and overall number of breeding attempts per group were also analyzed. These calculations were performed to determine whether there were differences between treatments in the number of fish able or willing to breed under experimental conditions.

A final measure of reproductive output evaluated egg production in context of female body size. Egg production for a replicate tank was divided by the amount of time females were present in the tank (egg production/female days in tank) to account for mortality. This data was analyzed using two separate tests (ANCOVAs) where female body weight and fork length were used as covariates.

2.7 Analysis of survival and morphometrics

Survival was analyzed at the end of the experiment as a percentage per replicate tank (surviving fish/total fish x 100). Morphometric measurements were taken on the last day of the study and included measurements of fish mass, fork length, liver and gonad weight, as well as secondary sexual characteristics. These data were used to evaluate liver somatic index (LSI=[liver weight/total body weight]*100), gonoadosomatic index (GSI=[gonad weight/total body weight]*100), condition factor (CF=[total body weight/fork length^3]*100), fork length, and total body weight. Each of these endpoints was measured independently for both sexes. For female fish, liver weight and gonad weight were each analyzed with both fork length and total body weight as a covariate.

Secondary sex characteristics were assessed according to the protocol of Parrot and Wood (2001). Each fish was assigned a score for individual characteristics by visual means, based on the size or conspicuousness of these characteristics. For males, the fin dot, banding, and nuptial tubercles were rated 0 or 1 depicting presence or absence of each characteristic. The fat pad was scored between 0 and 3 based on size, where zero indicated the absence of a pad and 3 indicated an extremely large pad. For female fish, ovipositor size was assigned a score between 0 and 3, where 0 indicated the lack of an ovipositor and 3 indicated an extremely pronounced ovipositor. As in previous endpoints, female data for each replicate tank was averaged, producing a single number for each tank. The total scores for each treatment were then presented as a proportion of the total possible score.

2.8 Analysis of reproductive behaviour

Reproductive behaviour was monitored using video cameras and was recorded in a subset of eight tanks (four per treatment) for 20 minutes each morning between 9:00 and 9:20 am. Each day, the cameras were moved to record a different subset of tanks until all trios had been recorded. At this time, the cycle began again. Due to mortality, some trios were not recorded (if one of the trio died early in the study) while others may have been recorded as many as four times. In either case, all observations were totaled for each trio and the percentage of time engaged in each type of behaviour was calculated. The percentage of time engaged in each behaviour rather than the absolute value was used to control for differences in numbers of observations for each trio.

Digitally recorded videos were scored for behaviours with the aid of behavioural observation software (Observer_{xt} by Noldus). Use of this observational software allowed the accurate scoring of multiple behaviours simultaneously via a specialized keypad, as well as providing complete summaries, both graphical and in tabular format, following each observation. The software also enables extremely slow play-back of video, as well as proving a high resolution full screen view of each trio, again allowing for accurate recording of discrete behaviours. Behavioural observations included time spent in the nest by males, time spent in nest care (cleaning the underside of the nest in preparation for egg attachment), and courtship behaviour between males and females (male attempting to manipulate the ovipositor and push the female toward the nest, spawning, and males pursuing females). All the above mentioned behaviours are well known typical behaviours of breeding male fathead minnows (Scott & Crossman 1998, Cole & Smith, 1987) and have been used in similar studies (e.g. Weber & Bannerman 2004).

Due to the fact that several fish died, behavioural analysis was conducted in a second way. A "survivors only" behavioural analysis was conducted with the intention of correlating reproductive behaviour with egg production, which could only be collected from surviving individuals.

2.9 Histology and hormone analysis

Gonads from all surviving individuals in experiment 1 were weighed, halved, and stored for histopathology or hormone analysis. Gonads to be used for hormone analysis were flash frozen in liquid nitrogen then stored in a – 80°C freezer. Vitellogenin (males) and hormone analysis (testosterone and estradiol for both genders) were conducted using

kits designed specifically for use in the fathead minnow (Fathead minnow vitellogenin kit, Biosense laboratories; Estradiol and testosterone EIA kits, Cayman chemicals). Tissues from each fish were homogenized and ether extraction was utilized. Vitellogenin analysis was performed as per Jenson et al., 2001. Gonad samples used for testosterone and estradiol analysis were diluted such that they fell between the reliable boundaries of the standards. The standards and diluted samples were then incubated with Estradiol EIA antiserum for one hour at room temperature. Wells were then emptied and rinsed with wash buffer, and 200 μl of Ellman's reagent was added. Plates were then developed for 90 minutes and were read using a plate reader between 405 and 420 nm. Samples which read below the detection limit of the kit were recorded as one half the detection limit value.

Gonads used for histopathology were preserved in 10% formalin and processed at Prairie Diagnostic Services, Western College of Veterinary Medicine, University of Saskatchewan. Histological analyses were performed using a quantitative method. For female fish, one cross section of the gonad was photographed under a light microscope. Oocytes in early (1-3) and late (4 and 5) stages of development and the number of atreitic follicles were counted within this cross section. These categories were subsequently analyzed as percentages of the total eggs within the cross section. For male fish, a similar method was used. Ten randomly selected fields of view were assessed using a light microscope for each fish. Nests of spermatogonia, and the number of cysts containing spermatocytes and mature sperm were counted and expressed as a percentage of total cysts. For both sexes, appearance of histopathology (eosinophilia or fibrosis) was recorded, but occurred in very low incidence (<1%) and was not analyzed. As in previous

endpoints, female results from each tank for both histopathology and hormone analysis were averaged.

2.10 Experiment 1: The effects of various levels of dissolved oxygen on fathead minnows.

This research was conducted in July 2007. Males and females were held in separate but identical conditions until two weeks prior to the study, at which time they were introduced into a single 529L holding tank (same temperature, light and flow rate). This was done to enhance breeding performance in the upcoming study as minnows are socially facilitated breeders (Ankley et al., 2001) thus, more likely to breed if recently exposed to active breeding events in their environment.

The study was conducted for 17 days and was divided into two portions, the pre-exposure (normoxic conditions: 7 days) and exposure (hypoxic conditions: 11 days). The exposure phase of this experiment was designed to proceed for 21 days, however was terminated early due to high mortality in some treatment groups so that sufficient sample size could be retained for statistical analysis. Target levels of DO used in the study were 3.5, 4.5, 5.5 and 7.5(control) mg/L.

2.11 Experiment 2: The threshold for reproductive impairment in fathead minnows exposed to several concentrations of dissolved oxygen

This experiment was conducted in June and July of 2008. Males and females were held under the same holding conditions as in the first experiment, with one exception.

Fish were housed such that males and females could view each other through a mesh screen but were not permitted contact. This was assumed to enhance breeding condition of fish through visual and chemical stimuli, but prevent them from exhausting breeding potential before the pre-exposure was commenced. Aside from this one change, fish handling and maintenance were performed exactly as outlined for the previous experiment.

A pre-exposure phase of 8 days was followed by a 21-day exposure period. Levels of DO used in this experiment were targeted to be 5.0, 5.7, 6.4 and 7.5(control) mg/L. These levels were chosen based on the concentrations used in the previous experiment to determine the threshold level where reproductive impairment occurs in fathead minnows.

Additional changes involved the addition and omission of several endpoints. First, hormonal and histological endpoints were not analyzed in this study. A second change involved measuring egg size, which was not included in the first experiment. Fertilized eggs were collected every morning and a 10 mm scale was added to the petri dish in which the eggs were digitally photographed. 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). These eggs were later analyzed using ImageJ, a software program which can measure the dimensions of items in images. Egg area was measured, then from this measurement diameter was calculated ([square root (area/π)] x 2). Diameter was chosen as a measurement because three-dimensional measurements of each egg could not be performed on such small eggs. Measurements were taken for a random sub-sample of 10

eggs for each brood. Where broods were smaller than 10 eggs, all available eggs were measured.

2.12 Statistical analysis

One-way ANOVAs and ANCOVAs were used for all endpoints with continuous data. To test assumptions necessary for parametric statistics, a Shapiro-Wilk test was used to determine normality of data, and a Levene's test was used to assess homogeneity of variance. If the assumptions for a parametric test could not be met, data were either log10 or arcsine transformed. If data still did not meet assumptions, a non-parametric equivalent (Kruskal-Wallis test) was used rather than an ANOVA. For all endpoints aside from DO, a Tukey post-hoc test was performed to determine which treatments were different from the control. For DO, each treatment was compared to the one adjacent to ensure that levels of DO used in the experiment were statistically different.

A test for multiple proportions was used to determine whether survivorship and secondary sex characteristics differed among treatments. For data that demonstrated change in a variable over time, such as cumulative egg production or cumulative spawning events, a two-sample Kolmogorov-Smirnov test was used to compare treatments to the control.

Statistics for all variables were calculated using Systat 11.0 and SPSS 11.0. Regardless of the statistic used, all tests were conducted using two-tailed predictions with an alpha value of 0.05.

Chapter 3: Results

Experiment 1 The effects of various levels of dissolved oxygen on fathead minnows

3.1 Water quality

Water temperature and DO concentration were analyzed by averaging all 65 daily measurements to provide one measurement per day per treatment (n=11). The DO levels were different for all treatments (Kruskal-Wallis: $X^2_{(3)} = 40.3$, $p<0.001$; non-parametric Tukey post hoc comparisons: 7.5 vs 5.5 mg/L, 5.5 vs. 4.5 mg/L and 4.5 vs. 3.5 mg/L; q=0.781, $p<0.01$ in all cases) (Figure 3.1.1.). A significant difference in water temperature was observed between treatments (Kruskal-Wallis test, $X^2_{(3)} = 24.2$, $p<0.001$). Non-parametric Tukey post-hoc tests revealed that neither the 5.5 nor the 4.5 mg/L treatments differed significantly from the control, however the temperature in the 3.5 mg/L treatment was significantly lower than in the control (q=7.63, $p<0.001$) while the other two treatments did not significantly differ from the control (7.5 vs 5.5 mg/L: q=0.06, $p>0.5$; 7.5 vs 4.5 mg/L: q=0.13, $p>0.05$) (Figure 3.1.2). Despite the statistical difference in temperature, the magnitude of the difference was small (approximately 0.5° C).

Ammonia concentration and pH were analyzed from daily measurements starting on the second day of the experiment, with a sample size of 10 for each treatment. The conductivity meter malfunctioned after day 4 of the experiment, leaving only three conductivity measurements (n=3). The results from a one-way ANOVA showed that the pH was higher in the control than in all other treatments ($F_{(3,31)} = 12.6$, $p<0.001$; Tukey post hoc tests: 7.5 vs 5.5mg/L, 4.5 and 3.5 mg/L: all $p \leq 0.005$) (Table 3.1.1). Analyses of conductivity and ammonia concentration showed no significant difference among

treatments (Kruskal-Wallis: conductivity: $X^2_{(3)} = 1.05$, $p>0.7$; ammonia concentration: $X^2_{(3)} = 0.49$, $p>0.9$) (Table 3.1.1).

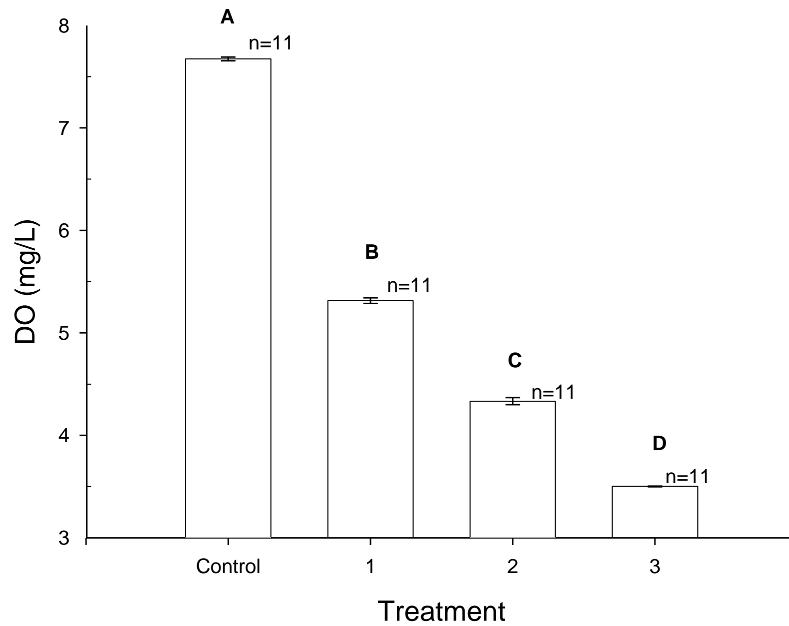


Figure 3.1.1. Mean (± standard error) dissolved oxygen concentration measured from four treatment groups during an 11-day exposure period. Different letters indicate a significant difference using a Tukey post-hoc test.

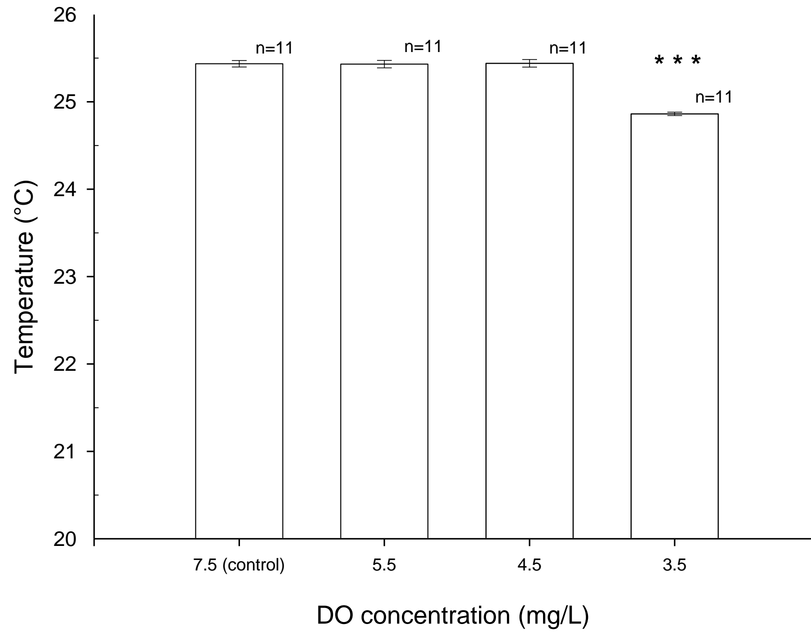


Figure 3.1.2. Mean (+ standard error) water temperature over an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5 (control) mg/L of dissolved oxygen. Asterix denote a significant difference from the control (*** $p < 0.001$).

Water quality parameter	n	7.5 mg/L (control)	5.5 mg/l	4.5 mg/l	3.5 mg/l
pH	10	7.86 + 0.06	7.58 + 0.04 ***	7.46 + 0.05***	7.44 + 0.68***
Conductivity (µS/cm)	3	377.9 + 9.3	407.7 + 1.7	406.4 + 2.2	408 + 0.2
Ammonia (mg/L)	10	0.03 + 0.01	0.03 + 0.01	0.02 + 0.01	0.02 + 0.01

Table 3.1.1. Water quality parameters measured daily over an 11-day exposure to 3.5, 4.5, 5.5, and 7.5 (control) mg/L of dissolved oxygen. Mean values + standard error are represented. Significant differences ($p < 0.05$) are denoted by an asterix (** = p>0.01, *** = $p < 0.001$).

3.2 Reproductive endpoints

Fertilization success and hatching success were analyzed for all tanks which produced broods (n=5 for control, and n=4 for 5.5 mg/L group). Larval deformities and days required to hatch were assessed for all tanks where larvae successfully hatched (n=5 for control, and n=3 for 5.5 mg/L group). There were no significant differences from the control in fertilization success (Figure 3.2.1) or days required for eggs to hatch (Figure 3.2.2) (Kruskal-Wallis, fertility: $X^2_{(1)}=3.05$, $p>0.05$; hatch time: $X^2_{(1)}=0.92$, $p>0.3$). One-way ANOVAs showed no significant difference in hatching success (Figure 3.2.3) or larval deformities (Figures 3.2.4, 3.2.5) (hatching success: $F_{(1,7)}=1.96$, $p>0.2$; larval deformities: $F_{(1,6)}=0.50$, $p>0.5$).

A Kolmogorov-Smirnov test (n=11) showed a significant difference in cumulative eggs produced by female per day between the control and the 5.5 mg/L treatment ($p< 0.001$) (Figure 3.2.6). This measurement accounts for mortality. Testing the other two treatments was not possible as no egg production occurred, and the test cannot properly function when all data points are zero.

Egg production per female per day was analyzed based on the average number of eggs produced per replicate tank (Figure 3.2.7), and based on the total number of eggs produced per tank (Figure 3.2.8) (n=9 for all treatments). A Kruskal-Wallace test found that in both cases, egg production in the 4.5 and 3.5 mg/L treatment groups was significantly lower than that of the control (average egg production: $X^2_{(3)}=11.77$, $p< 0.01$; Tukey non-parametric post hoc: 7.5 vs 4.5mg/L $q=3.80$, $p<0.05$; 7.5 vs 3.5 mg/L: $q=3.80$, $p<0.05$. Total egg production: $X^2_{(3)}=12.03$, $p<0.01$; Tukey non-parametric post hoc: 7.5

vs 4.5mg/L: q=3.99, p<0.05; 7.5 vs 3.5 mg/L: q=3.99, p<0.05). However, there was no difference in egg production between the 7.5 and 5.5 mg/L treatments (average egg production: 7.5 vs 5.5 mg/L: q=0.92, p>0.5; total egg production: 7.5 vs 5.5 mg/L: q=1.18, p>0.2)

Eggs produced per female per day were also analyzed using one-way ANCOVAs (n=9 for each treatment). Two tests were performed, using body weight and fork length as covariates. A significant interaction was found between egg production and the covariate in both cases (egg production with body weight as covariate: $F_{(3,26)}$=10.203, p<0.001; egg production with fork length as covariate: $F_{(3,26)}$=5.448, p=0.005 respectively) (Data not shown).

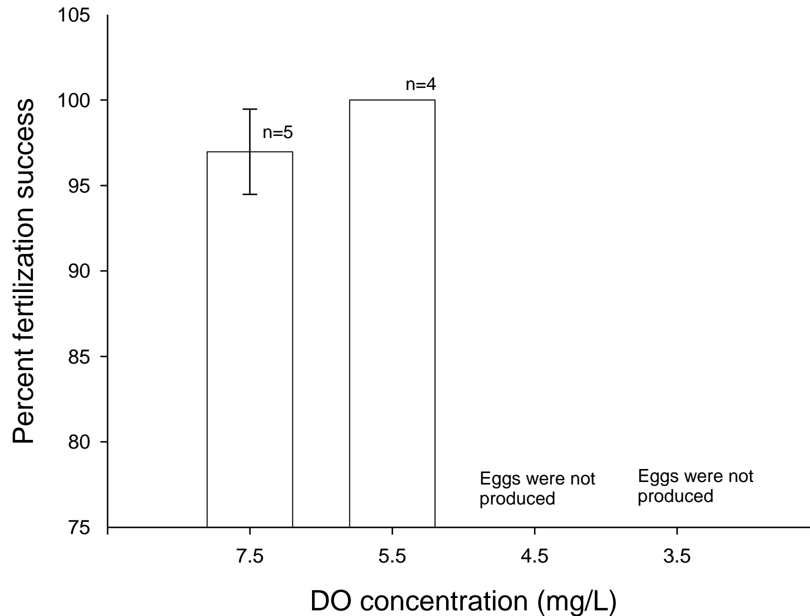


Figure 3.2.1. Percent fertilization success (± standard error) for eggs laid by fathead minnows over an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5 mg/L of dissolved oxygen.

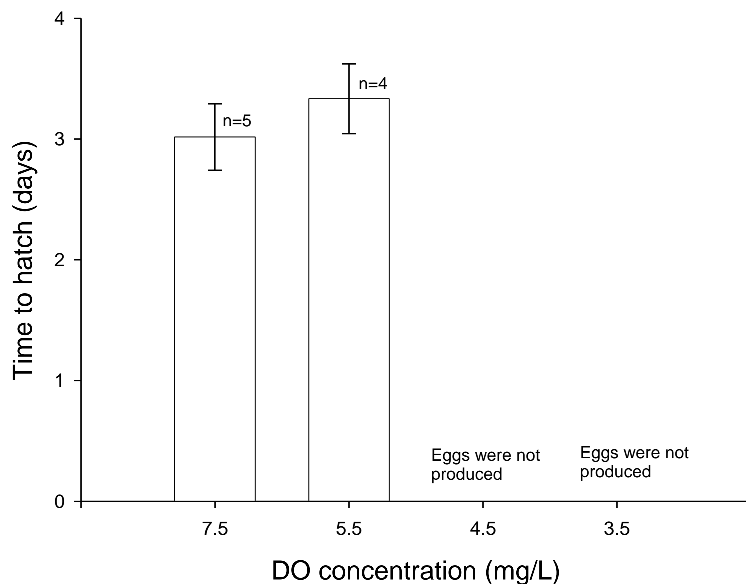


Figure 3.2.2. Mean number of days required to hatch (± standard error) for eggs laid by fathead minnows over an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen.

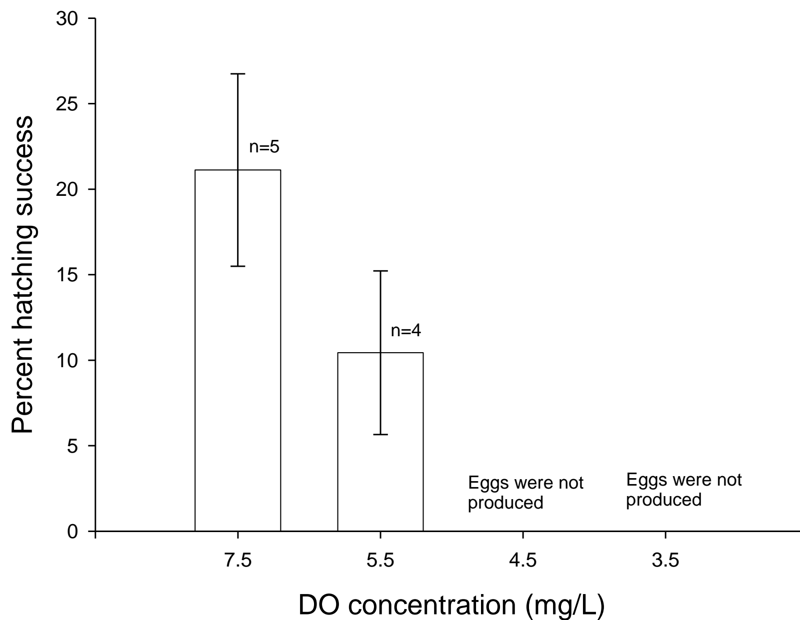


Figure 3.2.3. Percent hatching success (± standard error) for eggs laid by fathead minnows over an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen.

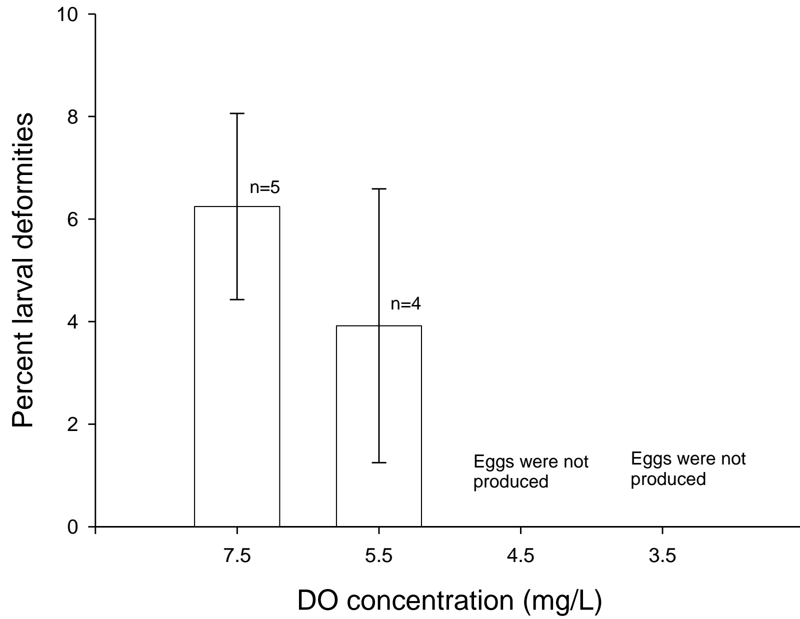
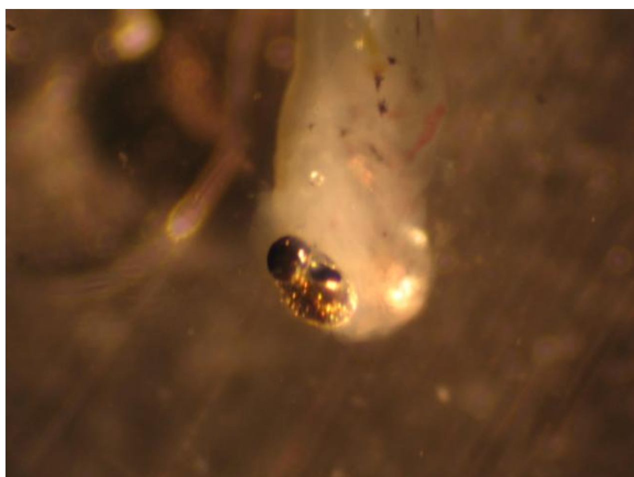


Figure 3.2.4. Percent larval deformities (+ standard error) for newly-hatched offspring produced by fathead minnows over an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen.

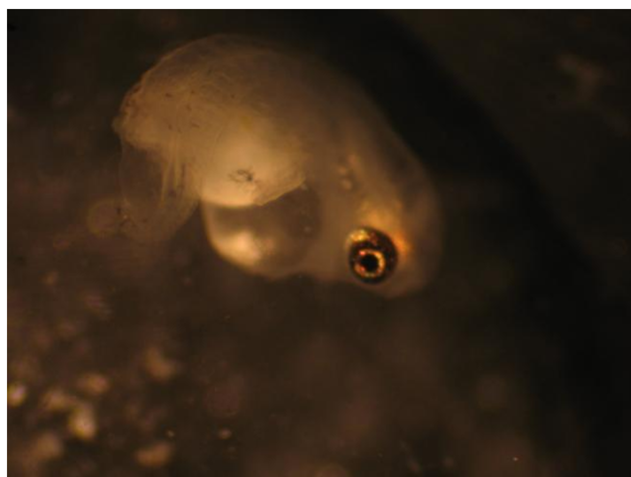
A

B

C



D



E

Figure 3.2.5 Deformities observed in fathead minnow larvae

A: Scoliosis
B: Yolk sac edema
C: Craniofacial deformity
D: Deformity of spine and yolk sac
E: Normal fathead minnow larva

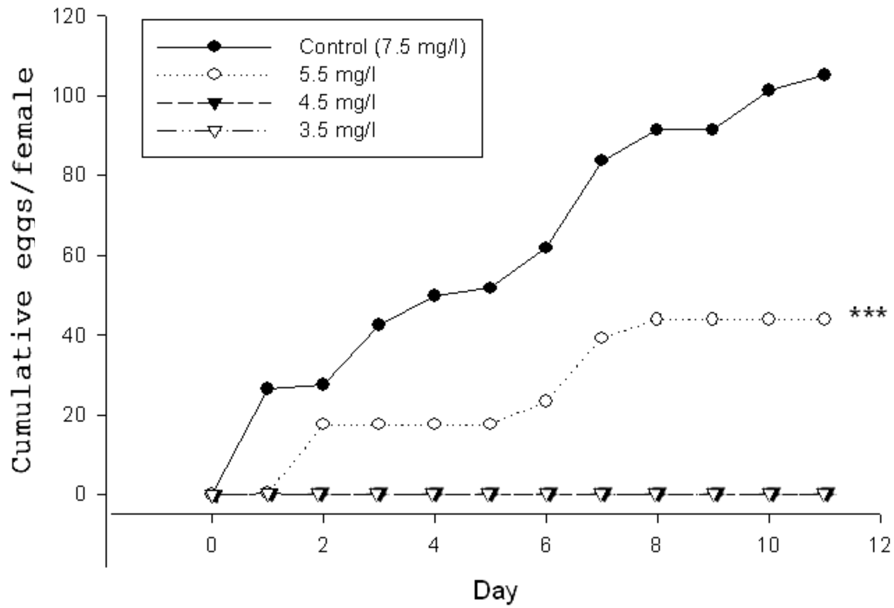


Figure 3.2.6. Cumulative eggs/female produced by fathead minnows over an 11- day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen. Asterix denotes a significant difference from the control (*** = $p < 0.001$). Statistics could not be performed on the 3.5 and 4.5 mg/L group because no eggs were produced.

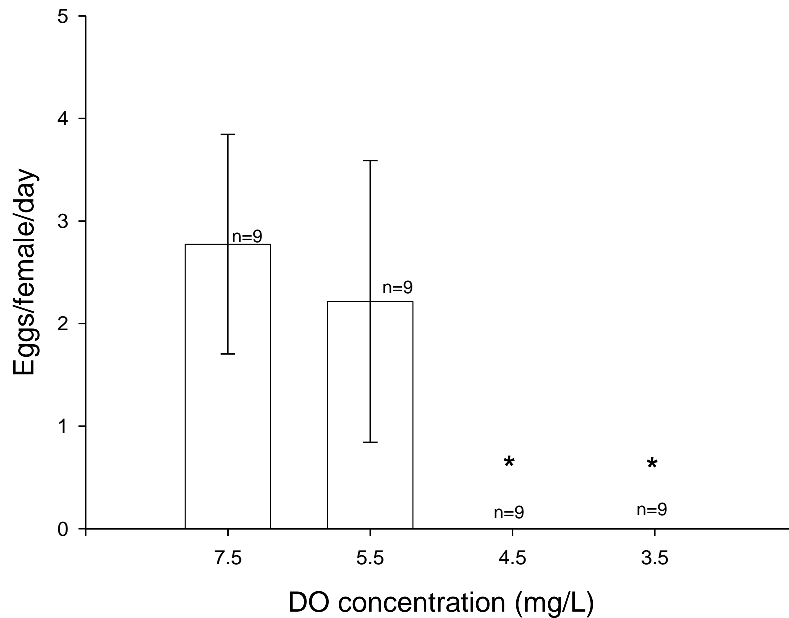


Figure 3.2.7. Mean (+ SE) of the average number of eggs produced per female per day over an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen. Asterix (*) denotes a significant difference from the control ($p< 0.05$).

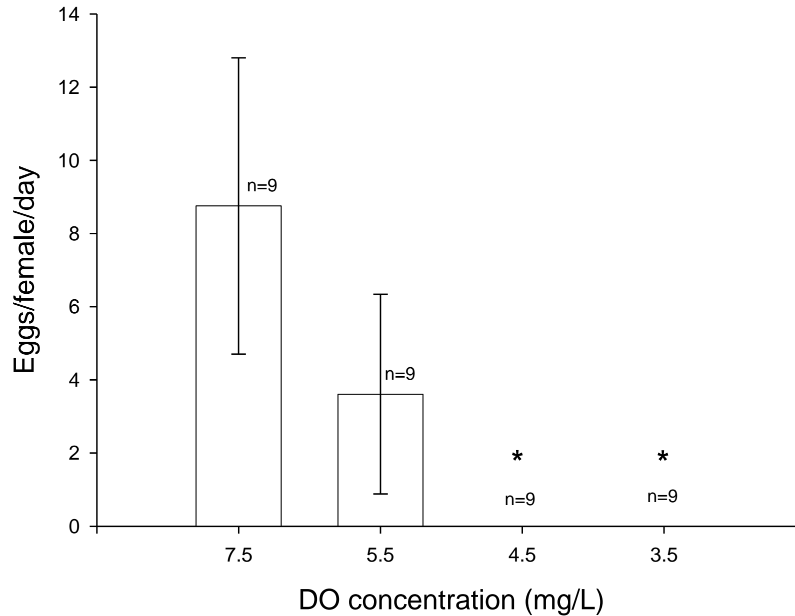


Figure 3.2.8. Mean (+ SE) of the total number of eggs produced per female per day over an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen. Asterix (*) denotes a significant difference from the control ($p < 0.05$).

3.3 Survival and morphometrics

Adult survival was analyzed using a test for multiple proportions (Zar, 1999; pg 562) (n=27 in each treatment). There was no significant difference among treatments ($X^2_{(3)}=7.82$, $p>0.05$) (Figure 3.3.1).

Morphometric measurements were analyzed for fish surviving until the termination of the experiment. (Males: control and 3.5 mg/L treatment: n=6, 5.5 and 4.5 mg/L treatment: n=3; Females: control and 4.5 mg/L treatment: n=9, 5.5 and 3.5 mg/L treatment: n=8). One-way ANOVAs showed no significant differences in male or female body weight (Male: $F_{(3,14)}=1.06$, $p> 0.3$; Female: $F_{(3,30)}=0.44$ $p>0.7$ respectively) (Table 3.3.1). There was also no significant difference in fork length among treatments for male

or female fish (Male: Kruskal-Wallis test: $X^2_{(3)}$=1.13, p>0.7; Females: One-way ANOVA, $F_{(3,30)}$=0.67, p>0.5 respectively) (Table 3.3.1). No significant differences among treatments were found in GSI (Males: Kruskal-Wallis, $X^2_{(3)}$=1.56, p>0.6; Females: One-way ANOVA, $F_{(3,30)}$=0.35, p>0.7) or in LSI (Males: One-way ANOVA; $F_{(3,14)}$=0.09, p>0.9; Females: Kruskal-Wallis; $X^2_{(3)}$=3.44, p>0.3) (Table 3.3.1). Condition factor also indicated no significant differences between treatments (Males: One-way ANOVA, $F_{(3,14)}$=0.68, p>0.5; Females: Kruskal-Wallis, $X^2_{(3)}$=0.40, p>0.9) (Table 3.3.1).

Analysis of secondary sex characteristics was conducted using a test for multiple proportions, where actual scores over total possible scores were compared for each treatment (Zar, 1999; pg 562). There were no significant differences in male (Q=0.89, p>0.7) or female (Q=1.45, p>0.7) sex characteristics (Data not shown).

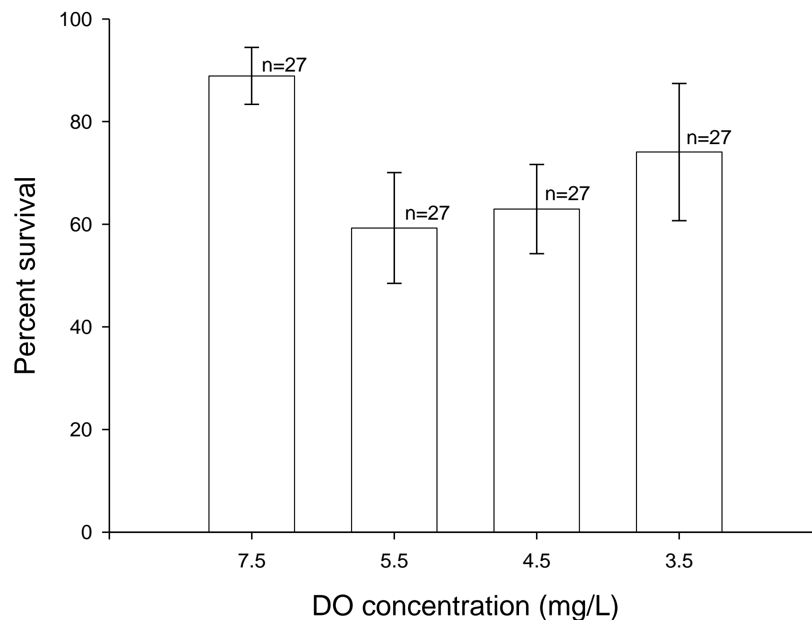


Figure 3.3.1. Percent survival (± standard error) of fathead minnows over an 11-day exposure to 3.5, 4.5, 5.5 and 7.5(control) mg/L dissolved oxygen.

MALES	7.5 mg/L (n=6)	5.5 mg/l (n=3)	4.5 mg/l (n=3)	3.5 mg/l (n=6)
Body weight (g)	3.93 ± 0.45	4.77 ± 0.20	3.77 ± 0.70	4.63 ± 0.36
Fork length (mm)	64 ± 2	66 ± 0	64 ± 3	66 ± 3
GSI %	0.89 ± 0.23	0.94 ± 0.19	1.27 ± 0.43	0.96 ± 0.24
LSI %	1.35 ± 0.41	1.26 ± 0.31	1.43 ± 0.75	1.57 ± 0.33
CF (g/cm^3)	1.47 ± 0.14	1.66 ± 0.07	1.42 ± 0.13	1.61 ± 0.10
FEMALES	**7.5 mg/L** (n=9)	**5.5 mg/l** (n=8)	**4.5 mg/l** (n=9)	**3.5 mg/l** (n=8)
Body weight (g)	2.57 ± 0.17	2.61 ± 0.17	2.61 ± 0.25	2.86 ± 0.18
Fork length (mm)	55 ± 1	55 ±1	55 ±1	56 ± 1
GSI %	9.51 ± 1.18	9.86 ± 1.44	8.28 ± 1.01	8.70 ± 1.32
LSI %	2.09 ± 0.14	1.53 ± 0.21	2.39 ± 0.50	1.98 ± 0.28
CF (g/cm^3)	1.54 ± 0.05	1.54 ± 0.06	1.56 ± 0.10	1.57 ± 0.06

Table 3.3.1. Morphometric endpoints from fathead minnows after an 11-day exposure to 3.5, 4.5, 5.5 and 7.5(control) mg/L dissolved oxygen. Values represent means ± standard error.

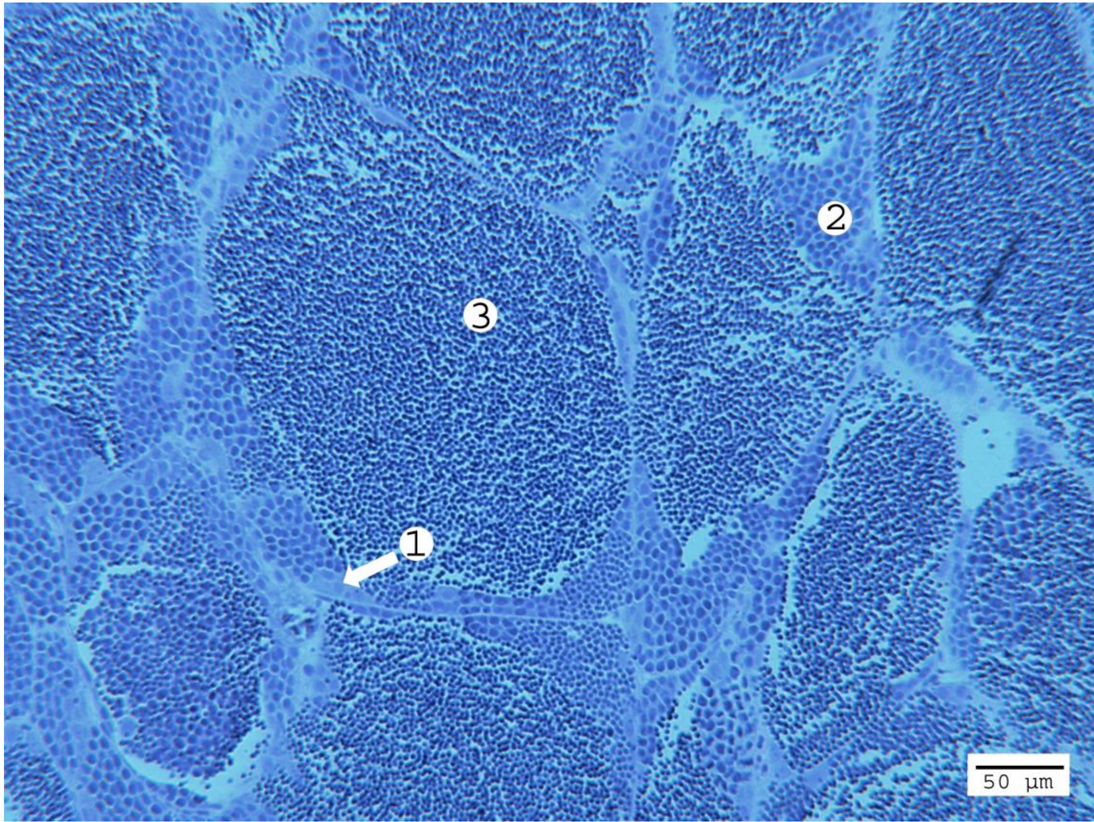
3.4 Histology

Histological analysis was performed on the gonads of fish surviving to the end of the experiment. Several slides were deemed unusable due to poor sectioning, reducing sample size for control males (Males: control: n=4, 5.5 and 4.5 mg/L:n=3, 3.5 mg/L n=6; Females: control and 4.5 mg/L: n=9, 5.5 and 3.5 mg/L n=8).

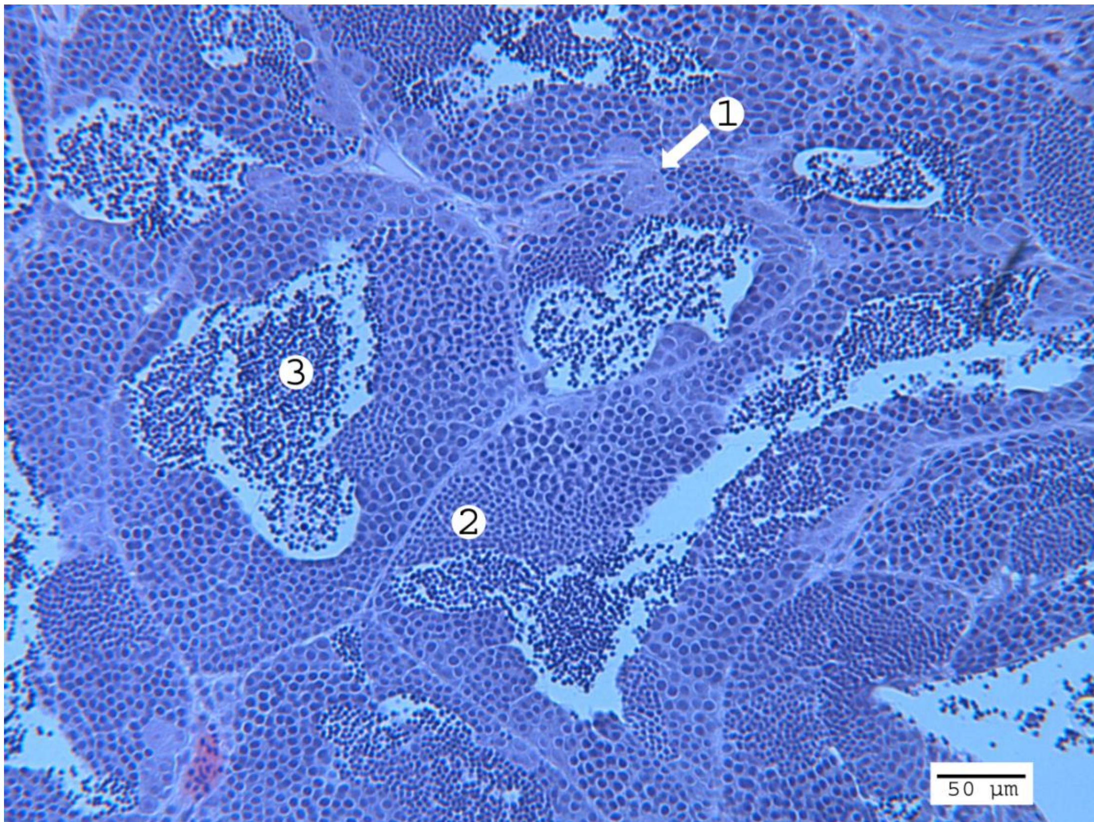
Male histological analysis (Figure 3.4.1) showed no significant differences among treatments with regard to percentage of spermatogonia (Kruskal-Wallis, $X^2_{(3)}=0.79$, $p>0.7$) (Figure 3.4.2) or spermatocytes (One-way ANOVA, $F_{(3,12)}=3.45$, $p>0.05$) (Figure 3.4.3), nor was there a difference in the development of mature sperm (One-way ANOVA, $F_{(3,12)}=0.66$, $p>0.5$) (Figure 3.4.4). Low occurrences of fibrosis (0% occurrence rate in all treatments) and eosinophilia (<5%) (presence of white blood cells indicating infection) did not allow for statistical analysis.

Female histological analysis (Figure 3.4.5) revealed no significant differences in oocyte development. No significant differences were found in the percentages of early stage (Figure 3.4.6) and late stage (Figure 3.4.5) oocytes among treatments (Early: One-way ANOVA, $F_{(3,30)}= 1.51$, $p>0.2$; Late: Kruskal-Wallis, $X^2_{(3)}=1.42$, $p>0.7$). A Kruskal-Wallis test also showed no difference in the percentage of atretic eggs ($X^2_{(3)}=0.97$, $p=0.8$) (Figure 3.4.8). As with the male histopathology, very low incidence of eosinophilia (0%) and fibrosis (<5%) prevented statistical analysis.

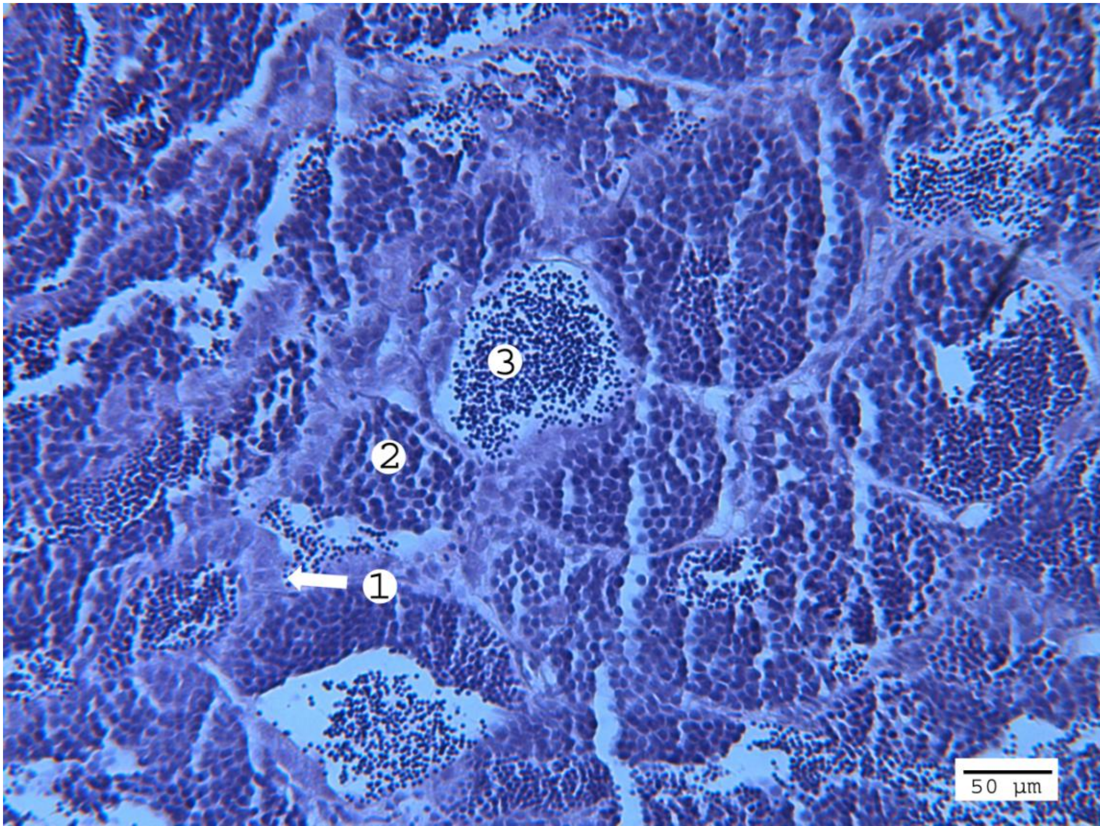
A



B



C



D

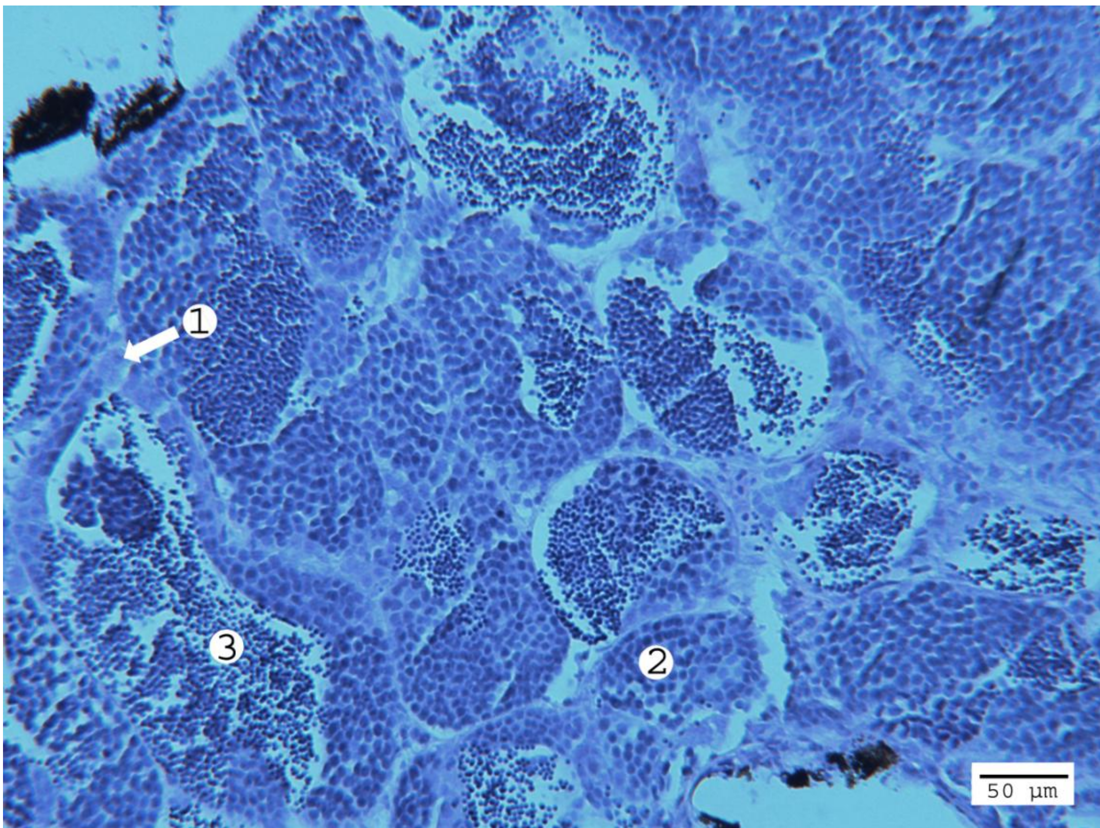


Figure 3.4.1. Representative cross sections of fathead minnow testes after exposure to 3.5 (A), 4.5 (B), 5.5 (C) and 7.5 (D) mg/L DO. Spermatogonia (1), spermatocytes (2), and spermatids (3) are shown.

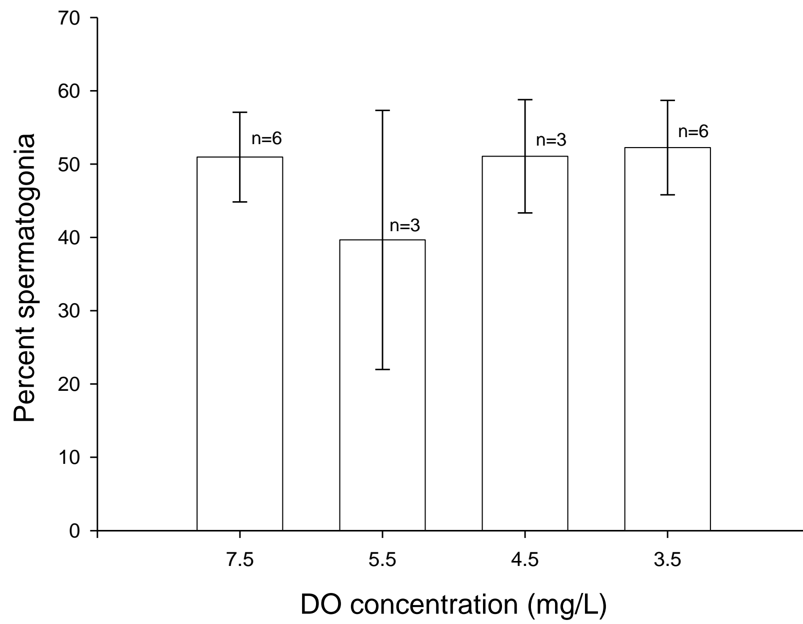


Figure 3.4.2. Percentage of spermatogonia (± standard error) present within cross sections of fathead minnow testis after an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen.

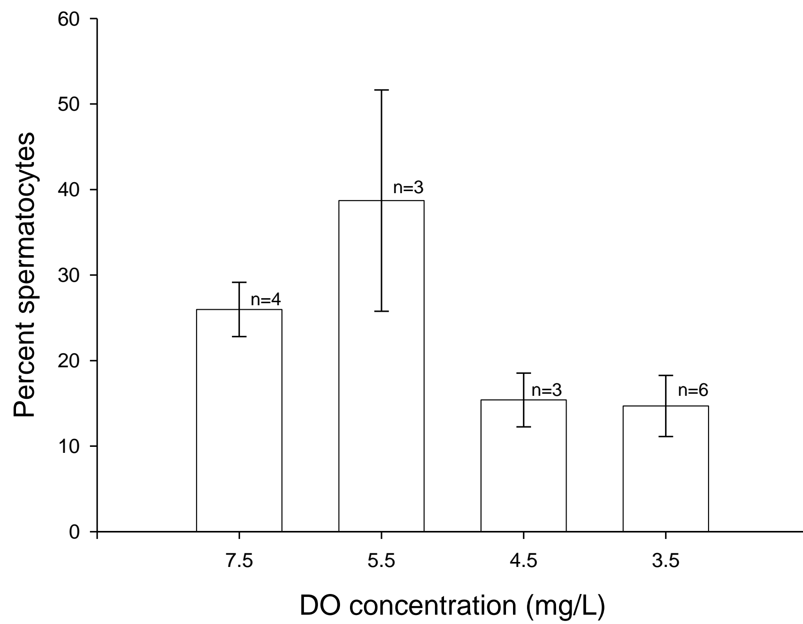


Figure 3.4.3. Percentage of spermatocytes (± standard error) present within cross sections of fathead minnow testis after an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen.

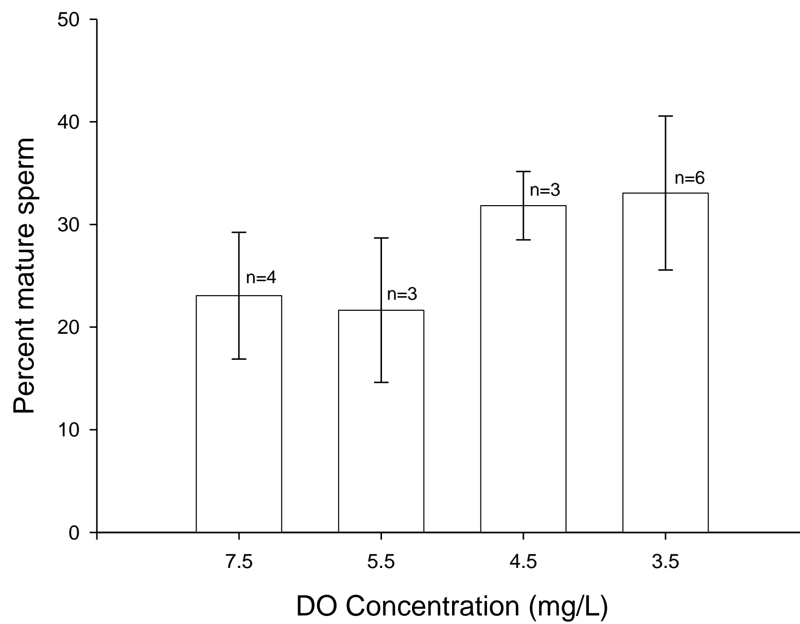
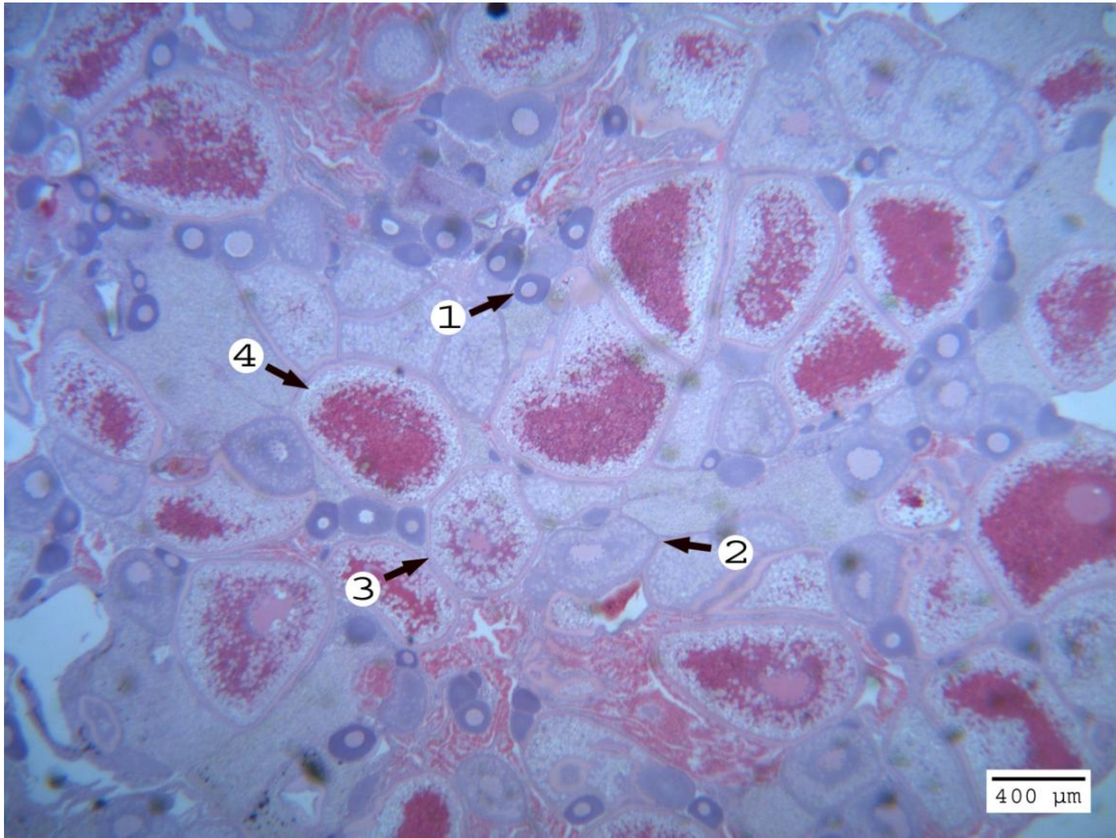
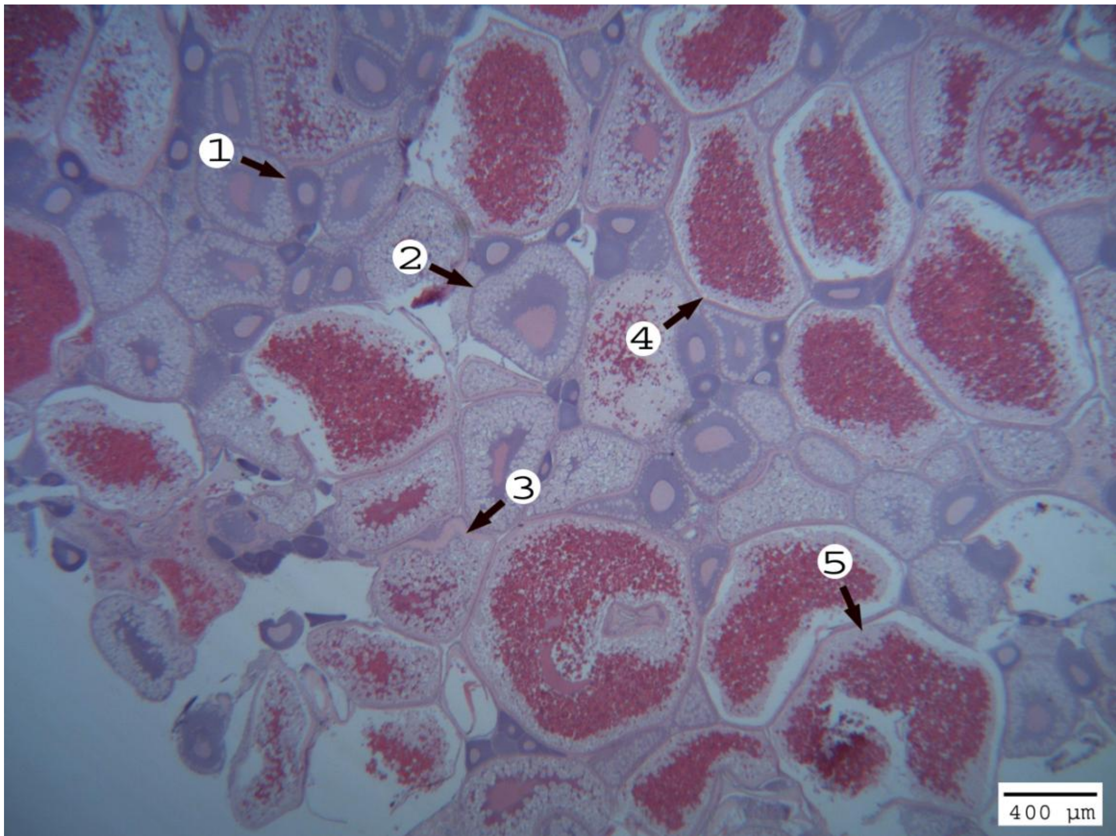


Figure 3.4.4. Percentage of mature sperm (± standard error) present within cross sections of fathead minnow testis after an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen.

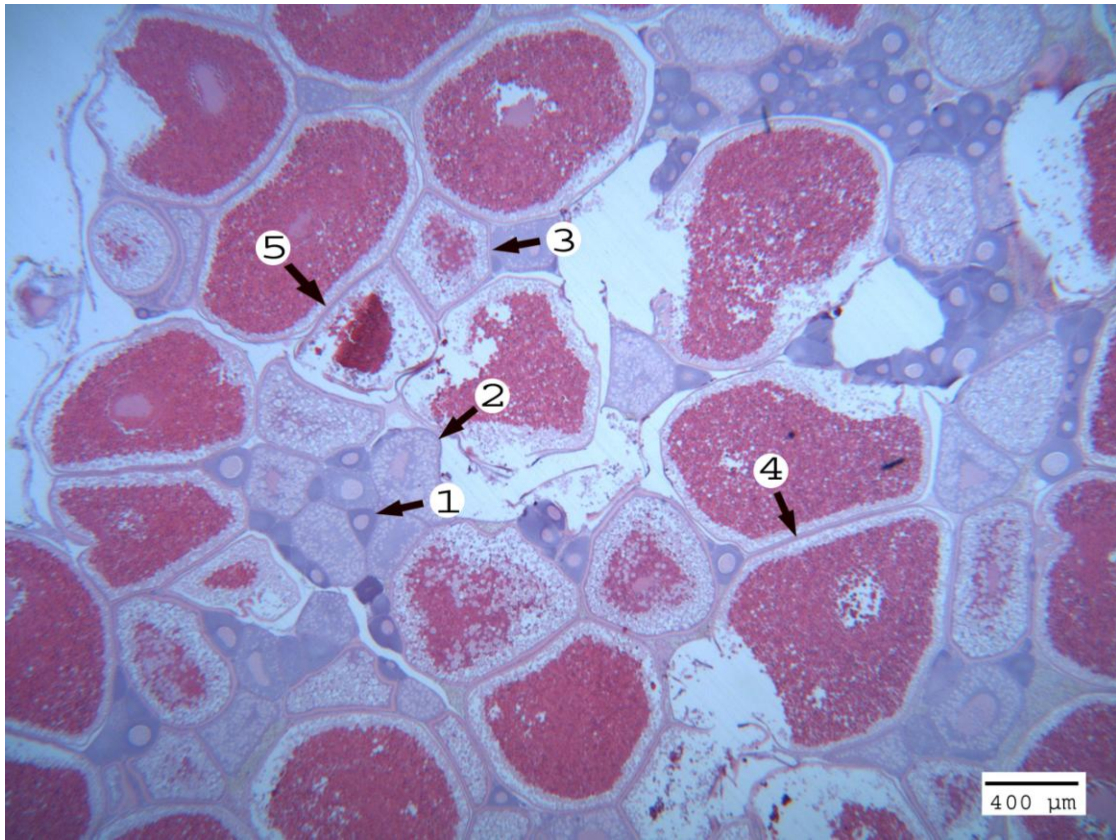
A



B



C



D

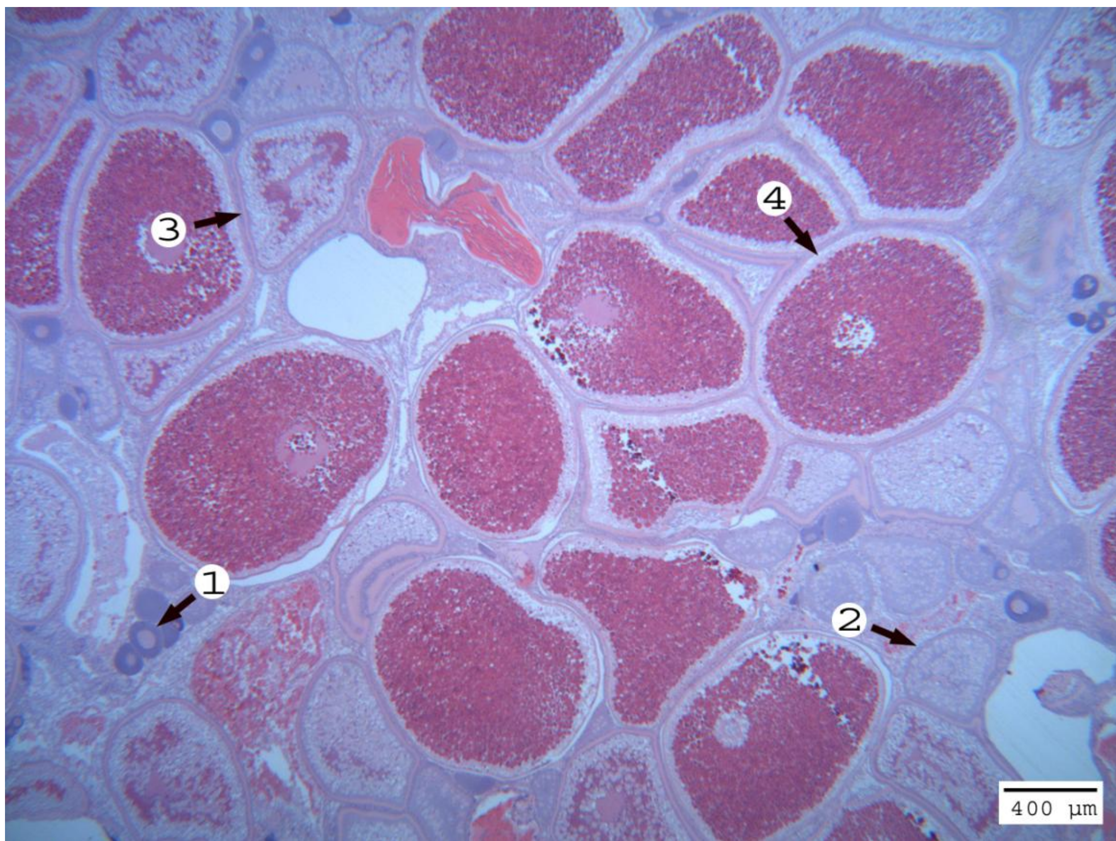


Figure 3.4.5. Representative cross sections of fathead minnow ovaries after exposure to 3.5 (A), 4.5 (B), 5.5 (C) and 7.5 (D) mg/L DO. Primary growth (1), cortical alveolus (2), early vitellogenic (3), late vitellogenic (4), and atretic (5) oocytes are shown.

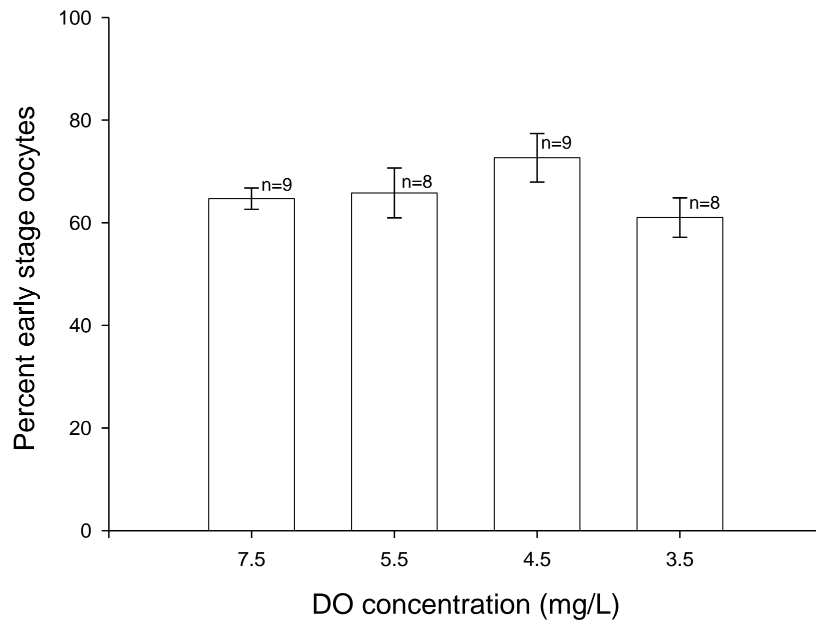


Figure 3.4.6. Percentage of early stage(1-3) oocytes (± standard error) within cross sections of fathead minnow ovaries after an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen.

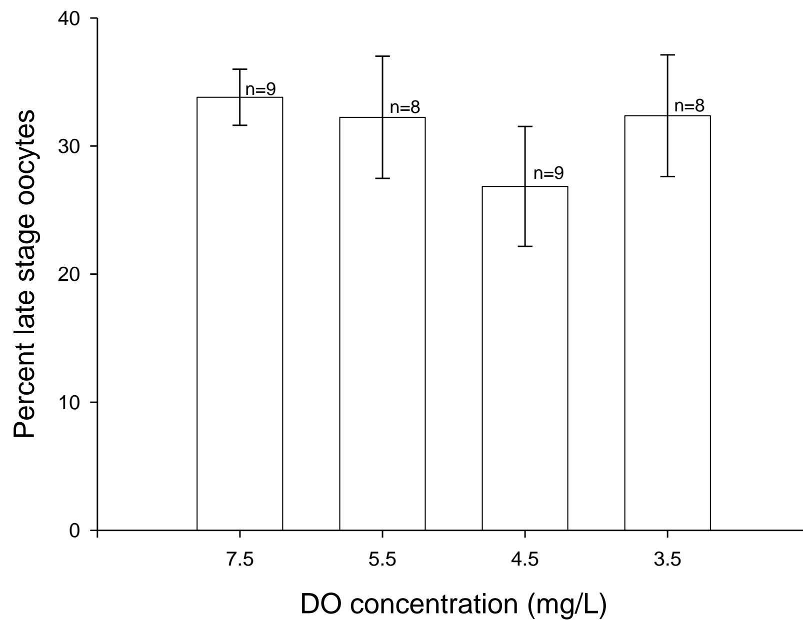


Figure 3.4.7. Percentage of late stage(4-5) oocytes (± standard error) within cross sections of fathead minnow ovaries after an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen.

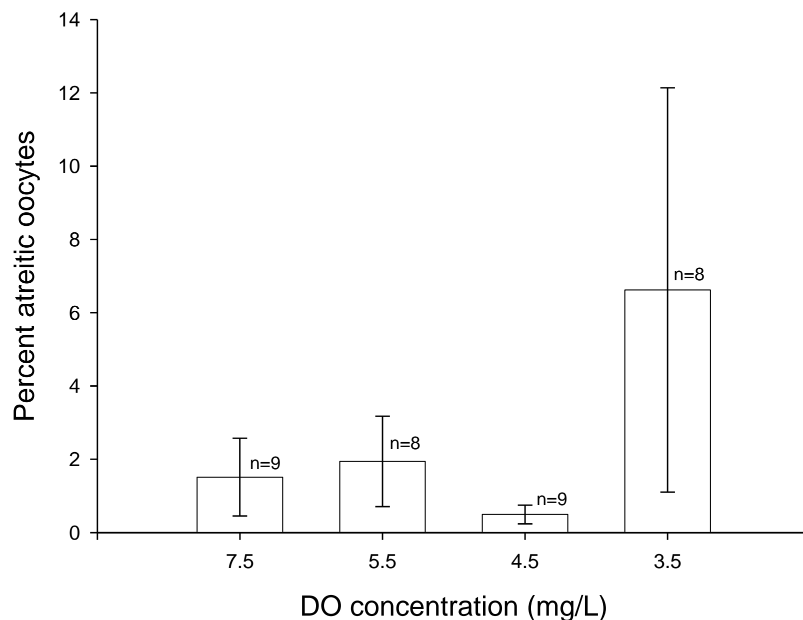


Figure 3.4.8. Percentage of atretic oocytes (± standard error) within cross sections of fathead minnow ovaries after an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen.

3.5 Hormone and vitellogenin analysis

Estradiol and testosterone was measured in the gonads of fish surviving until the termination of the experiment. In some cases, insufficient tissue samples prevented analysis, lowering sample size (Males: control: n=6, 5.5 and 4.5 mg/L: n=3, 3.5 mg/L: n=5; Females: control and 4.5 mg/L: n=9, 5.5 and 3.5 mg/L: n=8). Vitellogenin was measured in the liver of male fish. In one fish, insufficient tissue prevented analysis (control: n=5, 5.5 and 4.5 mg/L: n=3, 3.5 mg/L: n=6).

Hormone analysis using Kruskal-Wallis tests showed no significant difference in levels of estradiol (Figure 3.5.1) or testosterone (Figure 3.5.2) in female fish (Estradiol:

$X^2_{(3)}$=0.19, p>0.9; Testosterone: $X^2_{(3)}$=0.54, p>0.9). Similarly, Kruskal-Wallis tests did not find significant differences among treatments in male levels of estradiol (Figure 3.5.3) or testosterone (Figure 3.5.4) (Estradiol: $X^2_{(3)}$=0.99, p>0.8; Testosterone: $X^2_{(3)}$=2.68, p>0.4). A Kruskal-Wallis test showed no differences in vitellogenin levels among treatments ($X^2_{(3)}$=2.93, p>0.4) (Figure 3.5.5).

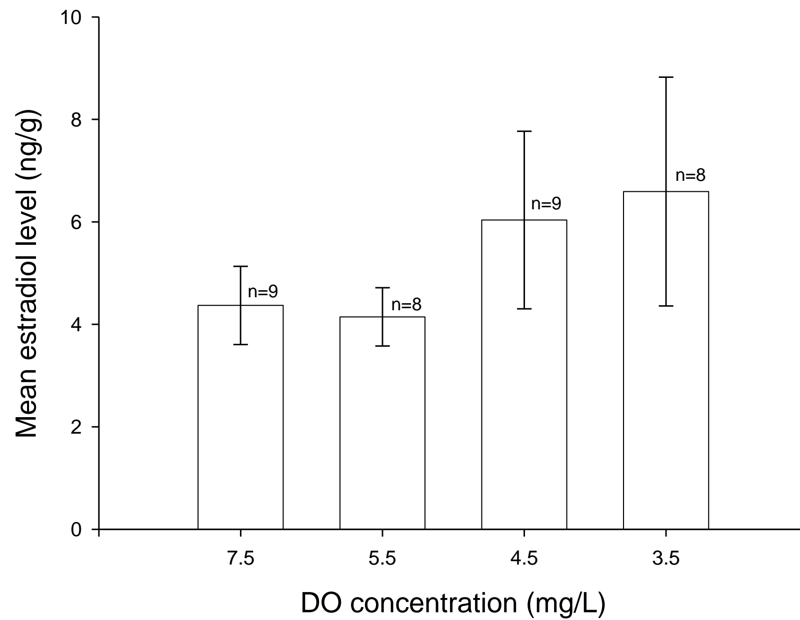


Figure 3.5.1. Mean estradiol level (± standard error) in the gonad tissue of female fathead minnows after an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen.

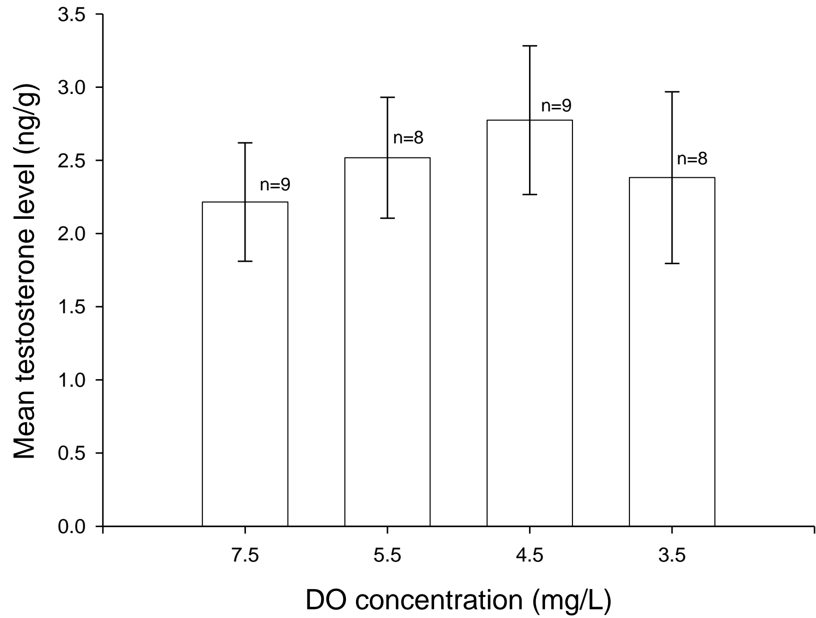


Figure 3.5.2. Mean testosterone level (± standard error) in the gonad tissue of female fathead minnows after an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen.

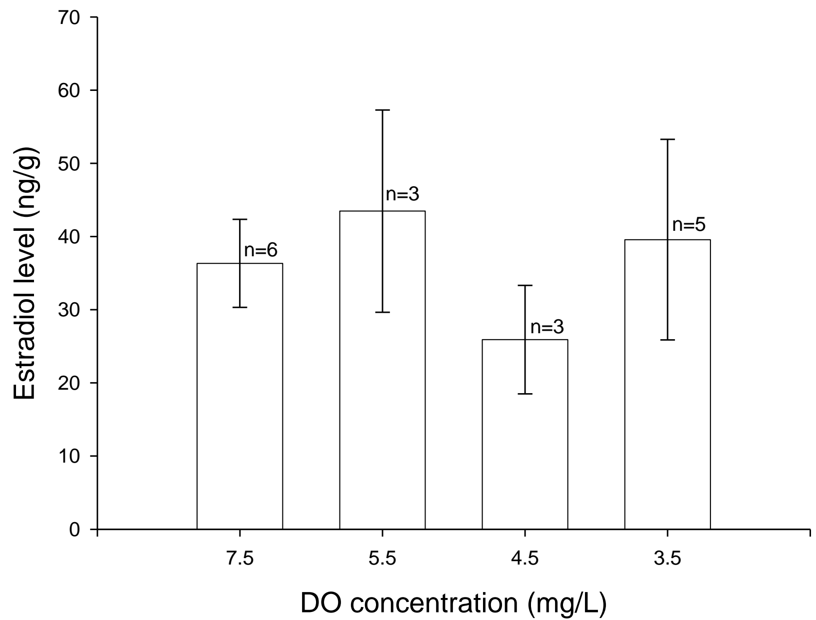


Figure 3.5.3. Mean estradiol level (± standard error) in the gonad tissue of male fathead minnows after an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control)

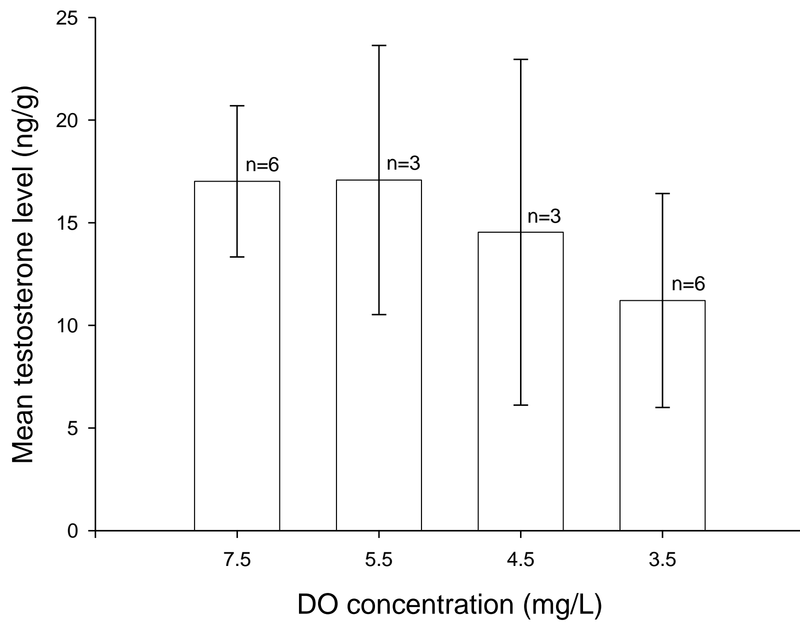


Figure 3.5.4. Mean testosterone level (± standard error) in the gonad tissue of male fathead minnows after an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen.

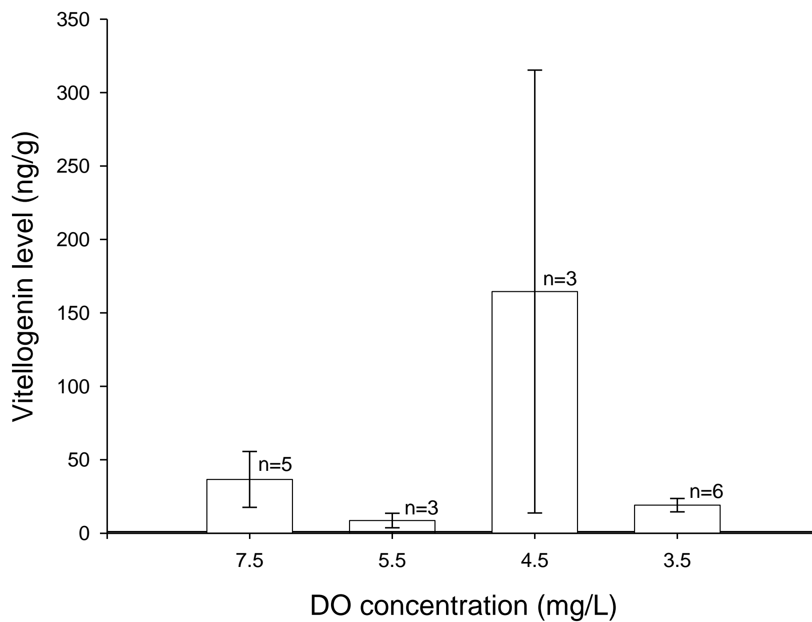


Figure 3.5.5. Mean vitellogenin level (± standard error) in the liver tissue of male fathead minnows after an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5(control) mg/L of dissolved oxygen.

3.6 Behavioural data

Behavioural data was recorded daily in a subset of 2 tanks per treatment. Mortality of male or both females in a tank prior to the first recording reduced sample size (control: n=9, 5.5 and 3.5 mg/L: n=8, 4.5 mg/L: n=7).

A one-way ANOVA showed no significant differences among treatments in the time spent in the nest by males ($F_{(3,28)}=1.33$, $p>0.2$) (Figure 3.6.1). A Kruskal-Wallis test showed a significant difference in time spent on nest maintenance by male fish ($X^2_{(3)}=11.30$, $p=0.01$) (Figure 3.6.2). A non-parametric post-hoc test for unequal sample sizes was not powerful enough to find this difference (7.5 vs 5.5 mg/L: $Q=1.37$, $p>0.2$; 7.5 vs 4.5 mg/L: $Q=0.83$, $p>0.5$; 7.5 vs 3.5 mg/L: $Q=1.37$, $p>0.2$). Analysis of courtship behaviour also showed a significant difference among treatments (One-way ANOVA: $F_{(3,28)}=11.86$, $p<0.001$) (Figure 3.6.3). Subsequent post-hoc tests showed that males in all treatment groups significantly decreased time spent in courtship compared to the control (control vs: 5.5 and 3.5 mg/L, $p<0.001$; 4.5 mg/L, $p<0.01$).

Pearson correlations examining relationships between reproductive behaviours (time in nest, time in nest care and courtship behaviour) and egg production revealed a statistically significant correlation. The amount of time males spent engaging in courtship behaviour significantly correlated with the number of eggs produced (Pearson correlation, $p=0.006$) All other behaviours failed to significantly correlate with reproductive output (Time in nest: $p=0.065$; Time spent in nest maintenance: $p=0.743$) (Data not shown).

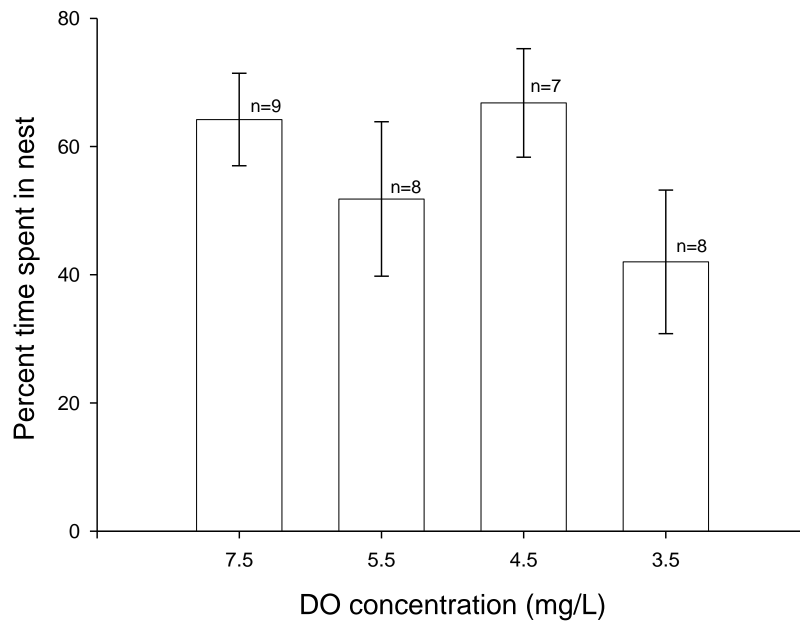


Figure 3.6.1. Percent time spent in the nest (+ standard error) by male fathead minnows over an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5 (control) mg/L dissolved oxygen

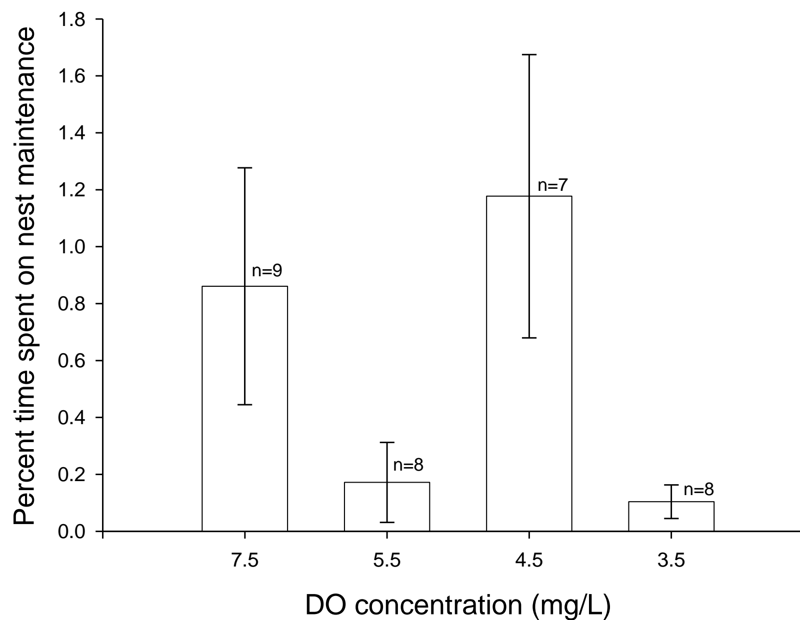


Figure 3.6.2. Percent time spent on nest maintenance (+ standard error) by male fathead minnows over an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5 (control) mg/L dissolved oxygen.

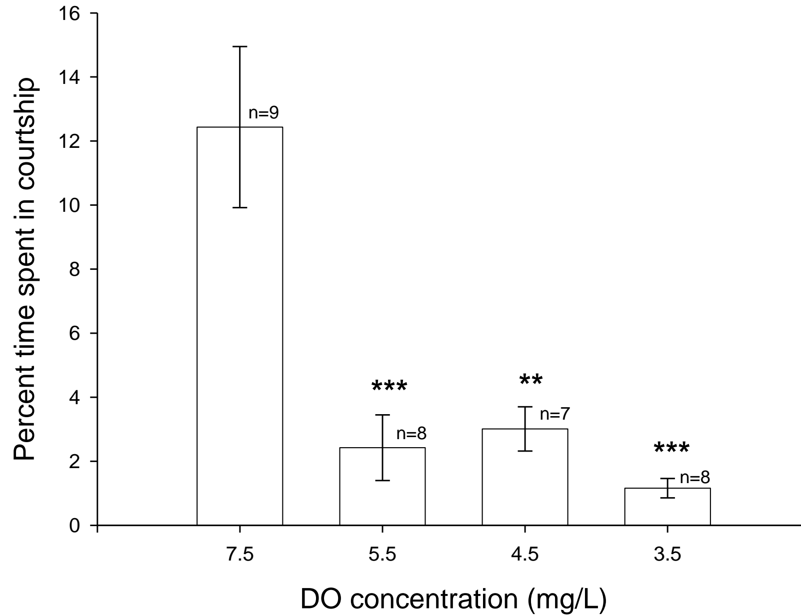


Figure 3.6.3. Time spent in courtship (± standard error) by male fathead minnows over an 11-day exposure period to 3.5, 4.5, 5.5, and 7.5 (control) mg/L dissolved oxygen. Asterix denotes significant difference from the control (**=p<0.01;*** = p < 0.001).

Experiment 2: The effects of various levels of dissolved oxygen on fathead minnows

3.7 Water quality

All water quality measurements were analyzed as described in experiment 1, such that sample size for each treatment group was equal to the duration of the experiment (n=21). All treatment levels of DO were significantly different from each other (ANOVA, p = <0.001; Tukey post-hoc test: p<0.001 for all treatments) (Figure 3.7.1). A Kruskal-Wallis test showed no significant difference in water temperature between treatments ($X^2_{(3)} = 0.78$, p>0.8) (Figure 3.7.2). The results from a Kruskal-Wallis test showed no significant difference in ammonia concentration or pH among treatments

(Ammonia: $X^2_{(3)} = 5.37$, $p>0.1$; pH: $X^2_{(3)} = 1.97$, $p>0.5$) (Table 3.7.1). There was also no significant difference in conductivity among treatments (Kruskal-Wallis: $X^2_{(3)} = 0.41$, $p>0.9$).

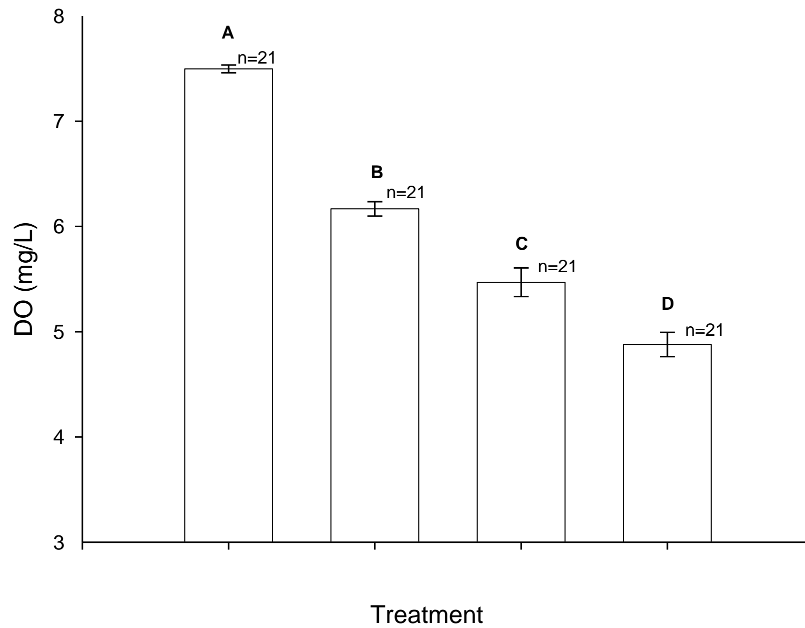


Figure 3.7.1. Mean (± standard error) dissolved oxygen concentration measured from four treatment groups during a 21-day exposure period.

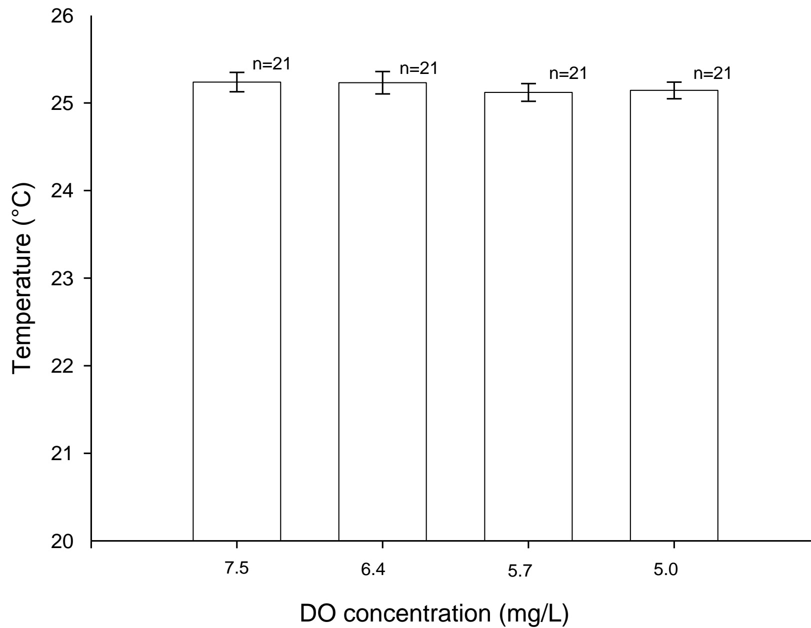


Figure 3.7.2. Mean (± standard error) water temperature over a 21-day exposure period to 5.0, 5.7, 6.4, and 7.5 (control) mg/L of dissolved oxygen.

Water quality parameter	n	7.5 mg/L (control)	6.4 mg/l	5.7 mg/l	5.0 mg/l
pH	21	7.56 ± 0.08	7.52 ± 0.08	7.56 ± 0.08	7.61 ± 0.09
Conductivity (µS/cm)	21	326.3 ± 22.6	318.3 ± 23.3	323.8 ± 23.8	333.5 ± 23.9
Ammonia (mg/L)	21	0.01 ± 0.00	0.05 ± 0.02	0.08 ± 0.03	0.05 ± 0.02

Table 3.7.1. Water quality parameters measured daily over an 21-day exposure to 5.0, 5.7, 6.4, and 7.5 (control) mg/L of dissolved oxygen. Mean values ± standard error are represented.

3.8 Reproductive endpoints

Fertilization success, hatching success, larval deformities, and days required for eggs to hatch were analyzed as in experiment 1. Egg diameter was also analyzed for this experiment using a sub-sample of 10 eggs/brood. Sample size for these endpoints varied with the number of tanks per treatment which produced broods (control, 5.0 mg/L: n=4; 6.4 mg/L: n=6; 5.7 mg/L: n=8). A Kruskal-Wallis test found no significant difference in fertilization success ($X^2_{(3)}=2.67$, $p>0.4$) (Figure 3.8.1) and a one-way ANOVA showed no difference in the hatching success ($F_{(3,18)}=0.61$, $p>0.6$) (Figure 3.8.2). There was no significant difference in the number of days required for eggs to hatch (Kruskal Wallis: $X^2_{(3)}=4.08$, $p>0.2$) (Figure 3.8.3) or larval deformities (one-way ANOVA: $F_{(3,18)}=0.65$, $p>0.5$) (Figure 3.8.4). A one-way ANOVA showed no difference in egg diameter ($F_{(3,18)}=1.69$, $p>0.2$) (Figure 3.8.5).

A Kolmogorov-Smirnov test showed that the cumulative eggs produced per female was higher in the control than in the 5.0 and 6.4 mg/L treatments, but lower than the 5.7 mg/L treatment (7.5 vs 6.4 mg/L: $p<0.05$, 7.5 vs 5.7 $p<0.001$; 7.5 vs 5.0 mg/L: $p<0.001$)(Figure 3.8.6).

Egg production per female per day was analyzed as in experiment 1 (n=9 for all treatments). When analyzed based on the mean brood size per replicate tank, a Kruskal-Wallis test showed no significant difference between the control and all treatments ($X^2_{(3)}=3.42$, $p>0.3$) (Figure 3.8.7). A significant difference was not found when egg production was additionally analyzed based on the total number of eggs produced per tank (Kruskal-Wallis: $X^2_{(3)}=4.06$, $p>0.2$) (Figure 3.8.8).

Eggs produced per female per day were analyzed using one-way ANCOVAs, as in experiment 1 (n=9 for each treatment). No significant differences among treatments were found when body weight or fork length were used as a covariates (egg production with body weight as covariate: $F_{(3,31)}=1.04$, $p>0.3$; egg production with fork length as covariate: $F_{(3,31)}=1.21$, $p>0.3$) (Data not shown).

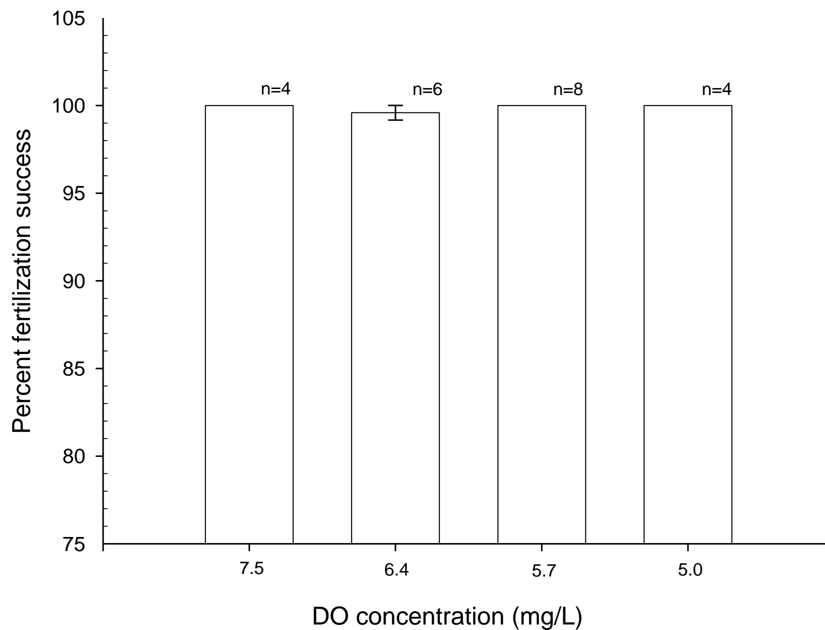


Figure 3.8.1. Percent fertilization success (± standard error) for eggs laid by fathead minnows over a 21-day exposure period to 5.0, 5.7, 6.4, and 7.5 mg/L of dissolved oxygen.

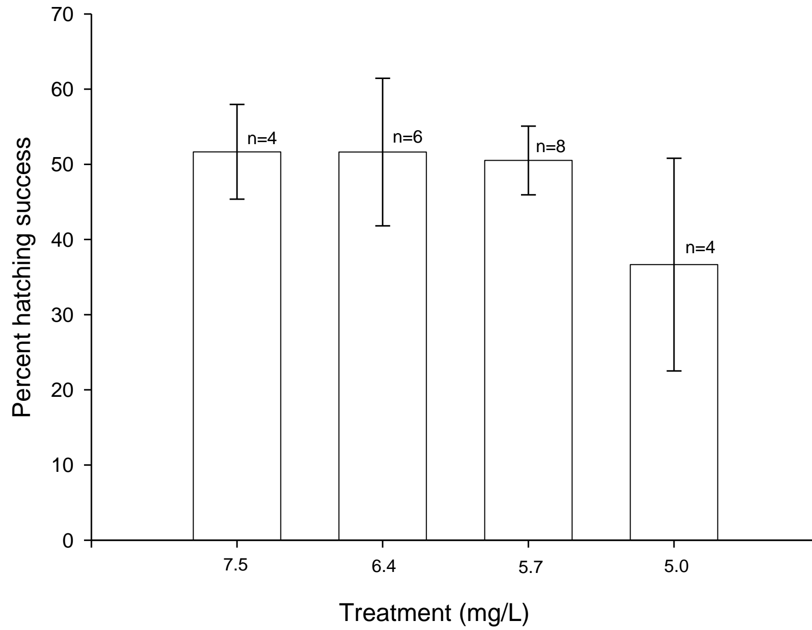


Figure 3.8.2. Percent hatching success (± standard error) for eggs laid by fathead minnows over a 21-day exposure period 5.0, 5.7, 6.4, and 7.5(control) mg/L of dissolved oxygen.

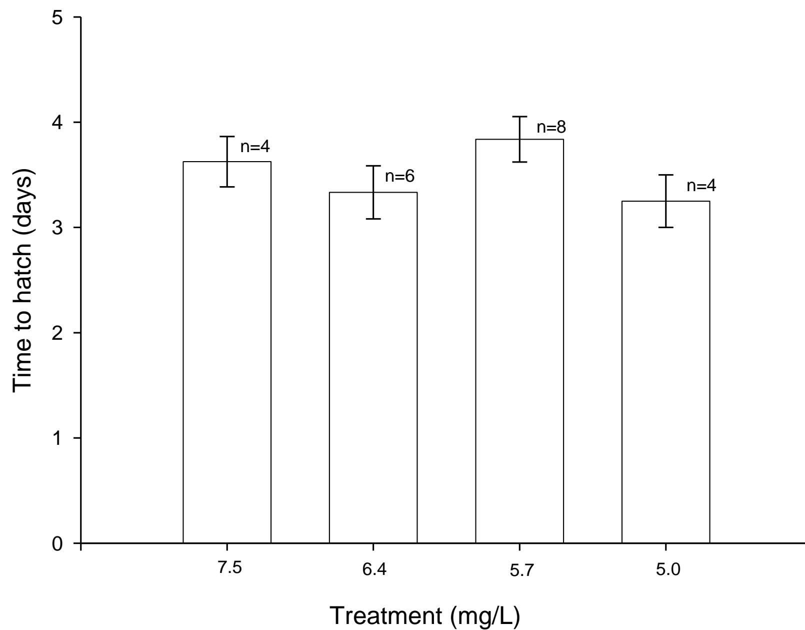


Figure 3.8.3. Mean number of days required to hatch (± standard error) for eggs laid by fathead minnows over a 21-day exposure period to 5.0, 5.7, 6.4, and 7.5(control) mg/L of dissolved oxygen.

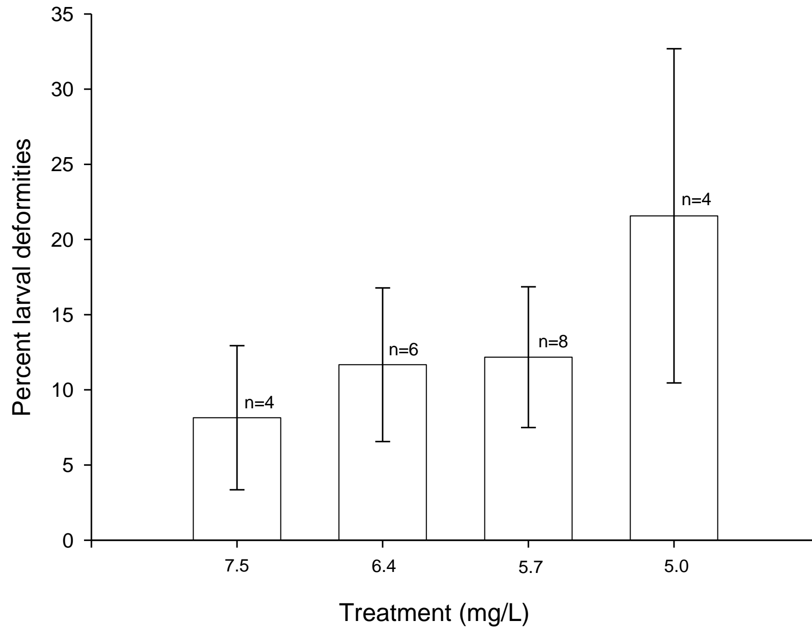


Figure 3.8.4. Percent larval deformities (± standard error) for newly-hatched offspring produced by fathead minnows over a 21-day exposure period to 5.0, 5.7, 6.4, and 7.5(control) mg/L of dissolved oxygen.

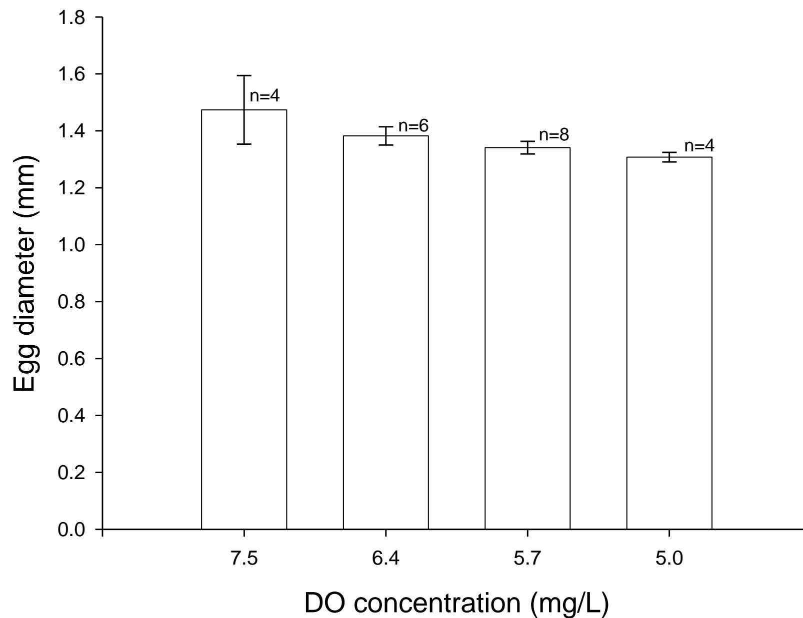


Figure 3.8.5. Mean diameter (± SE) of newly-laid fathead minnow eggs during a 21-day exposure period to 5.0, 5.7, 6.4, and 7.5(control) mg/L of dissolved oxygen. Eggs were measured within several hours of laying.

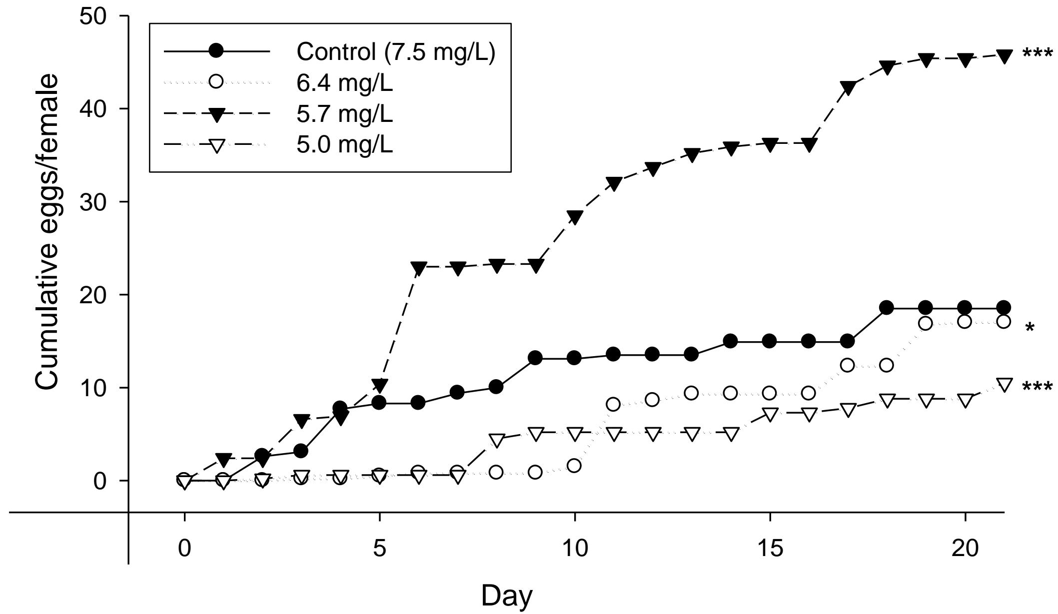


Figure 3.8.6. Cumulative eggs/female produced by fathead minnows over a 21- day exposure period to 5.0, 5.7, 6.4, and 7.5(control) mg/L of dissolved oxygen. Asterix denotes a significant difference from the control (*** = p < 0.001).

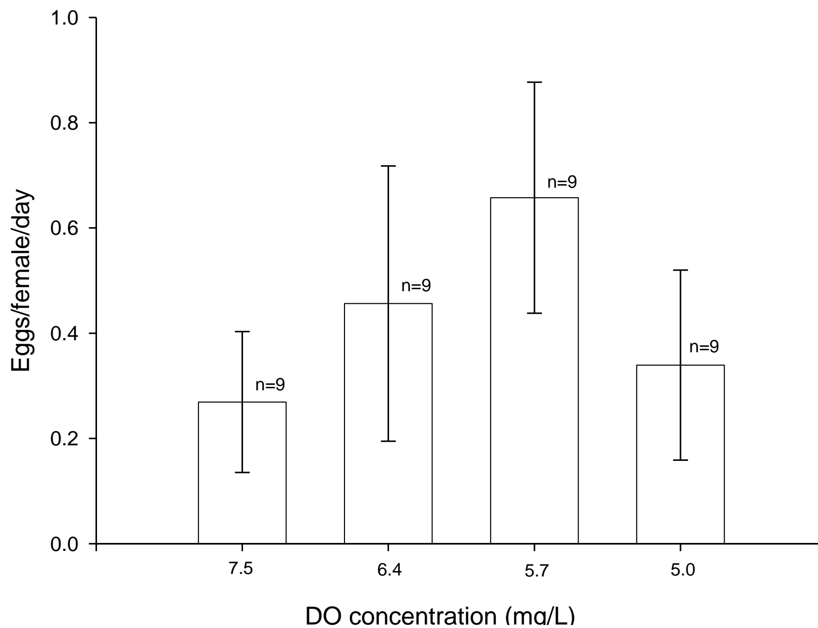


Figure 3.8.7. Mean (+ SE) of the average number of eggs produced per female per day over a 21-day exposure period to 5.0, 5.7, 6.4, and 7.5(control) mg/L of dissolved oxygen.

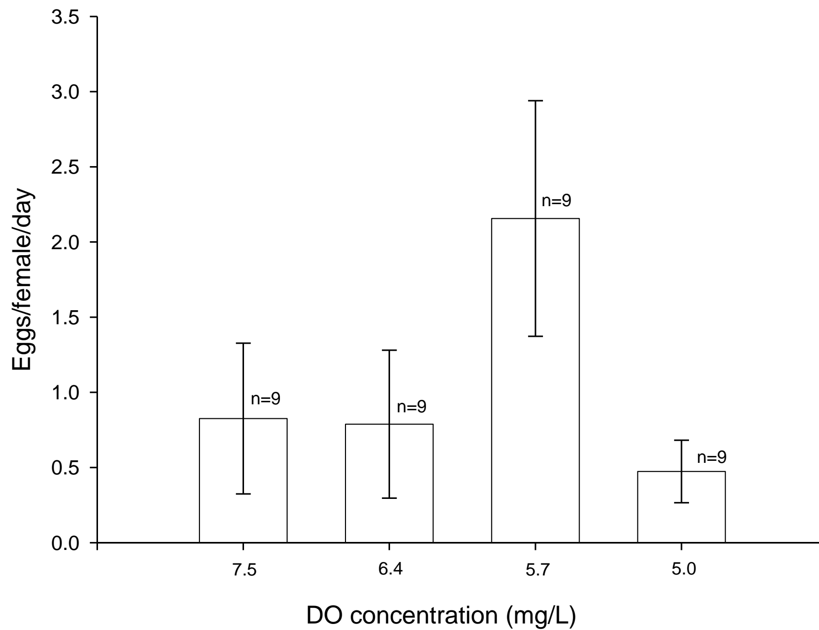


Figure 3.8.8. Mean (+ SE) of the total number of eggs produced per female per day over a 21-day exposure period to 5.0, 5.7, 6.4, and 7.5(control) mg/L of dissolved oxygen.

3.9 Survival and morphometrics

Adult survival was analyzed as in experiment 1 (n=27 in all treatments). No significant differences were found among treatments ($X^2_{(3)}$ =0.63, p>0.7) (Figure 3.9.1).

Morphometric measurements were analyzed in the same manner as in experiment 1 (n=9 for all treatments). A Kruskal-Wallis test showed no significant differences in male body weight ($X^2_{(3)}$=1.17, p>0.7) and a one-way ANOVA demonstrated no significant difference in female body weight ($F_{(3,32)}$=2.11, p>0.1) (Table 3.9.1). There was no significant difference in fork length for male or female fish (One-way ANOVAs, Males: $F_{(3,32)}$=2.31, p>0.05; Females: $F_{(3,32)}$=2.73, p>0.05). (Table 3.9.1) No significant differences among treatments were found in GSI (Kruskal-Wallis, Males: $X^2_{(3)}$=4.44, p>

0.2; Females: $X^2_{(3)}$=2.60, p>0.5) or in LSI (Males: Kruskal-Wallis, $X^2_{(3)}$=2.54, p>0.4; Females, One-way ANOVA: $F_{(3,32)}$=0.74, p>0.5) (Table 3.9.1). There was no significant difference in condition factor between treatments (One-way ANOVAs, Males: $F_{(3,32)}$=2.36, p>0.05; Females: $F_{(3,32)}$=0.63, p>0.6) (Table 3.9.1).

Secondary sex characteristics were analyzed as in experiment 1, and there were no significant differences in either males (Q=0.08, p>0.2) or females (Q=0.63, p>0.7) (Data not shown).

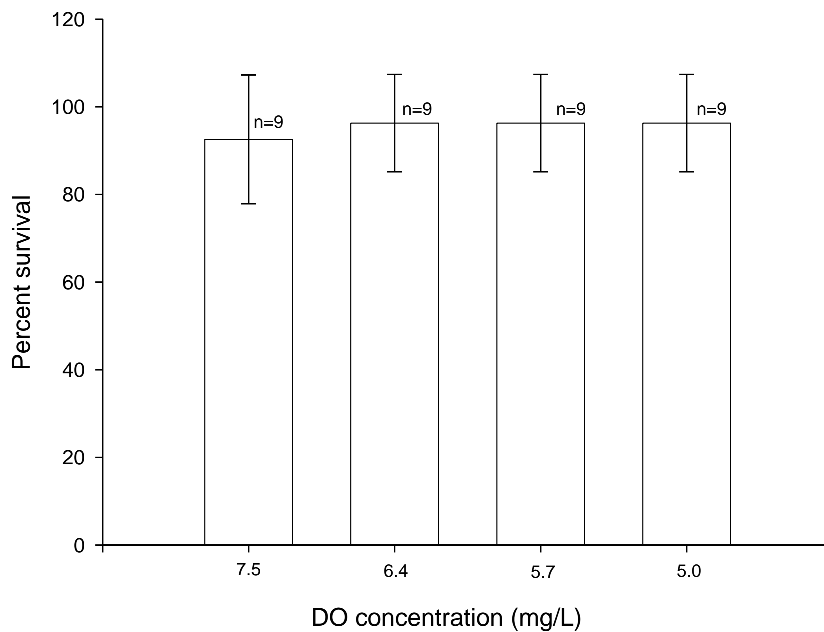


Figure 3.9.1. Mean survival (± standard error) of fathead minnows over an 11-day exposure to 5.0, 5.7, 6.4, and 7.5(control)mg/L dissolved oxygen.

MALES	7.5 mg/L (n=9)	6.4 mg/l (n=9)	5.7 mg/l (n=9)	5.0 mg/l (n=9)
Body weight (g)	3.41 ± 0.26	3.68 ± 0.30	3.27 ± 0.21	3.62 ± 0.27
Fork length (mm)	64 ± 2	68 ± 2	62 ± 1	64 ± 2
GSI %	1.50 ± 0.35	0.77 ± 0.10	0.75 ± 0.13	0.72 ± 0.11
LSI %	1.30 ± 0.23	0.98 ± 0.14	1.62 ± 0.26	1.43 ± 0.26
CF (g/cm^3)	1.28 ± 0.07	1.18 ± 0.06	1.39 ± 0.04	1.36 ± 0.06
FEMALES	**7.5 mg/L** (n=9)	**6.4 mg/l** (n=9)	**5.7 mg/l** (n=9)	**5.0 mg/l** (n=9)
Body weight (g)	1.73 ± 0.06	1.89 ± 0.08	1.56 ± 0.10	1.70 ± 0.12
Fork length (mm)	51 ± 1	53 ± 1	49 ± 1	50 ± 1
GSI %	9.67 ± 0.88	8.45 ± 0.87	9.12 ± 1.05	7.76 ± 0.92
LSI %	2.03 ± 0.17	1.84 ± 0.20	1.58 ± 0.19	1.90 ± 0.27
CF (g/cm^3)	1.32 ± 0.06	1.30 ± 0.04	1.36 ± 0.05	1.39 ± 0.03

Table 3.9.1. Morphometric endpoints from fathead minnows after a 21-day exposure to 5.0, 5.7, 6.4, and 7.5 (control) mg/L dissolved oxygen. Values represent means ± standard error.

3.10 Behavioural data

Behavioural data was recorded daily in one tank per treatment from day 2 to 18, until all tanks had been recorded twice. The two recordings were then averaged so that sample size for all treatments was 9.

The amount of time spent in courtship was not significantly different between treatments (Kruskal-Walis: $X^2_{(3)}=2.66$, $p > 0.4$) (Figure 3.10.1). No significant differences were found in the amount of time spent in nest by males (One-way ANOVA: $F_{(3,32)}=0.63$, $p>0.6$) (Figure 3.10.2). A Kruskal-Wallis test also showed no significant difference in time spent in nest maintenance ($X^2_{(3)}=3.46$, $p > 0.3$) (Figure 3.10.3).

Pearson correlations revealed a statistically significant correlation between time spent in courtship behaviour and egg production (p=0.005). Time spent by males in the nest and time spent on nest maintenance failed to correlate with reproductive output (Time in nest: p=0.096; Time spent in nest maintenance: p=0.099) (Data not shown).

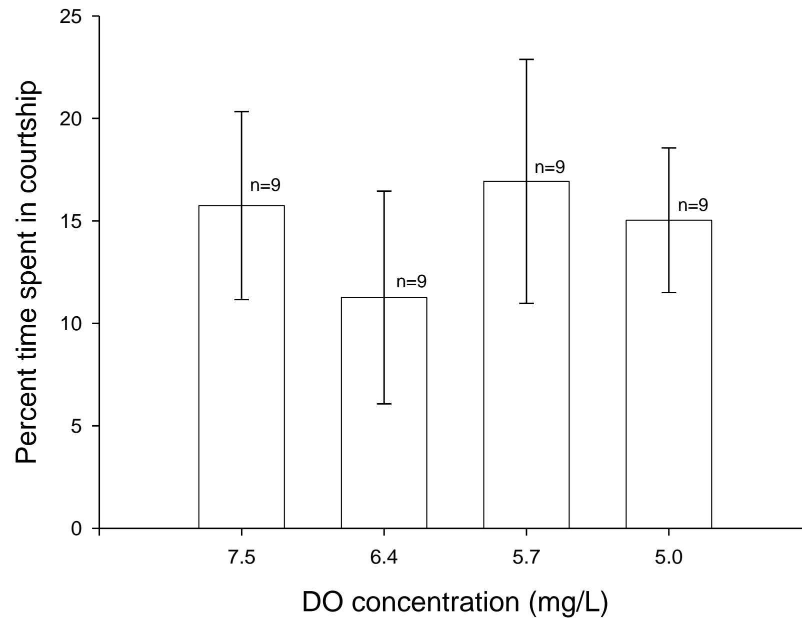


Figure 3.10.1. Percent time spent on courtship (± standard error) by male fathead minnows over a 21-day exposure period to 5.0, 5.7, 6.4, and 7.5 (control) mg/L dissolved oxygen

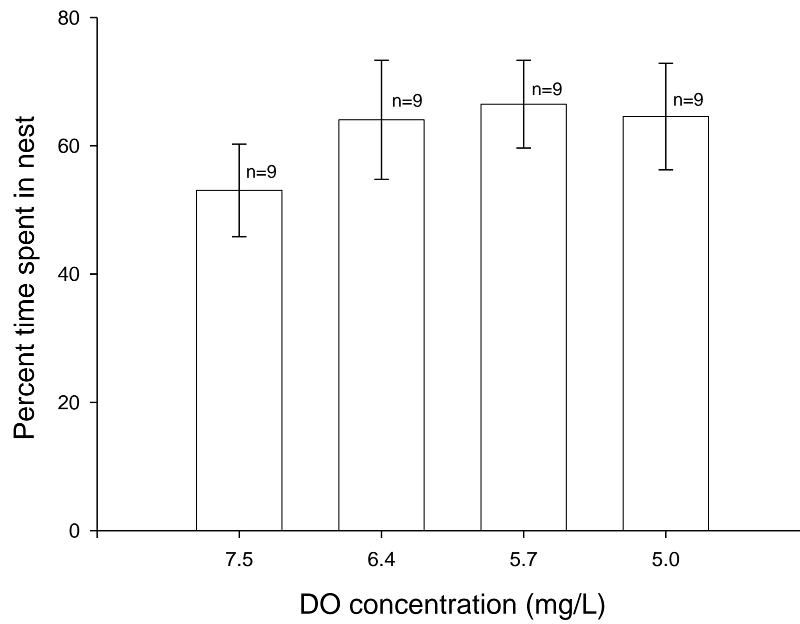


Figure 3.10.2. Percent time spent in the nest (± standard error) by male fathead minnows over a 21-day exposure period to 5.0, 5.7, 6.4, and 7.5 (control) mg/L dissolved oxygen

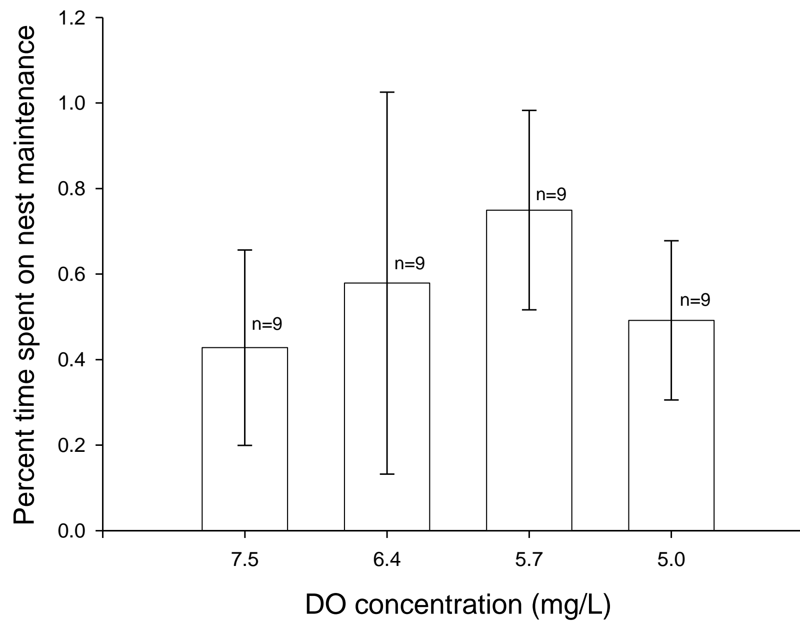


Figure 3.10.3. Percent time spent on nest maintenance (± standard error) by male fathead minnows over a 21-day exposure period to 5.0, 5.7, 6.4, and 7.5 (control) mg/L dissolved oxygen

Chapter 4: Discussion

4.1 Water Quality

In experiment 1, there were several differences in water quality between treatments. While the temperature was lower in the 3.5 mg/L treatment group than in the control, this difference represented only 0.6°C. Although this difference was statistically significant, it is unlikely that such a small difference in temperature is biologically relevant. A significant difference was also found in pH, where the pH was significantly higher in the control than in all other groups. Once again, these differences were small (0.4 pH unit or less) so while statistically relevant, they likely did not produce a biological effect. While pH has shown to influence breeding in fathead minnows, Mount (1973) found that pH levels as low as 6.6 did not affect reproductive performance. Ellis (1937) found that healthy wild populations of fathead minnows generally inhabited areas with pH levels ranging from 6.7 and 8.6, indicating that fathead minnows have a fairly wide range of tolerance for pH. No significant differences were found in any of the other water quality parameters that were measured in either experiment.

4.2 Reproductive endpoints

Reproductive output is an integral endpoint in fish toxicology studies, and is an important aspect of risk assessment for aquatic ecosystems (Ankley et al., 2001). Many chemicals or other stressors impair reproduction in fishes. A prediction of reproductive impairment is therefore essential to estimate recruitment, which is imperative to the maintenance of fish populations (Ankley et al., 2001). For the purposes of this research, reproductive output was the primary concern.

In my first experiment, egg production decreased with decreasing DO. Both mean and total egg production did not differ significantly between the control and 5.5 mg/L groups. When the distribution of egg production was analyzed taking into account mortality and using experimental day as a replicate, a significant difference was found. As Kolmogorov-Smirnov tests analyze both differences in magnitude and distribution, a significant result indicated that the production of eggs over time differed between 7.5 and 5.5 mg/L groups. These data indicate a possibility of reproductive impairment when DO is maintained at 5.5 mg/L. In the 3.5 and 4.5 mg/L groups, spawning did not occur over the course of the experiment. This suggests that complete reproductive failure can occur at levels of DO lower than 4.5 mg/L.

In the second experiment, no significant differences in mean or total egg production were detected when DO was maintained at 6.4, 5.7, and 5.0 mg/L. Using the cumulative approach, however, the distribution of egg production in all treatment groups was significantly different from the control. Egg production was higher in the 5.7 mg/L group, and lower in both the 6.4 and 5.0 mg/L groups. These results are contradictory to those of the first experiment, and two explanations can be proposed. First, the DO level in the second experiment was less constant than in the first due to equipment malfunction. These fluctuations in DO level reduce confidence in the relevance of this experiment. Also, overall egg production was much lower in the second experiment than in the first. The control in experiment 1 produced 3.6 times more eggs than the control in experiment 2, in approximately half the time. This low egg production further reduces confidence in the results of experiment 2.

A second possibility is that a hormetic response to DO concentration exists in fathead minnows. Hormesis occurs when a biphasic dose-response exists in the presence of some chemical or stressor, and is characterized by inhibition at high doses, and enhancement at smaller doses (Calabrese, 2008). This may be caused by an over-reaction of detoxification or coping-mechanisms (Weltje et al., 2005). If one considers oxygen deprivation as a stressor, my data shows a pattern where higher stress (4.5 mg/L and lower) causes a negative effect, and moderate stress (5.7 mg/L) may cause enhancement. While this response was not consistent between experiments, hormetic responses may often vary between individuals or populations (Calabrese, 2008). This predicts that high egg production would be expected when DO concentration is near saturation, low or no egg production at lower DO concentrations (below about 4.5 mg/L), and that responses to intermediate DO concentrations may vary from enhancement to inhibition. One should however use caution when interpreting the word "enhancement" in this context; while it does indicate increased egg production, authors have warned that this may result in lower quality eggs or larvae (Giesy, 2001). This may be reflected in smaller egg size or hatching success, or lower rates of larval survival (Giesy, 2001).

Finally, it is important to note that the holding conditions of my fish were slightly modified between experiments. In the first experiment, male and female fish were allowed contact with each other for two weeks prior to the start of the pre-exposure. For my second experiment, however, males and females were only allowed contact through a mesh screen. This difference in holding conditions may explain some of the variability in egg production between the two experiments.

There are several mechanisms by which depressed DO could conceivably cause reproductive impairment or failure. One likely explanation is the occurrence of metabolic stress, as oxygen is required for the production of ATP (Wilson et al., 1988). The energy derived from ATP is essential to most animals for biosynthesis, transport, and mechanical function (Wilson, 1988). Oxygen is potentially as essential to energy production in animals as the nutrients derived from food intake (Kramer, 1987), so with decreasing available oxygen, one would expect fish to conserve energy behaviourally or physiologically. Given that egg production represents a considerable energetic investment (Dewsbury, 1982), depressed DO has the potential to limit reproductive output.

While not all fish spawned, upon dissection most females in all treatment groups had post-vitellogenic eggs within their ovaries. In cultured fish, fully-grown oocytes can develop without the occurrence of subsequent spawning (Wang et al., 2008). It is possible that females can choose not to release their eggs into an environment with insufficient oxygen for survival. This contradicts the fact that hatching success was not significantly different between the control and the 5.5 mg/L treatment, although long-term larval survival could not be evaluated.

It also possible that the physiological state of fathead minnows under conditions of depressed DO is responsible for the failure to spawn. Wang et al. (2008) found that hypoxia (1.0 ± 0.2mg/L) did not prevent gonad development or oocyte formation in common carp (*Cyprinus carpio*), however, final oocyte maturation was retarded and spawning did not occur. Oocytes developed to the post-vitellogenic stage, but final oocyte maturation and subsequent ovulation was impaired. This disruption in development was related to a decrease in serum luteinizing hormone (LH), which

regulates final oocyte maturation. The presence of post-vitellogenic oocytes that were never spawned within the ovaries of fish in the 4.5 and 3.5 mg/L groups makes impairment of final oocyte maturation a likely explanation for reproductive failure at these DO concentrations.

Neither experiment showed an effect of DO concentration on fertilization success, hatching success, time required to hatch or larval deformities. Previous studies found that under true hypoxic conditions (below 2 mg/L), fertility and hatching success was decreased in common carp (*Cyprinus carpio*) and atlantic croaker *(Micropogonias undulatus)* (Wu et al., 2003, Thomas et al., 2006). My data, however, suggests that fertility, hatching time, and larval deformities in fathead minnow larvae are not affected by DO levels as low as 5.0 mg/L. Hatching success, on the other hand, was low in all treatments that I tested (11-21% in experiment 1 and 36-52% in experiment 2). This low level of hatching success was likely due to the presence of fungal infections among the clutches produced in all treatments. While infected eggs were removed daily to prevent the spread of this problem, fungal infections remained a common occurrence throughout both experiments.

The results from this research indicate that fathead minnow eggs produced in DO levels of 5.0 and higher are likely to develop with equal viability to those in environments near saturation. As no eggs were produced in the 4.5 and 3.5mg/L treatments, these DO levels were not tested. Future research could investigate egg viability at 3.5 and 4.5 mg/L DO to determine the sensitivity of the early life-stages in fathead minnows. Limitations of the experimental apparatus also prevented an analysis of long-term larval survival and

development; however this represents another important aspect of the fathead minnow life-cycle, and hence determines recruitment.

The implications of lowered egg production at the tested levels of DO are severe; if reproductive failure is consistently observed at DO concentrations as high as 4.5 mg/L, then depressed DO may represent a greater threat to fish populations than previously imagined. Furthermore, the high degree of variability in egg production among replicate tanks and the small sample size likely presented an obstacle in detecting differences between treatments. Further experimentation with larger sample sizes would be required to determine more accurately the DO concentration at which reproductive impairment is first observed in lab populations. This notwithstanding, the minimum DO standard for warm water environments to the Canadian Water Quality Guidelines is currently 5.0 mg/L. These guidelines are calculated using data from the most sensitive species in Canadian ecosystems. Given that I observed a significant decline in egg production at a DO concentration higher than this guideline, and complete reproductive failure at half a mg below this number in a tolerant species, revision of current standards may be desirable during spawning periods.

4.3 Survival and Metrics

Hypoxic conditions in natural aquatic ecosystems have long been associated with fish kills (Paerl et al., 1998; Brietburg, 2002; Portnoy, 1991). Mortality in relation to more moderate degrees of oxygen deprivation, however, has not received as much attention. In both experiments, mortality showed no significant difference between the

control and all treatments. This indicates that the tested levels of DO are unlikely to directly cause increased mortality in the absence of other stressors.

While previous studies using gulf killifish or mummichog (*F. heteroclitus)* have demonstrated decreased growth in response to hypoxia (Landry et al., 2007, Stierhoff et al., 2003), I found no differences between treatments in fish length or body weight. This may be due to the short duration of my experiment, and higher levels of DO as compared to those used by other authors, however it is more likely that the fish used in my research had attained near full growth by the time my experiment was initiated.

The body condition of a fish is thought to be an indicator of energy reserves, which may vary in response to environmental stressors (Morgan, 2004). Fish in poor condition may have reduced fitness due to low egg production, poor quality offspring, and a higher risk of mortality (Morgan, 2004). There were no significant differences in condition factor between treatment groups and control in either experiment indicating that short-term (11-day) exposure to DO levels as low as 3.5 mg/L, and 21-day exposure to DO levels as low as 5.0 mg/L does not significantly reduce energy stores in fathead minnows. These results are not surprising, given that oxygen is required to metabolize fats, and metabolic rate may decline with decreasing available oxygen (Muusze, 1998).

As most fish store energy in their liver, (Lambert & Dutil, 1997; Morgan et al., 2004) liver somatic index (LSI) is also an indicator of overall fish condition (Morgan et al., 2004). I found no significant differences in LSI from the control in either experiment, indicating that the tested DO levels do not significantly affect energy storage in the liver of fathead minnows.

Gonadosomatic index (GSI) represents a measure of fecundity in female fish, and previous work has shown that fish with a lower GSI may have smaller larvae with a lower rate of survival (Wu et al., 2003). As fathead minnows are fractional spawners, female GSI is expected to vary with position in the spawning cycle (Jenson et al. 2001) and to decline throughout the breeding season (McCormick et al., 1989). Male GSI, however, remains relatively constant with respect of position in breeding cycle (Jenson et al. 2001).

Wu et al (2003) found that GSI was reduced in common carp that were maintained long term (12 weeks) at 1.0 ± 0.2 mg/L DO. In my experiments, no significant differences were found in male or female GSI from the control. Ankley (2001) reported that GSI for fathead minnows in control groups should be between 8-13% for females, and 1-2% for males. In experiment 1, mean male GSI in the controls was slightly lower than these predictions (0.89 ± 0.23), however all other control GSI calculations were within this range. Jenson et al. 2001 found that GSI in female fathead minnows reached its peak at about 2 days post-spawn, immediately before the period of maximum spawning activity. As all fish were sacrificed on the same day, they were likely in different stages within their spawning cycle, greatly adding to the variability between replicates. If an effect was present at any of the tested levels, it would therefore be difficult to diagnose unless it was very great in magnitude.

4.4 Histology

The ovaries of actively reproducing fathead minnows contain oocytes at a variety of stages of development, ranging from primary growth, cortical alveolus, early

vitellogenic, and later vitellogenic stages (Jenson et al., 2001; Leino et al., 2005). The relative number of oocytes present in each stage can vary according to position in the spawning cycle. For example, immediately post spawn, a relatively higher proportion of primary growth oocytes are present (Jenson et al., 2001). Within two days post-spawn, a higher percentage of vitellogenic oocytes were found, and fewer primary growth oocytes are present as development progresses (Jenson et al., 2001). Stage 5 oocytes are not commonly observed unless fish are sampled precisely during spawning (Leino et al., 2005). Evaluating percentages of oocytes in each stage allows for assessment of developmental delay or interruption, although this type of analysis likely only reveals very profound impairment in gonad development (Leino et al., 2005). No significant differences in the percentage of early and late oocytes were found between treatments in experiment 1, indicating that delays in oocytes development up to the late vitellogenic stages do not occur in fathead minnows at DO levels as low as 3.5 mg/L. Due to the rarity of stage 5 oocytes and uncertainty in their identification, it cannot be determined whether final oocytes maturation was affected by DO levels in my experiment.

Atretic oocytes are produced when oocytes undergo inter-ovarian resorption (McCormick et al., 1989). Oocyte atresia results in energy consumption without any reproductive payoff, as oocytes must be produced but are never spawned (McCormick et al., 1989). High incidence of atresia (>20%) may result in a reduction or failure of recruitment (McCormick et al., 1989).While atretic oocytes are often found in normal fish, they are more common in stressed fish (from McCormick et al., 1989), and few atretic oocytes (less than 12%) are found in healthy fathead minnows (Leino et al., 2005; Jenson et al., 2001). I found that percentages of atretic oocytes in all treatments were low

(<7%) and there was no significant difference between any of the treatment groups and the control. This may either indicate a lack of resorption, or sampling may have occurred before the majority of oocytes were ready to begin resorption. The ideal sampling period for diagnostic ability is likely within one to three weeks of peak reproductive activity (McCormick et al., 1989). As experiment 1 ended after only 11 days, it is likely that sampling occurred before this optimal period, and oocytes were not sufficiently developed for atresia to occur (McCormick et al., 1989).

Five stages of spermatogenesis can be identified in fathead minnows including primary and secondary spermatogonia, primary and secondary spermatocytes, and spermatozoa (Jenson et al., 2001). As in ovarian staging, the percentage of cysts at each stage of development may be counted to reveal impairment of gonad maturation (Leino et al., 2005). For the purposes of this research, percentages of spermatogonia, spermatocytes and mature spermatozoa were analyzed, and no significant differences were found between the control and all treatments. This suggests that for short-term (11-day) exposure to DO levels as low as 3.5 mg/L, testicular development is not impaired in fathead minnows.

4.5 Hormone and vitellogenin analysis

The results of the hormone assays were enigmatic, as estradiol and testosterone levels were approximately an order of magnitude higher in males than in females. Previous hormone assays performed on fathead minnows have typically used plasma or whole body samples, so direct comparison to the literature is difficult (Ankley et al., 2001; Jenson et al., 2001). The large difference between male and female samples was

nonetheless unexpected. Additionally, plasma levels of estradiol are usually very low, and often undetectable in male fathead minnows, whereas I found levels of estradiol that were on average higher than testosterone levels. There are several possibilities regarding why these results were observed. First of all, testosterone is secreted by tissues in the gonad (Devlin & Nagahama, 2002). Therefore numerous eggs within the ovaries may dilute the amount of hormone present within a sample. Furthermore, the viscosity of some substances such as yolk proteins may have prevented the detection of either hormone by the assay in female fish. This does not, however, explain why estradiol levels in male fish were higher than testosterone levels.

A second possibility which may explain the high hormone levels in males is the possibility of accidental inclusion of adipose tissue in the hormone assay. Due to the small size of the testis in fathead minnows, it is possible that some of the adipose tissue was not removed before the testes were processed for hormone extraction. In mammals, adipose tissue has been demonstrated to be hormonally active, producing sex steroids (Siiteri, 1987; Kershaw & Flier, 2004). It is unknown whether this also occurs in fishes, however, extra tissue containing steroid hormones could account for the higher levels of estradiol and testosterone found in males.

While it is not entirely clear why the male hormone levels were so much higher than the females or why estradiol levels in males were higher than testosterone levels, no treatment effect was found for either reproductive hormone in male or female fathead minnows. This indicates that the tested concentrations of DO did not disrupt the production of steroid hormones in either sex. Furthermore, the processing of gonad tissue in hormone assays is a novel process, as previous studies have utilized plasma or whole

body samples. Therefore the normal levels of testosterone and estradiol in gonad tissue of fathead minnows have not been previously studied. It is possible that the hormone levels measured in this experiment reflect normal levels present within fathead minnow gonads.

Vitellogenin was detected in the liver tissue of male fish in all treatments. Vitellogenin induction in male fathead minnows has been considered a biomarker for exposure to estrogenic compounds, and is not produced under normal circumstances (Folmer et al., 1996). Once again, however, no treatment effect was found as vitellogenin was detected in the liver tissue of males in all treatment groups. Estrogenic compounds such as biphenol A are present in many types of plastics, and may leech out upon contact with water (Sajiki & Yonekubo, 2003). Furthermore, water systems experiencing input from municipal waste water or other contaminants may contain estrogenic substances (Folmer et al., 1996; Leino et al., 2005; Ankley et a;., 2001). As much of the experimental system was composed of plastics and treated municipal water from the South Saskatchewan River was utilized, it is possible that male vitellogenesis was induced by estrogenic compounds present within the experimental system.

4.6 Behaviour

While the physiological effects of countless industrial chemicals have been evaluated, fish behaviour is an endpoint that is less commonly used in toxicological studies. Although many authors have suggested that behavioral components be added to standard toxicity tests (Atchison et al., 1987; Scherer, 1992), few authors have comprehensively studied the effects of toxicants on reproductive behaviour (Atchison et al., 1987; Scott & Sloman, 2004). Behaviour has the potential for use in toxicological

studies as a sensitive indicator for reproductive impairment or other manifestations of toxicity. Behavioural studies can provide insight regarding an organism's ability to persist in an ecological context, rather then merely demonstrating levels of physiological tolerance to a chemical, as in acute lethality testing (Scott & Sloman, 2004). The majority of behavioural research in toxicological studies published to date encompasses simple reactions to pollutants such as coughing, tremors or preference and avoidance (Scott & Sloman, 2004). There is, however, a trend towards the analysis of the effects of stressors on environmentally-relevant behaviours such as those associated with foraging, dominance hierarchies, or reproduction (Scott & Sloman, 2004).

I chose to investigate reproductive behaviour (courtship, nest cleaning, and time spent in the nest) to determine if depressed DO elicits changes in behavioural patterns. I also wished to determine whether correlations between behaviour and reproductive events occur in fathead minnows. In both experiments, I found a correlation between the amount of male courtship and egg production, reinforcing the idea that behaviour can be utilized as a sensitive indicator of reproductive impairment.

In fathead minnows, male courtship consists of a variety of behaviours including the approach of females, displays, leading or pushing females towards the spawning site, and nudging of the flank region with the head (Cole & Smith, 1987). In experiment 1, I found that male courtship behaviour was reduced with decreasing DO. While this difference was only statistically significant in the 3.5 and 4.5 mg/L treatments, there was a trend towards decrease in the 5.5 mg/L treatment. In experiment 2, however, no significant differences were found, likely because the tested DO levels were so similar in magnitude. In both experiments there was no statistical difference in the time spent

within the nest by either sex. In experiment 1, a significant difference in time allotted to nest care was detected; however the post-hoc test was not powerful enough to determine where this difference occurred. Visual inspection of the data makes it seem likely that maintenance in the 5.5 and 3.5 mg/L groups was significantly lower than in the control.

The results of experiment 1 indicate that decreased courtship is a good indicator of reproductive impairment in fathead minnows. This is likely due to the large amount of time, and presumably energy, that courtship requires on the part of the male. It is possible that with decreasing available oxygen, male fathead minnows must conserve energy by limiting energetically-expensive behaviours which are not strictly necessary for survival. Under natural conditions, a tradeoff may exist between present and future breeding opportunities. By limiting courtship under conditions of low DO, it is possible that males increase their probability of survival, allowing for future breeding opportunities when conditions improve. It is also a possibility that limited courtship is not facultative, but obligate, if strenuous activity becomes physiologically impossible when DO is depressed.

Wang et al. (2008), found that male courtship in common carp was absent under conditions of true hypoxia. The author proposed lack of female pheromone production as a possible reason for a lack of courtship on the part of the male. While my results showed that courtship was performed in all treatments, it is possible that a decrease in female stimulation had an effect on male behaviour.

To my knowledge, reproductive behaviour has not previously been evaluated under moderately depressed DO levels. While numerous anthropogenic chemicals including endocrine disrupting chemicals, pesticides, metals, and sewage have been shown to alter reproductive behaviour (Bell, 2001, Bjerselius et al., 2001, Matthiesson &

Logan, 1984, Schoenfuss et al., 2002, Schroder & Peters, 1988, Weber, 1993), other environmental stressors are often overlooked. This research demonstrates that even moderate decreases in DO may have detrimental effects on reproductive behaviour.

4.7 Conclusions

The results of my research have application in a variety of areas. This is the first research that comprehensively examines morphometrics, reproductive output, hormones, histology, mortality, and behavior in relation to depressed DO for fathead minnows. Additionally, it is the first research to investigate fish reproductive behaviour in relation to low DO. Therefore, my experiments provide a comprehensive view regarding the effects of depressed DO on a model fish species. The results have implications both in future DO research and from a regulatory perspective.

My research demonstrates that behaviour can be a sensitive and reliable indicator of reproductive impairment. My results indicate that behaviour may represent a more responsive marker than histological or hormone analysis. It is also reasonably inexpensive. Therefore utilizing behavioural endpoints has the potential to lend toxicological studies a greater resolution, as well as rendering them more cost-effective.

My results also indicate that depressed DO may have deleterious effects on reproduction in fathead minnows at levels that are currently considered acceptable in aquatic ecosystems. One must, however, remember that my control represents ideal circumstances, which are not common in most natural settings. In natural environments, it would be unusual for a water body to maintain 7.5 mg/L DO at 25 ° C, as this concentration is very near the saturation point. Additionally, difficulty can arise when

utilizing laboratory results to predict effects on a natural environment. For example, when confronted with depressed DO some fish may use alternate strategies of respiration, such as surface breathing (Kramer 1987). Additionally, fish may simply migrate to more suitable habitat if their current location does not meet their needs. Finally, the tolerance of different species, populations and individuals to depressed DO is likely quite variable. This research nonetheless demonstrates that even minor decreases in DO have the potential to cause detrimental effects in fishes. Caution should therefore be used when assigning regulatory guidelines to ecosystems with a history of a decline in DO due to anthropogenic factors.

Lowered DO has become a global concern, and my results provide a novel perspective regarding its consequences. Future research in this area is required to produce a finer threshold of DO concentrations over which reproductive impairment occurs in fathead minnows and other species.

Literature cited:

Ankley, G.T., Jensen, K.M., Kahl, M.D., Korte, J.J. & Makynen, E.A. (2001). Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry* 20, 1276–1290.

Atchison, G.J., Henry, M.G. & Sandheinrich, M.B. (1987). Effects of metals on fish behavior: a review. *Environmental Biology of Fishes* 18, 11–25.

Bell, A.M. (2001). Effects of an endocrine disrupter on courtship and aggressive behaviour of male three-spined stickleback, *Gasterosteus aculeatus*. *Animal Behaviour* 62, 775–780.

Bjerselius, R., Lundstedt-Enkel K., Olsén, H, Mayer, I. & Dimberg, K. (2001). Male goldfish reproductive behavior and physiology are severely affected by exogenous exposure to 17β-estradiol. *Aquatic Toxicology* 53, 139–152.

Breitburg, D. (2002). Effects of hypoxia, and the balance between hypoxia and enrichment, on coastal fishes and fisheries. *Estuaries and Coasts* 25, 767-781.

Calabrese, E.J. (2008). Hormesis: why it is important to toxicology and toxicologists. *Environmental Toxicology and Chemistry* 27, 1451–1474.

Clelland, E. & Peng C.. (2009). Endocrine/paracrine control of zebrafish ovarian development. *Molecular and Cellular Endocrinology* 312, 42-52.

Cole, K.S. & Smith, R.J.F. (1987). Male courting behaviour in the fathead minnow, *Pimephales promelas*. *Environmental Biology of Fishes* 18, 135–239.

Dalla Via, J., Van den Thillart, G., Cattani, O. & Cortesi, P. (1998). Behavioural responses and biochemical correlates in *Solea solea* to gradual hypoxic exposure. *Canadian Journal of Zoology* 76, 2108–2113.

Dean, T.L. & Richardson, J. (1999). Responses of seven species of native freshwater fish and a shrimp to low levels of dissolved oxygen. *New Zealand Journal of Marine and Freshwater Research* 33, 99–106.

Devlin, R.H. & Nagahama, Y. (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture* 208, 191–364.

Eby, L.A. & Crowder, LB. (2002). Hypoxia-based habitat compression in the Neuse River Estuary: context-dependent shifts in behavioral avoidance thresholds. *Canadian Journal of Fisheries and Aquatic Sciences* 59, 952–965.

Ellis M. M. (1937) Detection and measurement of stream pollution. *U.S. Dept. Comm. Bur. Fish. B*ull. 22, 364-437.

Folmar, L.C., Denslow, N.C., Rao, V., Chow, M., Crain, D.A., Enblom, J., Marcino, J. & Guillete, L.J. (1996). Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. *Environmental Health Perspectives* 104, 1096–1011.

Giesy, J.P. (2001). Hormesis — does it have relevance at the population, community or ecosystem levels of organization? *Human & Experimental Toxicology* 20, 517–520.

Grier, H.J. (1981). Cellular organization of the testis and spermatogenesis in fishes. *American Zoologist* 21, 345–357.

Hlohowskyj, I. & Chagnon, N. (1991). Reduction in tolerance to progressive hypoxia in the central stoneroller minnow following sublethal exposure to phenol. *Water, Air, & Soil Pollution* 60, 189-196.

Jensen, K.M., Korte, J.J., Kahl, M.D., Pasha, M.S. & Ankley, G.T. (2001). Aspects of basic reproductive biology and endocrinology in the fathead minnow (*Pimephales promelas*). *Comparative Biochemistry and Physiology – Part C: Toxicology & Pharmacology* 128, 127–148.

Jezierska, B., Lugowska, K., Witeska, M. & Sarnowski, P. (2000). Malformations of newly hatched common carp larvae. *Electronic Journal of Polish Agricultural Universities* 3, art. 1.

Karr, J.R. & Dudley, D.R. (1981). Ecological perspective on water quality goals. *Environmental Management* 5, 55–68.

Kershaw, E. E. & Flier, J. S. (2004). *Adipose tissue as an endocrine organ.* The Journal of Clinical Endocrinology & Metabolism *89,2548-2556.*

Klinger, S.A., Jmagnuson, J. & Gallepp, G.W. (1982). Survival mechanisms of the central mudminnow (*Umbra hi*), fathead minnow (*Pimephales promelas*) and brook stickleback (*Culaea inconstans*) for low oxygen in winter. *Environmental Biology of Fishes* 7, 113–120.

Kramer, D.L. (1987). Dissolved oxygen and fish behavior. *Environmental Biology of Fishes* 18, 81–92.

Lambert, Y. & Dutil J.-D. (1997). Can simple condition indices be used to monitor and quantify seasonal changes in the energy reserves of Atlantic cod (*Gadus morhua*)? *Canadian Journal of Fisheries and Aquatic Sciences* 54: Suppl. 1, 104–112.

Landry, C.A., Steele, S.L., Manning, S. & Cheek, A.O. (2007). Long term hypoxia suppresses reproductive capacity in the estuarine fish, *Fundulus grandis*. *Comparative Biochemistry and Physiology – Part A: Molecular & Integrative Physiology* 148, 317–323.

Leinoa, R.L., Jensen, K.M. & Ankley, G.T. (2005). Gonadal histology and characteristic histopathology associated with endocrine disruption in the adult fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Pharmacology* 19, 85–98.

Lloyd, D. S. (1987). Turbidity as a water quality standard for salmonid habitats in Alaska. *North American Journal of Fisheries Management* 7, 34-45.

Magnuson, J.J., Beckel, A.L., Mills, K. & Brandt, S.B. (1985). Surviving winter hypoxia: behavioral adaptations of fishes in a northern Wisconsin winterkill lake. *Environmental Biology of Fishes* 14, 241–250.

Matthiessen, P. & Logan, J.W.M. (1984). Low concentration effects of endosulfan insecticide on reproductive behaviour in the tropical cichlid fish *Sarotherodon mossambicus*. *Bulletin of Environmental Contamination and Toxicology* 33, 575–583.

McCormick, J.H., Stokes, G.N. & Hermanutz, R.O. (1989). Oocyte atresia and reproductive success in fathead minnows (*Pimephales promelas*) exposed to acidified hardwater environments. *Archives of Environmental Contamination and Toxicology* 18, 207–214.

McMillan, D.B. (2007). Ovulation, in Fish Histology: Female Reproductive Systems, D. B. McMillan (ed.), 209–284. Springer, Dordrecht.

Morgan, M.J. (2004). The relationship between fish condition and the probability of being mature in American plaice (*Hippoglossoides platessoides*). *ICES Journal of Marine Science: Journal du Conseil* 61, 64–70.

Mount, D. (1973). Chronic effect of low pH on fathead minnow survival, growth and reproduction. *Water Research* 7, 987-993.

Muusze, B., Marcon, J, Thillart, G & Almeida-Val, V. (1998). Hypoxia tolerance of Amazon fish: Respirometry and energy metabolism of the cichlid *Astronotus ocellatus. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* 120, 151-156.

Paerl, H.W., Pinckney, J.L., Fear, J.M. & Peierls, B.L. (1998). Ecosystem responses to internal and watershed organic matter loading: consequences for hypoxia in the eutrophying Neuse River Estuary, North Carolina, USA. *Marine Ecology Progress Series* 166, 17–25.

Parrott, J. & Wood, C. (2002). Fathead minnow lifecycle tests for detection of endocrine-disrupting substances in effluents. *Water Qualilty Research Journal of Canada* 37, 651–667.

Pihl, L., Baden, S.P. & Diaz, R.J. (1991). Effects of periodic hypoxia on distribution of demersal fish and crustaceans. *Marine Biology* 108, 349–360.

Pollock, M.S., Fisher, S.E., Squires, A.J., Pollock, R.J., Chivers, D.P., & Dube, M.G. (2008). Relative body size influences breeding propensity in fathead minnows: Implications for ecotoxicology testing procedure. *Water Quality Research Journal of Canada* 43, 257-264.

Portnoy, W. (1991). Summer oxygen depletion in a diked New England estuary. *Estuaries and Coasts* 14, 122-129.

Rickwood, C.J., Dube´, M.G., Hewitt, M., Kovacs, T.G., Parrott, J.L., & Maclatchy, D. L. (2006). Use of paired fathead minnow (*Pimephales promelas*) reproductive test. Part 1: Assessing biological effects of final bleached kraft pulp mill effluent using a mobile bioassay trailer system. *Environmental Toxicology and Chemistry* 25, 1836-1846.

Rickwood, C.J., Dube´, M.G., Hewitt, M., Kovacs,T.G., & Maclatchy, D. L. (2006). Use of paired fathead minnow (*Pimephales promelas*) reproductive test. Part 2: Source identification of biological effects at a bleached kraft pulp mill. *Environmental Toxicology and Chemistry* 25, 1847-1856.

Robb, T. & Abrahams, M.V. (2003). Variation in tolerance to hypoxia in a predator and prey species: an ecological advantage of being small? *Journal of Fish Biology* 62, 1067–1081.

Rosenberg, R., Hellman, B. & Johansson, B. (1991). Hypoxic tolerance of marine benthic fauna. *Marine Ecology Progress Series* 79, 127–131.

Sajiki, J. & Yonekubo, J. (2003). Leaching of bisphenol A (BPA) to seawater from polycarbonate plastic and its degradation by reactive oxygen species. *Chemosphere* 51, 55-62.

Scherer, E. (1992). Behavioural responses as indicators of environmental alterations: approaches, results, developments. *Journal of Applied Ichthyology* 8, 122-131.

Schoenfuss, H.L., Levitt, J.T., Van Der Kraak, G. & Sorensen, P.W. (2002). Ten-week exposure to treated sewage discharge has relatively minor, variable effects on reproductive behaviour and sperm production in goldfish. *Environmental Toxicology and Chemistry* 21, 2185–2190.

Schroder, J.H. & Peters, K. (1988). Differential courtship activity of competing guppy males (*Poecilia reticulata* Peters; Pisces: Poeciliidae) as an indicator for low concentrations of aquatic pollutants. *Bulletin of Environmental Contamination and Toxicology* 40, 396-404.

Schulz RW, de França LR, Lareyre JJ, LeGac F, Chiarini-Garcia H, Nobrega RH, Miura T (2010) Spermatogenesis in fish. *General and Comparative Endocrinology* 165, 390-411.

Scott, G.R. & Sloman, K.A. (2004). The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. *Aquatic Toxicology* 68, 369–392.

Scott, W. B. & Crossman, J. (1998). Freshwater Fishes of Canada. Fisheries Research Board of Canada, Ottawa, 996 p.

Siiteri, P.K. (1987). Adipose tissue as a source of hormones. *American Journal of Clinicial Nutrition* 45, 277–282.

Stierhoff, K.L., Targett, T.E. & Grecay, P.A. (2003). Hypoxia tolerance of the mummichog: the role of access to the water surface. *Journal of Fish Biology* 63, 580–592.

Suwa, K. & Yamashita, M. (2007). Regulatory mechanisms of oocyte maturation and ovulation, *in* P.J. Babin et al. (eds.), The Fish Oocyte: From Basic Studies to Biotechnological Applications, 323–347. Springer, Dordrecht.

Thomas, P., Rahman, M. S., Khan, I.A. & Kummer, J.A. (2007). Widespread endocrine disruption and reproductive impairment in an estuarine fish population exposed to seasonal hypoxia. *Proceedings of the Royal Society*, ser. B, 274, 2693–2701.

Thomas, P., Rahman, M.S., Kummer, J. A. & Lawson, S. (2006). Reproductive endocrine dysfunction in Atlantic croaker exposed to hypoxia. *Marine and Environmental Research* 62, Suppl. 1, S249–S252.

Wang, S., Yuen, S.S., Randall, D.J., Hung, C.Y., Tsui, T.K., Poon, W.L., Lai, J.C., Zhang, Y. & Lin, H. (2008). Hypoxia inhibits fish spawning via LH-dependent final oocyte maturation. *Comparative Biochemistry and Physiology – Part C: Toxicology & Pharmacology* 148, 363–369.

Weber, D. N. (1993). Exposure to sublethal levels of waterborne lead alters reproductive behavior patterns in fathead minnows (*Pimephales promelas*). *Neurotoxicology* 14, 347-358.

Weber, D. N. & Bannerman, R. (2004). Relationships between impervious surfaces within a watershed and measures of reproduction in fathead minnows (*Pimephales Promelas*). *Hydrobiologia* 525, 215-228.

Weltje L, vom Saal F.S., & Oehlmann J. (2005). Reproductive stimulation by low doses of xenoestrogens contrasts with the view of hormesis as an adaptive response. *Human and Experimental Toxicology* 24, 431–437.

Wilson, D.F., Rumsey, W.L., Green, T.J. & Vanderkooi, J.M. (1988). The oxygen dependence of mitochondrial oxidative phosphorylation measured by a new optical method for measuring oxygen concentration. *The Journal of Biological Chemistry* 263, 2712–2718.

Wu, R.S.S., Zhou, B.S., Randall, D.J., Woo, N.Y.S. & Lam, P.K.S. (2003). Aquatic hypoxia is an endocrine disruptor and impairs fish reproduction. *Environmental Science and Technology* 37, 1137–1141.

Young, G., Kusakabe, M., Nakamura, I., Lokman, P.M. & Goetz, F.W. (2005). Gonadal steroidogenesis in teleost fish, *in* P. Melamed & N. Sherwood (eds.), Hormones and Their Receptors in Fish Reproduction, 155–223. Molecular Aspects of Fish and Marine Biology, v. 4. World Scientific, Singapore.

Zar, J. H. (1999). Biostatistical Analysis, 4th edn. Prentice-Hall, Englewood Cliffs, 663 p.