Effects of the interaction of environmental factors (hypoxia and ammonia) on fish

THESIS

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Abstract

Hypoxia is a common phenomenon in aquatic environments which most frequently occurring during late summer or late winter. Hypoxic events are increasing in intensity as water temperatures increase, and it is important to understand how hypoxia affects aquatic ecosystems, particularly fish. Additionally, increases in ammonia concentrations have been shown to occur during periods of hypoxia. Ammonia is converted to nitrate in aquatic environments through nitrification, via the nitrogen cycle. However, this process is carried out by aerobic bacteria and is depressed by hypoxia. Ammonia has been shown to be more toxic to fish during hypoxia than during normoxia, indicating that it could have large implications on fish foraging activity in hypoxic waters. While fish have reduced growth in hypoxic water, the simultaneous accumulation of ammonia may result in death. Therefore, evaluation of how hypoxia affects fish simultaneously exposed to ammonia is essential to understand how fish are affected during natural hypoxic events, such as those that occur in the hypolimnion of Lake Erie.

Juvenile yellow perch were reared under varied oxygen conditions to examine how hypoxia affects growth and survival of fish. Yellow perch are a physoclistous fish which can result in irreversible failure to inflate the swim bladder. Therefore, fish with both inflated and uninflated swim bladders were tested at different concentrations of oxygen for survival, growth, and oxygen consumption to gain a better understanding of how hypoxia affects yellow perch. Fish were stocked into 12 tanks with three levels of
oxygen: 3, 4, and >7 mg O$_2$/L (33, 45, & >78% saturation at 21°C). Fish were fed *ad libitum* with live brine shrimp nauplii over a 14-day period. Oxygen consumption was measured at the end of the experimental trial. Survival was not affected by lowered dissolved oxygen, but growth was reduced significantly in fish with both inflated (at 32% oxygen saturation) and uninflated swim bladders (at 33% and 48% oxygen saturation). This indicated that fish without swim bladders were less tolerant of hypoxia. Oxygen consumption was dependent on oxygen level, but did not differ between fish with inflated and uninflated swim bladders. The critical oxygen level causing a decrease in oxygen consumption of yellow perch was 3.5 mg/L O$_2$. Results from this study indicated that larval/juvenile yellow perch are able to survive lowered dissolved oxygen levels, but are faced with 50% and 24% reduced growth in fish with uninflated and inflated swim bladders, respectively.

Elevated ammonia is commonly associated with hypoxia in aquatic environments, and it is important to study the interactive effects of simultaneous exposure of hypoxia and ammonia on fish. Hypoxia has been shown to cause gill remodeling in some species of fish, which involves reduction of interlamellar cell mass to increase respiratory surface area. However, ammonia has been shown to cause severe damage to gills, limiting the respiratory, ionregulatory, and excretory efficiency of the gills. In this study, common carp (*Cyprinus carpio*) were exposed simultaneously to ammonia and a range of oxygen concentrations to assess how oxygen concentrations might affect ammonia toxicity. Histology of gill tissue was examined to observe how the gills of carp responded to hypoxia, hypoxia and ammonia simultaneously, and how gills recovered in normoxic
conditions. Carp were exposed to three levels of oxygen 8.8 ± 1.1, 2.7 ± 0.5 and 1.6 ± 0.6 mg O₂/L. The 24-h LC₅₀ for the three respective levels of oxygen were 0.66 (0.55-0.81), 0.50 (0.38-0.66), and 0.45 (0.35-0.52) NH₃-N/L. The histological results showed that gill remodeling was not present in carp. Gills however did undergo severe histopathological changes when exposed to ammonia and hypoxia simultaneously, emphasizing the importance of monitoring water quality when the effects of lowered dissolved oxygen on fish are examined.

This work has serious ecological implications for distribution and growth of yellow perch in the Great Lakes due to changes in hypolimnion oxygen and ammonia levels under current conditions and simulations that forecast more frequent and severe hypoxic events.
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CHAPTER 1: Overview of the effects of environmental hypoxia and ammonia on fish

1.1 Environmental hypoxia and ammonia

1.1.1 Causes of hypoxia

Severe depletion of dissolved oxygen in the water during the summer (high water temperature) is caused by large algae blooms respiring during the night. Sedimentation of dead algae creates oxygen depletion in the hypolimnion of water bodies. Dense populations of algae switch from photosynthesis to respiration which results in depletion of oxygen during the night (Boyd 1973). The severity of this phenomenon increases throughout the summer with increasing temperatures, as the rate of phytoplankton oxygen consumption has been shown to increase between 15 and 30 °C (Boyd 1973). Stratified bodies of water experience hypoxia in the hypolimnion due to the sedimentation of dead phytoplankton and the lack of mixing with the epilimnion above the thermocline. The decomposition of the dead plankton causes rapid depletion of the oxygen (Nicholls et al. 1980). This phenomenon can also lead to oxygen depletion in the entire water column due to sudden turnover of the water caused by low air temperatures, high winds, or heavy rain (Swingle 1968).

Winter hypoxia often causes winterkill, suffocation of animals due to low levels of oxygen. Hypoxia is caused when thick accumulation of ice and snow prevent renewal of oxygen which causes the water to become hypoxic or anoxic. Lakes with
ice cover are unable to acquire oxygen from the atmosphere, and the snow diminishes light penetration into the water (Dabrowski 1985) thusly inhibiting photosynthesis. Without either oxygen supply or photosynthesis, decomposition and respiration by plants and animals deplete the oxygen in the water causing hypoxia.

Winterkill occurs mostly in lakes and ponds that are highly productive and less than 3 meters in depth (Nickum 1970). Ice formation, in addition to snow on the pond, not only needs to be thick enough to block light from entering the water, but for it to remain on the water long enough for oxygen to be depleted (Weglenska et al. 1979). In Lake Warniak, a small lake in Poland, hypoxic conditions were reached after nearly 2 months of ice cover (Weglenska et al. 1979). Similarly, oxygen decreased at an average rate of 0.19 mg O$_2$/L. in ice covered largemouth bass aquaculture ponds located in Ontario (Johnson 1965). Oxygen consumption in large part is done by decomposition of organic material in the sediment, and therefore, it is common for a shallow lake with a high sediment area to lake volume ratio to have a rapid oxygen depletion rate creating the winterkill conditions (Mathias and Barica 1980).

1.1.2 Causes of elevated ammonia

Ammonia enters the water through anthropogenic sources (e.g. agricultural run-off) (Schindler et al. 2006), from metabolic waste of aquatic organisms (e.g. waste generated by catabolism of protein in fish) and decomposition of organics in the sediment. Ammonia is removed from the water by aquatic plant uptake (Toetz 1974) and nitrification (aerobic process in sediments), as part of the nitrogen cycle. However, hypoxia has been shown to result in elevated ammonia concentration in aquatic
environments. Ammonium is converted to nitrate via nitrification which is an aerobic process. The lack of oxygen inhibits ammonia from being converted to nitrates resulting in elevated ammonia during hypoxic events. Hypoxia has been shown to result in elevated ammonia levels during winter (Cong et al. 2009; Johnson 1965) and summer (Grochowska and Gawronska 2004; Hietanen et al. 2012). Cong et al. (2009) found that ice formation inhibited the mixing of water and caused the water near the sediment to become anoxic, which resulted in an increase in ammonia in the Fenhe Reservoir, China. The authors showed that when dissolved oxygen concentrations of less than 2 mg/L the release of ammonia from the sediment was greater than during normoxic conditions.

1.2 Respiration and hypoxia tolerance

The majority of fish respire across the surface of their gills using a countercurrent system in which the blood flows through their secondary lamellae in the opposite direction of the flow of water. This is highly efficient process and can remove up to 80% of the oxygen from normoxic water (Kisia and Onyango 2007). The ability for a fish to remove oxygen from the water varies from species to species, distinguishing of hypoxia intolerant and tolerant species (Davis 1975). The pO$_2$ of the water in which the blood of the fish ceases to be saturated (critical pO$_2$) indicates that the fish is becoming limited by oxygen, and at this point, the fish is required to make behavioral or physiological changes to meet the oxygen requirement (Davis 1975).

1.2.1 Hypoxia tolerance and temperature

Fish are poikilothermic and their metabolic rate is a function of water temperature. Therefore, fish have a very low metabolism during winter and require less
oxygen which enables them to be more tolerant of lower dissolved oxygen levels at temperatures of 2-4 °C (Wilding 1939). Yellow perch were shown to survive at oxygen concentrations as low as 0.25 mg O₂/L in water temperatures of 2-4 °C (Petrosky and Magnuson 1973). Alabaster et al. (1979b) suggested that fish that are acclimated to low dissolved oxygen can tolerate hypoxia longer than those faced with sudden hypoxia. Based on this conclusion, fish acclimated to hypoxia become more tolerant to hypoxia in winterkill lakes considering that oxygen decreases at a slow rate (0.19 mg/L per day) (Johnson 1965). Moore (1942) found that oxygen levels less than 3.5 mg/L were lethal within 24 hours to most fish species at temperatures ranging from 15-26 °C. However, fish were more tolerant of low dissolved oxygen in temperatures of 0-4 °C; oxygen levels of 2 mg/L were fatal to most species after 48 hours and 1 mg/L being lethal to all species except black bullhead (*Ameiurus melas*) (Moore 1942).

1.2.2 Life stages and hypoxia tolerance

Fish during early life stages, have a mass specific metabolism that is much higher than in adult fish (Nilsson and Östlund-Nilsson 2008). Yager and Summerfelt (1993) found that metabolic rate is inversely related to body weight in juvenile walleye (*Sander vitreus*), explaining 86% of variation in the data set. Therefore, smaller/younger fish are likely to be more susceptible to hypoxia due to a high metabolic demand. Almeida-Val et al. (2000) showed that larger oscar (260 g) (*Astronotus ocellatus*) could survive hypoxia for a longer period of time than smaller individuals (13 g). This was deemed to be caused by an increase in muscle anaerobic capacity and blood carrying O₂ capacity as the fish becomes larger (Almeida-Val et al. 2000; Sloman et al. 2006).
Nilsson and Östlund-Nilsson (2008) pointed out that there are conflicting results when it comes to the size of fish and hypoxia tolerance. While some species are more susceptible to hypoxia related death during early life stages (pre-metamorphosed) other species are not (Nilsson and Östlund-Nilsson 2008). This may be related to differences in proportion of respiratory partitioning between skin and gills (Nilsson and Östlund-Nilsson 2008; Rombough and Moroz 1997). Common carp (Cyprinus carpio) larvae have an initial gill surface area of 7.066 mm$^2$/gram of fish weighing 1.6-2.8 mg that decreases to 0.794 mm$^2$/gram of fish weighing 0.33-2250g (Oikawa and Itazawa 1985). In walleye one day after post hatch gills surface area was 5 mm$^2$ per gram of fish and increased to 1100 mm$^2$ per gram of fish weighing 200 mg (Rombough and Moroz 1997). Meanwhile, the surface area of the skin at 1 day post hatch was 8500 mm$^2$/gram of fish (Rombough and Moroz 1997). Pelster and Burggren (1996) found the amount of oxygen in the blood of zebra fish larvae (Danio rerio) did not disrupt oxygen supply to tissues, indicating that diffusion of oxygen through the skin was adequate to maintain metabolic normal functions.

1.2.3 Behavioral and physiological response to hypoxia

Fish make behavioral and physiological changes to maintain required oxygen levels when dissolved oxygen concentrations decrease. Maintaining oxygen levels is first attempted by increasing ventilation of water over the gills (Itazawa and Takeda 1978; Kisia and Onyango 2007; Marvin and Heath 1968; Petrosky and Magnuson 1973; Randall 1982; Scott and Rogers 1980) and is accompanied by an increased of level of “stress hormones” (e.g. cortisol) in the blood stream (Vanlandeghem et al. 2010) that
results in reduced appetite in carp (Bernier et al. 2012). Itazawa and Takeda (1978) found that common carp increased their respiratory frequency during normoxic conditions from 16.9 to 53.7 times/min during slight hypoxia (51.6 % saturation). Along with increasing ventilation, proliferation of lamellar epithelium cells takes place within one day of hypoxia exposure in order to increase surface area of the gills to remove oxygen from the water more efficiently (Sollid et al. 2003). However, when oxygen becomes severely limited, ventilation will become inadequate enough to supply oxygen and may cause sudden respiratory failure (Marvin and Heath 1968). Therefore, additional physiological changes are necessary for a fish to survive hypoxia.

The amount of oxygen that blood can transport can be improved by increasing the number of red blood cells and the concentration of hemoglobin within the red blood cells. Scott and Rogers (1981) found that channel catfish exposed to hypoxia for 72 hours had an increase in the amount of hemoglobin, from 7.74 to 10.42 g/100 ml. Heath (1995) illustrated this phenomenon by comparing the oxygen dissociation curve for hemoglobin of two species, common carp and rainbow trout (*Oncorhynchus mykiss*). Based on the graph provided in his chapter, carp, a species generally considered somewhat tolerant of hypoxia were able to maintain oxygen saturation in their blood at lower environmental oxygen concentrations than trout. This indicates that carp hemoglobin has a greater affinity toward oxygen than that of trout (Heath 1995).

Bradycardia takes place in many species of fish when severe hypoxia occurs. Marvin Jr and Burton (1973) found that rainbow trout heart rate decreased from 71 beats/min in pre-hypoxic stressed fish to a rate of 24 beats/min when faced with hypoxia.
stress (3.9 mg O$_2$/L). Similarly, brown bullhead catfish ($Ictalus nebulosus$) and bluegills ($Lepomis macrochirus$) had the same response to hypoxic stress, where heart rates reduced from 67 beats/min to 32 beats/min and 104 beats/min to 36 beats/min, respectively (Marvin Jr and Burton 1973). The decrease in stroke rate is compensated for by an increase in volume of blood flow per stroke, which gives blood more time at the respiratory surface, facilitating gas exchange (Holeton and Randall 1967). Heath (1964) proposed that heart tissue alone accounts for approximately 26% of metabolic demand in hypoxia and bradycardia is used as a way of decreasing metabolic demand.

1.2.4 Supply of O$_2$ to organs and tissues

Scott and Rogers (1980) suggest that blood flow is prioritized to the brain and heart when oxygen is limited, resulting in other tissues becoming anaerobic. Tissues become dependent on anaerobic respiration as the source of energy which results in an increase in lactic acid according to the authors. The decrease of pH in the blood due to the increase in lactic acid (Scott and Rogers 1981), causes hemoglobin’s oxygen combining strength to decrease (Root and Irving 1943), further reducing the ability of hemoglobin to bind oxygen resulting in greater hypoxic stress. However, Hughes et al. (1983) found that when common carp were exposed to hypoxia their blood pH initially fell, but then increased. They further suggested that some anaerobic mechanisms are used at the beginning of hypoxia to balance blood pH or even increase it as hypoxia continues. Following a return to normoxia, fish oxygen consumption is higher than prior to hypoxia in order to pay off the oxygen debt that occurred during hypoxia such as oxidizing lactic acid (Marvin Jr and Burton 1973; Vagner et al. 2008)
1.3 Growth and survival under hypoxic conditions

Hypoxia has been shown to reduce the growth rate of juvenile fish (Cech et al. 1984; Ebeling and Alpert 1966; Pichavant et al. 2000). Juvenile yellow perch reared at 2, 3.5, 5.0, 6.5 mg O₂/L and near saturation in 20°C for 67 days had significant reduced growth rates when the fish were reared in 2 mg/L in comparison to near saturation (Carlson et al. 1980). While not measured, fish were observed to not eating their entire rations when exposed to 2, or 3.5 mg O₂/L, but in all other oxygen concentration rations were fully consumed suggesting food intake was limited by low dissolved oxygen levels (Carlson et al. 1980). Moreover, feed intake reduction is common when fish experience hypoxia (Pichavant et al. 2000; Roberts et al. 2011). Roberts et al. (2011) concluded that hypoxia may potentially limit yellow perch consumption and growth by depression of physiological processes and reduction in metabolic scope. Brandt et al. (2009) showed that there is a critical level (threshold) in which fish performance quickly decreases.

Juvenile striped bass (*Morone saxatilis*) showed a rapid decrease in food consumption and growth at oxygen levels below 4 mg O₂/L at 20-30°C. These results are highly disputable as there was no difference in food consumption rate between 20 and 30°C, and for instance food intake in some cases decreased at O₂ saturation levels of 50% saturation.

Fish experience a decline in performance during hypoxia; however there is still a critical oxygen level in which fish are no longer able to tolerate hypoxic conditions resulting in reduced survival. Guppies (*Poecilia reticulata*) reared in conditions with 0.5-9.0 mg O₂/L at 25-26°C, without access to the water surface, showed decreased growth rates below 2-3 mg O₂/L and did not survive below 1 mg O₂/L (Weber and Kramer 1983).
Temperature also plays a role in the survival of fish during hypoxia. Atlantic Sturgeon \textit{(Acipenser oxyrinchus)} reared in 2-3 mg O$_2$/L had lower survival at 26°C compared to 19°C, 6.3% and 78.3%, respectively (Secor and Gunderson 1998). In the Secor and Gunderson (1998) experiment, metabolic rate (oxygen consumption) was found to be lower in hypoxic conditions compared to normoxic conditions. Furthermore, in normoxic conditions metabolic rate increased with temperature, however in hypoxia metabolic rate decreased with temperature. Secor and Gunderson (1998) concluded that at the higher temperatures the fish were unable to supply enough oxygen to their tissue (muscles) causing mortality.

1.4 Oxygen consumption (metabolism) and hypoxia

As dissolved oxygen in the water decreases, fish have been shown to increase frequency of respiration and respiratory volume (Glass et al. 1990) and decrease their metabolism (Pichavant et al. 2000; Schurmann and Steffensen 1997; Secor and Gunderson 1998; Sloman et al. 2006). Furthermore, fish can change their oxygen consumption strategies during different life stages. During early life stages Oscar use an oxygen conforming strategy, but larger fish become oxygen regulators (Sloman et al. 2006). Young Oscar reduce their oxygen consumption based on ambient dissolved oxygen levels, while adults are able to maintain oxygen in their blood when environmental oxygen begins to decrease (Sloman et al. 2006). Increases in water temperature also changes the metabolic rate of fish, for instance Atlantic cod \textit{(Gadus morhua)} have been shown to have increased oxygen consumption when temperatures increased (Schurmann and Steffensen 1997). However, Atlantic surgeon under hypoxic
conditions have shown an opposite trend having a decreased metabolism as temperatures increased (Secor and Gunderson 1998).

1.4.1 Fish activity and $O_2$ consumption

Fish activity drastically affects the metabolic rate in fish. Juvenile rainbow trout have been shown to increase metabolic rate, expressed as oxygen consumption or nitrogen (ammonia) and urea excretion as swimming speed increases (Alsop and Wood 1997). Oxygen consumption increased by 68% in fish fed to satiation compared to fasted trout, whereas an increase in swimming speed (1-4 times body length per second) led to exponential increase in oxygen consumption of 400-500% (Alsop and Wood 1997). Furthermore, Atlantic cod have the same response to swimming speed, an increasing metabolic rate by 250-400% (Schurmann and Steffensen 1997). Among other factors influencing fish metabolic rate would be the presence of a swim bladder and/or buoyancy. Larval yellow perch with uninflated swim bladders have been shown to have increased metabolic rate compared to individuals with a swim bladder (Czesny et al. 2005). Fish with uninflated swim bladders must continuously swim to maintain a position in the water column (schooling behavior), resulting in an increased metabolic rate, which Czesny et al. (2005) claimed was the reason for reduced growth rate of fish without swim bladders. However, increased in oxygen consumption caused by exercise and increased swimming speeds does not always have a negative effect on fish performance. It it has been shown that forced swimming at 1.2-2.4 body lengths (BL)/sec resulted in a significant increase in growth of young-of-the-year striped bass (Young and Cech Jr 1993) and also increased growth in many salmonids (Davison and Goldspink 1977;
Houlihan and Laurent 1987; Jorgensen and Jobling 1993). Forced exercise in juvenile rainbow trout and coho salmon (*Oncorhynchus kisutch*) resulted in drastic increases in plasma growth hormones (Barrett and McKeown 1988), which may explain increased growth rates from forced exercise at 20°C. Therefore, exercise actually has the ability to promote growth in fish even though it results in increased metabolic rate, given that adequate food is provided to the fish to compensate for the high energy costs associated with swimming. However, an increase in metabolic rate alone can result in a growth depression in rainbow trout because of the release of cortisol (De Boeck et al. 2001).

### 1.5 Ammonia toxicity

Fish are ammonotelic, meaning that they excrete ammonia as the final nitrogenous waste resulting from metabolism of amino acids. Ninety percent of all nitrogenous waste and specifically all ammonia is excreted via the gills (Sayer and Davenport 1987). Ammonia comes in two forms NH$_4^+$ (ionized) and gaseous NH$_3$ (un-ionized), the latter being the more toxic form. Toxicity of ammonia is dependent on the tolerance of the species. The 96-h LC$_{50}$ can vary from 0.47 mg/L NH$_3$-N for Gila trout (*Oncorhynchus gilae*) to 1.84 mg/L NH$_3$-N for common carp (Fuller et al. 2003; Hasan and Macintosh 1986).

#### 1.5.1 Mechanism of mortality

Sousa and Meade (1977) used coho salmon yearlings (*Oncorhynchus kisutch*) to evaluate the mechanism of ammonia toxicity using an experimental flow-through system with ammonia solution being added. Their hypothesis of the mechanism of ammonia toxicity was that an increase in NH$_4^+$ concentration increases the rate of glycolysis. This
results in an increase in acidic metabolites causing blood plasma acidemia. The drop in blood pH inhibits hemoglobin ability to bind oxygen causing it to release O\textsubscript{2} (i.e. the Borh and Root effect) leading to reduced efficiency for the fish to obtain and transport oxygen resulting in death by suffocation. Smart (1978) and Arillo et al. (1981) had similar findings in rainbow trout but concluded that the mechanism for ammonia toxicity was the same as that found in mammals. They hypothesized that unionized ammonia interrupts cerebral energy metabolism, depleting ATP in brain resulting in death.

1.5.2 Temperature and ammonia toxicity

The influence of water temperature and acclimation time on ammonia toxicity has many conflicting results. A literature review on ammonia toxicity by Randall and Tsui (2002) concluded that temperature has very little effect on toxicity between 3 and 30°C. However, Erickson (1985) showed that there is log-linear relationship between temperature and ammonia for several species of freshwater fish between 0-30°C. Fathead minnows (Pimephales promela) did show an increase of ammonia toxicity as temperatures decreased between 12 to 22 °C (Thurston et al. 1983). Studies by Arthur et al. (1987) on five species of fish showed only one species, channel catfish, had an increase in ammonia toxicity as water temperatures decreased. Furthermore, Thurston et al. (1983) using rainbow trout found that ammonia toxicity increased at lower temperatures, however, Arthur et al. (1987) findings were contrary.

1.5.3 Hypoxia and ammonia toxicity

Oxygen concentration has been found to influence the toxicity of unionized ammonia. Alabaster et al. (1979a) showed that 24-h LC\textsubscript{50} of un-ionized ammonia in
Atlantic salmon (*Salmo salar*) at a temperature of 11 °C decreased from 0.15 mg NH$_3$/L to 0.09 NH$_3$/L when dissolved oxygen in the water decreased from near saturation to 3.5 mg O$_2$/L (32% saturation). Similarly, ammonia toxicity dramatically increased as dissolved oxygen was lowered in golden dorado (*Salminus brasiliensis*) juveniles (De Leão Serafini et al. 2009). All duourado were kept in solution containing 0.927 mg NH$_3$/L with dissolved oxygen levels of 1.64, 1.99, 3.33, 5.10, and 7.77 mg O$_2$/L at 25°C. No fish in 1.64 and 1.99 mg O$_2$/L survived, but at 3.33, 5.10 and 7.77 mg O$_2$/L fish had survival rates of 12.5%, 55.0% and 86.3%, respectively. Thurston et al. (1981) found an increase in ammonia toxicity in lower dissolved oxygen (2.6 mg O$_2$/L) in rainbow trout. In contrast, Thurston et al. (1983) indicated that oxygen concentration did not influence the toxicity of ammonia in rainbow trout in acute exposure experiments. They argued that the contradicting results were because fish of a single parental origin were used in the first experiment that would have reduced variation amongst the fish. Furthermore, the levels of oxygen used in the experiments ranged between 6.1 and 9.4 mg/L (Thurston and Russo 1983) which is adequate oxygen for a fish to function, while Thurston et al. (1981) used 2.6 mg O$_2$/L and the pH was higher (7.95).

**1.6 Gill histology after hypoxia and hyperammonemia exposure**

**1.6.1 Hypoxia exposure**

Some fish species exposed to hypoxia undergo significant alteration of their gills in order to increase respiratory efficiency. Although it is not known how many species exhibit this ability, in the case of crucian carp (*Carassius carassius*) the process is called gill remodeling (Nilsson 2007). During this process fish reduce the amount of
interlamellar mass through apoptosis and halting mitosis resulting in an increased respiratory surface area (Sollid and Nilsson 2006). This is most notably seen in the crucian carp which lack protruding lamellae during normoxia (Sollid and Nilsson 2006). However, the interlamellar cell mass is reduced resulting in the protruding lamellae when water becomes hypoxic (Sollid and Nilsson 2006). Studies by Matey et al. (2008) using Qinghai scaleless carp (Gymnocypris przewalskii), showed when fish were exposed to 0.3 mg O$_2$/L that there is a reduction of gill filament epithelial thickness (>50%), elongation of respiratory lamellae (secondary lamellae), and overall increase of lamellar respiratory surface area (>60%). The remodeling of gills enhances the respiratory efficiency during hypoxia but comes with a cost (Matey et al. 2008; Nilsson 2007). There is an ionoregulatory cost of 10% and 15% reduction in plasma Na$^+$ and Cl$^-$ (Matey et al. 2008). When fish are returned to normoxic conditions, gills that have remodeled begin to return to their previous state (Matey et al. 2008).

1.6.2 Hyperammonemia exposure

The gills of fish are sites of ammonia excretion and uptake. Ammonia diffuses in and out of a fish gill vascular system across a gradient. Fromm and Gillette (1968) showed that there is a direct linear relationship between the concentration of ammonia in the blood and water. Ammonia concentrations were noted to always be higher in the blood than in water. This suggests that fish are not able to detoxify (glutamine synthesis) or excrete ammonia faster than it is produced due to the reduction of the blood-water NH$_3$ gradient.
Exposure to ammonia results in damage to the gills in salmonids (Smart 1976; Thurston et al. 1981; Thurston and Russo 1983) and in other species of fish (Lease et al. 2003; Milne et al. 2000; Spencer et al. 2008). Thurston et al. (1984) described extensive gill lesions in rainbow trout during chronic ammonia exposure that included lamellar epithelium hypertrophy and hyperplasia causing fusion of lamellae. Fish use mucus to prevent damage as a mechanism to reduce exposure to toxic substances (Wood 2001). Ammonia has been shown to increase the production of mucus in rainbow trout (Smart 1976). However, mucus production causes a reduction in the gill efficiency to transfer oxygen from the water by increasing the diffusion resistance and disturbing the flow of water of the lamellae (Ultsch and Gros 1979). Therefore, fish become more susceptible to hypoxia during ammonia exposure due to decreased respiratory efficiency.

Epithelial lifting has been noted in fish exposed to sub-lethal to lethal concentrations of ammonia in silver perch (Bidyanus bidyanus), Lost River suckers (Deltistes luxatus), rainbow trout, and slimy sculpin (Cottus cognatus) (Frances et al. 2000; Lease et al. 2003; Smart 1976; Spencer et al. 2008). Smart (1976) believes that epithelial lifting in rainbow trout was unlikely to reduce the effectiveness of the gills to remove oxygen from the water, but Lease et al. (2003) believes that increased diffusion distance caused by epithelial lifting in Lost River suckers may decrease the respiratory efficiency. Thurston et al. (1984) observed separation of epithelial from basement membrane and enlargement of lamellar capillaries (telangiectasia) in rainbow trout exposed chronically at ammonia concentrations as low as 0.02 – 0.04 mg NH$_3$/L. Further damage caused by ammonia exposure consists of lamellar fusion and hemorrhaging as
observed in slimy sculpin exposed to 0.8-1.6 mg NH₃/L (Spencer et al. 2008). Lamellar fusion is a significant symptom due to its role in reducing the surface area of the gills, exacerbating possible effects during hypoxic conditions. Although Smart (1976) speculated that ammonia toxicity is not caused by the suffocation of fish due to the damage caused to the gills, he concluded that it appears ammonia damage does reduce the fish’s ability to tolerate hypoxia and thus causing an additive effect on the toxicity of ammonia and hypoxia.

1.7 Justification

1.7.1 Effects of environmental hypoxia on yellow perch

Hypoxia is a major factor affecting all stages of yellow perch (*Perca flavescens*) life cycle in Lake Erie. The severity of hypoxia in Lake Erie has been increasing since the 1990s (Hawley et al. 2006). Studies have shown that adult yellow perch change their diet or behavior to minimize effects of hypoxia. Yellow perch will migrate to zones with dissolved oxygen as low as 1.5 mg O₂/L in order to feed (Roberts et al. 2012). A similar phenomenon was demonstrated as early as in 1942 by Beutel (2001) in Lake Mendota, Wisconsin using gill nets during the winter when the lake was frozen. Roberts et al. (2012) showed that yellow perch dive into the hypoxic hypolimnion to feed on benthic organisms and then return to normoxic waters above the oxycline. However, Suthers and Gee (1986) found that juvenile yellow perch had to leave their summer habitat, where vegetation provided protection from predation and higher abundance of their preferred diet, to escape hypoxia (0.2-0.4 mg O₂/L) in small lakes. Yellow perch need to make
metabolic sacrifices reducing both their activity level and oxygen consumption to cope with hypoxia. (Fry 1957).

Yellow perch and European perch without swim bladders have been observed in nature (Czesny et al. 2005; Egloff 1996). Yellow perch juveniles with uninflated swim bladders, exhibit decrease foraging efficiency, higher predation rate, increase metabolism, and slower growth rate (Czesny et al. 2005). However, no research has been done to determine how the metabolic demand of an uninflated swim bladder affects a fish exposed to hypoxia.

If juvenile yellow perch cannot avoid hypoxic water (<2 mg O₂/L), they begin decreasing their food consumption at 3.5 mg O₂/L and have significantly slower growth rates (Carlson et al. 1980). Current research has only examined at the effects of hypoxia on fully developed juvenile yellow perch: Roberts et al. (2011) used 100-180 mm fish and Carlson et al. (1980) used fish weighing approximately 3.6 g. It is important to understand how yellow perch are affected by low oxygen concentrations particularly at the larval to juvenile transitional life stage when fish are most vulnerable to hypoxia. Furthermore, determining the effects of uninflated swim bladders on performance of yellow perch in hypoxic water will provide useful information about their ability to persist in hypoxic conditions. Due to the increase in frequency and severity of hypoxic events it is critical to know how these events affect aquatic life to understand the possible impacts have on recruitment. The goal of this study was determine how hypoxia effects the survival, growth, and oxygen consumption of yellow perch with or without an inflated swim bladder.
1.7.2 Histological changes cause by environmental hypoxia and elevated ammonia

Many studies have been conducted on the effect of hypoxia, ammonia, and their interaction (Randall and Wright 1989; Sheehan and Lewis 1986; Watenpaugh et al. 1985), and on the histological changes of the gills caused by ammonia. Fish have been shown to undergo gill remodeling to increase the respiratory surface area of the gills when exposed to hypoxia (Sollid et al. 2003). However, hypoxia has been shown to increase toxicity of ammonia in Atlantic salmon (*Salmo salar*) (Alabaster et al. 1979a), golden dorado (*Salminus brasiliensis*) (De Leão Serafini et al. 2009), European perch and roach (*Rutilus rutilus*) (Merkens and Downing 1957). Mortality due to ammonia is not believed to be caused via suffocation from damage to gills, but Smart (1976) hypothesized that the damage caused by ammonia may limit gill efficacy reducing a fish’s ability to tolerate hypoxia. Even though ammonia and hypoxia have been extensively studied, studies focusing on the changes to the gill caused by simultaneous exposure to hypoxia and ammonia have not been conducted.

Understanding how gills respond to hypoxia is an important step to understanding how hypoxia affects ammonia toxicity. Recently gill remodeling has been discovered in several species of fish (Matey et al. 2008; Sollid et al. 2003), but is unknown if common carp exhibit this ability. The goal of this study was to gain a better understanding of how fish respond to multiple environmental stressors at the same time. It has been shown that fish are capable of surviving low doses of ammonia, but the addition of hypoxia results in death. Furthermore, ammonia is often overlooked when monitoring hypoxia (Conroy et al. 2011; Roberts et al. 2011; Roberts et al. 2009), but has been shown to accumulate during
hypoxic events (Beutel 2001; Cong et al. 2009; Hietanen et al. 2012). The results of the project highlight the importance of monitoring dissolve oxygen levels and ammonia simultaneously. We examined how the gills of common carp respond to hypoxia, simultaneous exposure to ammonia and hypoxia, and after recovery in normoxic water. These results provide the opportunity to address the question if common carp are able to remodel their gills and how ammonia alters the gill structure when fish are pre-exposed to hypoxia.
CHAPTER 2: Effects of oxygen saturation on growth and oxygen consumption of yellow perch (Perca flavescens) with un-inflated or inflated swim bladder

2.1 Abstract

Hypoxia and un-inflated swim bladders are issues affecting larval yellow perch populations in terms of growth, survival, and habitat usage. This study focused on examining the impacts of hypoxia over two experimental trials - one using fish with un-inflated swim bladders and a second using fish with inflated swim bladders. Fish were stocked into 12 tanks with three treatments 3, 4, and >7 mg O₂/L (33, 45, & >78% saturation at 21°C). Fish were fed ad libitum with brine shrimp nauplii over 14 day period. The oxygen consumption of the fish was measured at the end of the experimental trial. Survival was not affected by lowered dissolved oxygen. Growth was reduced significantly in fish with both inflated (at 32% saturation) and un-inflated swim bladders (at 33% and 48% saturation). Growth rate of un-inflated fish declined more rapidly as oxygen decreased compared to inflated fish, indicating that fish without swim bladders were less tolerant to hypoxia. The oxygen consumption did not differ between fish with inflated and un-inflated swim bladders. The critical oxygen level for yellow perch was 3.5 mg O₂/L for both inflated and un-inflated fish. Results from this study indicated that larval/juvenile yellow perch are able to survive lowered dissolved oxygen levels, but are faced with 50% and 24% reduced growth of fish with un-inflated and inflated swim bladders, respectively.
2.2 Introduction

Hypoxia is a major factor affecting yellow perch (*Perca flavescens*) life cycle in Lake Erie. The severity of hypoxia in Lake Erie has been increasing since the 1990s (Hawley et al. 2006). Studies have shown that adult yellow perch change their diet and behavior to minimize the effect of hypoxia by temporarily feeding and migrating to zones with dissolved oxygen as low as 1.5 mg O$_2$/L (Roberts et al. 2009). However, Suthers and Gee (1986), found that juvenile yellow perch had to leave habitat where their preferred diet is located in order to escape hypoxia in small lakes. If juvenile yellow perch do not leave hypoxic water (<2 mg O$_2$/L), they will have slower growth rates and decreased food consumption below oxygen concentrations of 3.5 mg O$_2$/L (Carlson et al. 1980). However, these results may be unreliable considering overall growth rates in these experiments were low (155-216% in 67 days) and these data might greatly underestimate oxygen limitations of fish fed to satiation.

It is likely that the dissolved oxygen in the epilimnion of Lake Erie may decrease and that the zone of Lake Erie hypoxia may increase in size and last longer due to increasing water temperatures (Blumberg and Di Toro 1990; Fang et al. 2004). Understanding the effects of hypoxia on larval yellow perch is important because it has been shown that during this period in other percid fish, particularly walleye (*Sander vitreus*), development of gills results during the change from cutaneous respiration to brachial respiration (Rombough and Moroz 1997). Furthermore, during this portion of their life history, walleye were shown to have their highest mass specific oxygen consumption rate which makes their oxygen threshold the highest it will be during the
fish’s life (Yager and Summerfelt 1993). Phillips and Summerfelt (1999) hypothesize that during this period of rapid gill differentiation, fish are more susceptible to toxins because of their increased surface area, but no work has been done looking at the susceptibility of fish to hypoxia during this life stage. Similarly, yellow perch gill morphology during early life was not described and no data is available on larval/juvenile yellow perch hypoxia threshold.

Yellow perch are a physoclistous fish, thus they only have a brief period during the larval stage to fill their swim bladder. Once the pneumatic duct degenerates fish, such as walleye, lose the ability to fill their swim bladder (Rieger and Summerfelt 1998). This notion was challenged in European perch (*Perca fluviatilis*) by Jacquemond (2004a) who claimed that 66% of fish filled swim bladder later during their life. Yellow perch and European perch without swim bladders have been observed in nature (Czesny et al. 2005; Egloff 1996). Yellow perch juveniles with uninflated swim bladders, exhibit decrease foraging efficiency, higher predation rate, increased metabolic rate, and slower growth rate (Czesny et al. 2005). However, no research has been done to determine how the metabolic cost associated with an uninflated swim bladder affects a fish exposed to hypoxia. This research examined if yellow perch during the larval stage with inflated or uninflated swim bladders are capable of surviving hypoxia (down to 30% saturation), and what are the impacts on growth and oxygen consumption. We hypothesized that hypoxia would result in a reduced growth rate in yellow perch. Fish with uninflated swim bladders growth would be more rapidly decreased by hypoxia due to the increase energy expended when swimming due to their negative buoyancy. This increase in the cost of
swimming will result in increased oxygen consumption at in all levels of oxygen tested. Survival of both fish with inflated and uninflated swim bladders in not expected to be affected due to previous research indicating that yellow perch can survive in a little as 2 mg O₂/L in similar temperatures.

2.3 Materials and Methods

Two trials were conducted, one using yellow perch larvae with uninflated swim-bladders and ones with inflated swim bladders. Initial rearing conditions for these larvae through the stage of swim bladder inflation were identical. Larvae were reared for one week in specialized system that included increased turbidity caused by supplemented algae (preserved *Nannochloropsis* spp.), water surface tension breakers (pressurized sprinklers), and fed live (rotifers then transitioned to *Artemia* nauplii). Larvae from seven rearing tanks were transferred to a single holding tank with no turbidity for three days and fed *Artemia* nauplii to recover from handling stress. From this tank, larvae were randomly collected and distributed to twelve tanks in the experimental hypoxia system. Prior to the trial using larvae with inflated swim bladders, were separated from those with uninflated swim bladders using Jacquemond (2004b) methods. This was not needed for the first trial as only 7% of larvae had inflated swim bladders due to the rate of low inflation during the initial larvae culture. The first trial used 100 larvae with uninflated swim bladders and the second used 55 larvae with inflated swim bladders per 40L tank due to variation in available larvae for each experiment.

Larvae were fed with *Artemia* nauplii (1-2 day after hatching) during the experiment. *Artemia* were added every two hours from 8:00-16:00. Continuous
availability allowed for larvae to feed *ad libitum*. Dissolved oxygen and temperatures were measured using an YSI 550 oxygen probe (YSI Inc., Yellow Springs, Ohio). Solid waste was removed from the tanks twice daily. All mortalities were recorded daily for each tank. Larvae/juvenile weight and length were measured at the beginning and at the end of the experiment. Length measurements were taken using digital calipers to the nearest 0.1 mm and weight was measured on a balance to the nearest 0.1 mg. Initial weight and length of larvae was 13.7 ± 3.4 mg and 12.89 ± 1.03 mm for uninflated fish and 20.5 ± 18.3 mg and 13.53 ± 2.56 mm for inflated fish. Initial larvae weights and lengths were determined by randomly sampling larvae from the source of larvae as the larvae were being stocked. Larvae being stocked to the tanks were not weighed to reduce mortality caused by handling stress. Weight and length of each larva was measured at the conclusion of experiments.

2.3.1 Experimental system

The semi-recirculating system consisted of twelve 40-L aquaria. Aquaria were allocated into 3 treatment groups, hypoxia, mid-hypoxia, and normoxia, consisting of four replicates each. The target dissolved oxygen in the three treatments were 3, 4, and >7 mg O₂/L, respectively (Figure 1). Actual dissolved oxygen achieved is presented in Table 1. Dissolved oxygen was adjusted using a large flow-through countercurrent column that deoxygenated water using nitrogen and degassed the water before being diverted to each tank (Blom et al. 1993). Water exiting the column would have a dissolved oxygen content of ≈4 mg O₂/L. Water in each tank was adjusted to treatment levels using an in-tank oxygen regulating column (Figure 2). The normoxic group used atmospheric air to
increase oxygen, and tanks designated for hypoxia used nitrogen to further decrease oxygen. No additional gas was injected into the in-tank column because water entering the tank was already at the desired oxygen level in the mid-hypoxic group. Water flowed into the column at a rate of 0.3L/min. Mixing with gas was done using countercurrent exchange by having water flow into the column at the top and gas bubbled into the bottom using an air-stone. The columns were packed full with K1 media bio-substrate (Kaldnes Media) to slow down water flow and increase surface area of water to increase gas exchange efficiency.

Water was heated using an immersed stainless steel heater (Process Technology; Mentor, Ohio) located in the system reservoir to keep water temperature at 21°C. The system had an exchange rate of 1.5 L/min to prevent the accumulation of metabolic waste. The source of the incoming water was city water that was de-chlorinated with activated charcoal filters and additionally treated with sodium thiosulfate (4mg/L) to keep chlorine levels below 0.1 mg/L. Water recirculating in the system flows through a filter for removing solids, and a biological filter for removable of metabolites.

2.3.2 Respirometry

Uninflated fish: Metabolic rate of juveniles was measured using the intermittent flow-through respirometer at the end point of both experimental trials. Fish were fasted for 24h before handling. All fish with swim bladders were removed using Jacquemond (2004b) methods prior to being placed in the respirometric chamber. Fish were anesthetized in 50 mg/L MS-222 and 5 g/L NaCl. Fish were then transferred to a 3000-ml respirometric chamber and given 30 minutes with flowing water to acclimate. Initial
dissolved oxygen was determined by taking the mean of water collected in three sample cups. Water was then turned off for 30-45 minutes depending on biomass. Final dissolved oxygen was measured by taking the mean of water collected in three sample cups. Consumed oxygen was determined by subtracting the final dissolved oxygen from the initial samples. Biomass was measured and consumed oxygen was converted to µmols O$_2$/g fish/hour following removal from the chamber. Slight modifications to the methods for the respirometry were made in the trials using fish with inflated swim bladders.

_Inflated Fish:_ uninflated fish during the first trial were observed occasionally laying on the bottom of the chamber and therefore modifications were made to the protocol to determine the oxygen consumption of active juveniles. Two respirometry trials were conducted consecutively on the juveniles from each tank before removing them from the chamber. Basal metabolic rate was measured using the same methods used with uninflated fish, except final dissolved oxygen samples were collected by turning the incoming water back on and collecting the water exiting the chamber. Only 300 ml of water was collected for the measurement (10% of chamber volume) thus the addition of oxygenated water did not affect final results. However, instead of removing fish from the chamber at this point, they were kept in the water and water was flushed through the chamber for 30 minutes to return oxygen to initial levels. An active metabolic rate was determined using a magnetic stirrer to create current in the chamber to force swimming (0.6 body lengths per second). Initial and final dissolved oxygen was measured using the same methods as the basal metabolic rate measurements. Swimming speed was
determined after larvae were removed by placing a neutrally buoyant ball into the chamber and timing 10 revolutions around the chamber.

2.3.3 Statistical analysis

Data were analyzed using SPSS statistical software (version 18, SPSS Inc., Chicago, IL). Results were considered significant at \( p=0.05 \). Data was checked for normality, constant variance, independence, and linearity. Any data that did not meet normality was transformed. One-way analysis of variance (ANOVA) was used to test for differences in survival within each trial. Data was significant differences among means of each treatment were found using the Tukey–Kramer method. All data was checked for normality, constant variance, independence, and linearity. Any data that did not meet normality was transferred by taking the natural log of the response. The effects of oxygen saturation on final weight and lengths was determined using a One-way ANOVA Tukey–Kramer method. Significance was only tested between treatments within each trial. The impact of growth by swim bladder inflation and oxygen content in the water was tested using a general linear model. Oxygen consumption was transformed by using the natural log of oxygen consumption and analyzed using a regression fitted with a Sigmoidal 3 parameter curve \( f = \frac{a}{1+\exp(-\frac{(x-x_0)}{b})} \) for the comparison of SB+ and SB-. Difference in oxygen consumption of resting versus swimming SB+ was done using linear regression and fitted to a polynomial curve. Oxygen consumption was calculated using the equation:

\[
\mu\text{Mols} \frac{O_2 \text{ g}^{-1}\text{fish}}{\text{hour}} = \left[\left(\frac{\Delta \text{mg L}^{-1}O_2}{\text{g of fish}} \div 32\right) \times 1000\right] \times \text{volume}
\]
2.4 Results

Survival in each of the two experimental trials (2 weeks) did not differ between treatment groups (uninflated trial $F_{2,9}=0.86$; $P=0.46$ & inflated trial $F_{2,9}=1.15$; $P=0.36$) (Figure 3). Oxygen concentration was lowered to $3.02 \pm 0.15$ and $2.84 \pm 0.17$ mg O$_2$/L, in the uninflated and inflated respectively, with no adverse effect on fish survival.

Using a Tukey-Kramer post-hoc test, growth rate of fish for both inflated and uninflated groups was significantly reduced by a decrease in water dissolved oxygen (Table 2). Fish with uninflated swim bladders had significantly reduced growth in both moderate hypoxia and sever hypoxia. Fish with inflated swim bladders only had as significant decreased growth in the hypoxia. The uninflated group had a more rapid decrease in growth when exposed to hypoxia then the inflated group (Figure 4). The general linear regression found that dissolved oxygen, uninflation, and the interaction of dissolved oxygen were significant confirming that the uninflated group’s growth rate was compromised significantly more than the inflated group by reduced oxygen (Table 3).

Oxygen consumption did not differ between inflated and uninflated swimbladders. Data followed a sigmoidal curve ($\ln$ O$_2$ consumption $= 3.18/(1 + \exp(-(\text{dissolved oxygen}-2.09)/0.34))$; $R^2=31.0$) with the oxygen threshold at 3.5 mg O$_2$/L (Figure 5). Comparison oxygen consumption of resting versus active fish did not fit the same relationship. Oxygen consumption of active fish in normoxia was not elevated and did not fit a sigmoidal curve (Figure 6). However, there is a clear reduction in oxygen consumption of resting fish in comparison to active fish at low oxygen levels, below 4.2 mg O$_2$/L (Figure 7). Using a linear model fitting a polynomial equation indicated that
active fish had a significantly higher oxygen consumption but decreased at the same rate as dissolved oxygen decreased in comparison to resting fish (ln O₂ consumption = - 4.14 - 0.53 Dissolved oxygen² + 3.98 Dissolved oxygen + 0.44 Swimming; R²=89.0).

2.5 Discussion

Oxygen was only lowered to 2.84 mg O₂/L with no difference in survival between hypoxic and normoxic treatments in the current study. These results confirmed previous research data of high tolerance of yellow perch to hypoxic conditions. Yellow perch at the size of 3.6 g are able to survive in water with as little as 2 mg O₂/L at 20°C (Carlson et al. 1980) and less than 0.5 mg O₂/L at 2-4°C (Petrosky and Magnuson 1973).

Growth was significantly reduced in lowered dissolved oxygen. Our results showed that fish with uninflated swim bladders had significantly smaller weight and lengths when exposed to oxygen below 3.02 ± 0.15 mg O₂/L and 4.32 ± 0.34, but fish with inflated swim bladders only experienced a significant decrease at 2.84 ± 0.17. However, Carlson et al. (1980) found that dissolved oxygen down to 3.0 mg O₂/L did not have a significant effect on fish growth, whereas 2.0 mg O₂/L depressed growth by 30%. Our results indicated that growth was significantly reduced at 3 mg O₂/L suggest that larval yellow perch have higher oxygen requirements than yellow perch of 3.6 g.

Food consumption was not measured as a part of our experiment due to fish being fed Artemia at a high frequency making accurate quantification unmanageable. However, fish in lowered dissolved oxygen were observed to have a reduced consumption and fish in normoxic conditions accepted larger rations. Carlson et al. (1980) found that yellow perch were unable to consume entire feeding rations below 3.5 mg O₂/L. Additionally,
Roberts et al. (2011) using yellow perch 100-180mm total length found that fish growth and food consumption was always lowest at 2 mg O$_2$/L compared to 5 and 8 mg O$_2$/L in 11, 20, and 26°C. Feeding behavior of perch might have a significant effect on the result as single fish behave (Roberts et al. 2011) differently from those in the school (Pitcher et al. 1982).

Growth rate uninflated fish was significantly decreased at 4.32 mg O$_2$/L, well above the dissolved oxygen concentrations that Carlson et al. (1980) found to be detrimental to growth. This could be a function of the increased energy expended to feed. Fish with uninflated swim bladders are claimed to have a significantly higher oxygen consumption than fish with inflated swim bladders due to their inefficient locomotion caused by lack of buoyancy (Czesny et al. (2005). These authors suggested that larval yellow perch with uninflated swim bladders not only have increased oxygen consumption, but also a decreased growth rate compared to fish with inflated swim-bladders (Czesny et al. (2005). However, the results presented by Czesny et al. (2005) have a considerable error as they differ by 2-3 order of magnitude in respect to oxygen uptake in yellow perch (24.7 ± 5.1umol O$_2$/g fish/hour) or European perch (Perca fluviatilis) (Wieser and Medgyesy 1991). We found oxygen consumption was not significantly different between fish with inflated and uninflated swim bladders (Figure. 6). This could be attributed to a “resting” behavior exhibited by fish with uninflated swim bladders. These fish would lie on the bottom and support themselves with their pectoral fins. Egloff (1996) described this behavior in populations of European perch in natural conditions that rested on the lake bottom. However, uninflated fish were unable to simply rest on the bottom during
feeding, and lack of buoyancy more energy would be consumed while feeding. Thus they would have a reduced net energy intake when foraging. To confirm this, more work would need to be done focusing on the difference in oxygen consumption of uninflated fish resting and during forced swimming as well as estimating the proportion of time spent “resting” and searching/feeding.

Data from this study suggest that juvenile yellow perch have a critical oxygen concentration of 3.5 mg O₂/L. These results corroborate findings by Fry (1957) who documented the oxygen level threshold for swimming performance of yellow perch. The growth data corresponds with this as the fish exposed to lowered dissolved oxygen exhibited a decreased in growth rate. Furthermore, the Carlson et al. (1980) data in part parallels our findings. Fish in his experiment began to decrease food consumption at 3.5 mg O₂/L and had a significantly reduced growth rate at 2.0 mg O₂/L at 20°C. This shows that fish at oxygen concentrations below 3.5 mg O₂/L were responding to the reduced oxygen indicating that oxygen was beginning to become limiting.

Oxygen consumption of active fish with inflated swim bladders had plateaued oxygen consumption. However, when the oxygen concentration was below 4.2 mg O₂/L active fish had increased oxygen consumption. We expected that active fish would have elevated oxygen consumption in all oxygen levels in comparison to resting fish. Studies examining the effects of exercise on oxygen consumption found that oxygen consumption, although to a different degree depending on fish size, rises with increased activity levels (Alsop and Wood 1997; Becker and Fishelson 1990; Schurmann and Steffensen 1997). The cause for the discrepancy in our results is unknown, but may be
explained by the lack of control of the swimming velocity of active fish. Beamish (1970) found that when largemouth bass were exposed to 80-150% oxygen saturation that there was no difference in oxygen consumption of active fish. In our experiment, there was no control over activity levels in the respirometer during the resting phase. Furthermore, the swimming speed used in our experiment (0.6 BL/sec) was not very high considering juvenile perch are able to withstand speeds up to 3.0 BL/sec (Houde 1969). Therefore, if the fish during the resting phase the fish were spontaneously active and oxygen was sufficient to meet metabolic demands, the lack of significance between resting and active fish is plausible.

The significant difference occurs between resting and active fish when the dissolved oxygen is nearing critical oxygen levels for yellow perch. Fish exposed to hypoxia have been shown to reduce their spontaneous movement when exposed to hypoxia as an energy saving strategy (Metcalf and Butler 1984; Schurmann and Steffensen 1994). Hence, we conclude that yellow perch juveniles in the “resting” phase would reduce oxygen consumption and rest to conserve energy resulting in a true long term energetically advantageous strategy. As a result of this response, a significant difference could be achieved in the resting and active fish in lowered dissolved oxygen.

2.6 Conclusions

Results of this study show that larval yellow perch are able to survive in water with a little as 2.84 mg O₂/L. However, hypoxia still negatively affects yellow perch larvae/juveniles resulting in a significantly lower growth rate. Hypoxia events in aquatic environments may not have direct impact in fish recruitment (i.e. mortality due directly to
hypoxia), but could result in reduced recruitment indirectly (i.e. longer time to sexual maturity, increased predation, etc). Swim bladder inflation is critical for a fish’s ability to cope with hypoxia. Fish with uninflated swim bladders did not have reduced survival but were significantly less tolerant to hypoxia in comparison to fish with swim bladders. Oxygen consumption of fish with uninflated and inflated swim bladders did not significantly decreased due to behavioral modification made by uninflated fish to conserve energy. Oxygen consumption data indicate that 3.5 mg O$_2$/L is the critical oxygen level for yellow perch.
2.7 Table and Figures

Table 1. Mean ± standard deviation of daily water temperatures (°C) and dissolved oxygen (mg O₂/L) throughout the experiments. Temperatures were control with submerged water heaters. Dissolved oxygen was lowered using in-tank oxygen stripping column using nitrogen to displace the oxygen.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment</th>
<th>Temperature</th>
<th>Dissolved oxygen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxia</td>
<td>Uninflated</td>
<td>21.3 ± 0.3</td>
<td>3.02 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>Inflated</td>
<td>22.1 ± 0.1</td>
<td>2.84 ± 0.17</td>
</tr>
<tr>
<td>Moderate hypoxia</td>
<td>Uninflated</td>
<td>21.4 ± 0.1</td>
<td>4.32 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>Inflated</td>
<td>22.1 ± 0.1</td>
<td>4.02 ± 0.29</td>
</tr>
<tr>
<td>Normoxia</td>
<td>Uninflated</td>
<td>21.3 ± 0.1</td>
<td>7.34 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Inflated</td>
<td>22.1 ± 0.1</td>
<td>7.22 ± 0.31</td>
</tr>
</tbody>
</table>
Table 2. Mean final weights (mg) ± standard deviation and total length (mm) ± standard deviation of yellow perch at the end of two week feeding trials in three different levels of dissolved oxygen. The effects of dissolved oxygen on final weights and lengths were determined using a one-way analysis of variance (ANOVA) followed by a posteriori means comparison using the Tukey–Kramer method (p=0.05). Significance was only tested between treatments within each trial.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Hypoxia Weight ± SD</th>
<th>Hypoxia Length ± SD</th>
<th>Moderate Hypoxia Weight ± SD</th>
<th>Moderate Hypoxia Length ± SD</th>
<th>Normoxia Weight ± SD</th>
<th>Normoxia Length ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninflated</td>
<td>41.3 ± 2.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.2 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>64.4 ± 10.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.76 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>96.2 ± 5.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>23.62 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Inflated</td>
<td>120.5 ± 19.1&lt;sup&gt;y&lt;/sup&gt;</td>
<td>22.4 ± 1.0&lt;sup&gt;y&lt;/sup&gt;</td>
<td>146.7 ± 20.4&lt;sup&gt;yz&lt;/sup&gt;</td>
<td>23.37 ± 0.7&lt;sup&gt;yz&lt;/sup&gt;</td>
<td>178.9 ± 22.4&lt;sup&gt;z&lt;/sup&gt;</td>
<td>25.25 ± 1.2&lt;sup&gt;z&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Analysis of variance table from a general linear model comparing the growth rates of fish with uninflated swim bladders or inflated swim bladders in three treatment levels of oxygen (hypoxia, moderate hypoxia, and normoxia) (R²=83.3). Fish were reared in the oxygen conditions for 14 days and fed ad libitum rations of brine shrimp nauplii. Results are considered significant at P=0.05.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Sum of Squares</th>
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<th>P</th>
</tr>
</thead>
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<td>0.17</td>
<td>34.3</td>
<td>0.000</td>
</tr>
<tr>
<td>Dissolved oxygen</td>
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</tr>
<tr>
<td>Uninflated*Dissolved oxygen</td>
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<td>0.04</td>
<td>4.1</td>
<td>0.033</td>
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<tr>
<td>Error</td>
<td>18</td>
<td>0.09</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

35
Figure 1. Mean daily dissolved oxygen (mg O$_2$/L) ± standard deviation (n=4) for each treatment throughout both experiments. Uninflated groups are indicated by grey lines and black lines represent inflated groups. Normoxia is represented by solid lines, moderate hypoxia by dashed lines, and hypoxia with dotted lines.
Figure 2. Picture of the experimental system used during the yellow perch hypoxia trials.

The large white column on the top of the picture is the large flow-through countercurrent column for deoxygenating and degasing the incoming water. Columns in each tank were used to adjust dissolved oxygen of each tank based on their respective treatment.
Figure 3. Results of the percent survival (mean ±SD (n=4)) of yellow perch juveniles with uninflated or inflated swim bladders reared at three different levels of oxygen concentrations. One-way ANOVA was conducted on uninflated and inflated groups followed by Tukey-Kramer test for comparison of means within groups. Mean is groups with similar letters are not significantly different (P> 0.05).
Figure 4. Mean growth rate (mm/day) from individual tanks of yellow perch juveniles with uninflated (○) or inflated swim bladders (●) from each experiment. Fish were reared at three levels of dissolved oxygen. Data points represent the mean values from each individual tanks within treatments. Significance was determined using a general linear model (uninflation P=0.0001, dissolved oxygen P=0.0001, uninflation*dissolved oxygen P=0.033; R² = 83.3) and fitted with a linear line for visual representation.
Figure 5. The relationship between oxygen consumption of yellow perch juveniles with uninflated (○) or inflated swim bladders (●) and dissolved oxygen of each experimental tank. Data is fitted with a sigmoidal curve (ln O₂ consumption = 3.18/(1 + exp(- (dissolved oxygen-2.09)/0.34)) ; R²=31.0)
Figure 6. Oxygen consumption of yellow perch juveniles resting (●) and forced to swim (○). All of the fish had inflated swim bladders. Fish were forced to swim by stirring with a magnetic stirrer at 60 rpm (0.6 body lengths per second).
Figure 7. Oxygen consumption of yellow perch juveniles with inflated or uninflated swim bladders resting or forced to swim in water with oxygen below 4.2 mg O$_2$/L. Resting group is indicated by (●) and swimming group is (○). Data was fitted with a polynomial equation (ln O$_2$ consumption = -4.14 - 0.534 Dissolved oxygen$^2$ + 3.98 Dissolved oxygen + 0.444 Swimming; $R^2$=89.0).
CHAPTER 3: Hypoxia and ammonia interactions effecting the survival and gill structure of common carp

3.1 Abstract

Hypoxia and elevated ammonia are common occurrences in aquatic environments. Hypoxia has been shown to cause gill remodeling in some species of fish such as crucian carp (*Carassius carassius*). Remodeling is the process of reducing interlamellar cell mass to increase respiratory surface area. However, ammonia has been shown to cause severe damage to gills and can limit the respiratory efficiency of gills. The objective of this study was to examine how hypoxia affects ammonia toxicity in common carp (*Cyprinus carpio*) and koi carp and how the gills respond to hypoxia, hypoxia and ammonia simultaneously, and how the fish recover in normoxic conditions. Carp were exposed to three levels of oxygen $8.8 \pm 1.1$, $2.7 \pm 0.5$ and $1.6 \pm 0.6$ mg O$_2$/L. The 24-h LC$_{50}$ for the three respective levels of oxygen were 0.66 (0.55-0.81), 0.50 (0.38-0.66), and 0.45 (0.35-0.52) NH$_3$-N/L for common carp. The histological results showed that gill remodeling was not present in koi carp. Gills however did undergo major changes when exposed to ammonia and hypoxia simultaneously indicating the importance of to take water quality when observing changes in gills induced by ammonia or dissolved oxygen.
3.2 Introduction

Several species of fish, crucian carp (*Carassius carassius*) and goldfish in particular, exposed to hypoxia undergo gill remodeling to increase respiratory surface area (access to secondary lamellae). During this process fish reduce the amount of interlamellar cell mass through apoptosis and halting mitosis resulting in increased respiratory surface area (Sollid and Nilsson 2006; Tzaneva et al. 2011). This is most notably seen in crucian carp that lack protruding lamellae during normoxia (Sollid and Nilsson 2006). However, interlamellar cell mass is reduced resulting in the protruding lamellae when water becomes hypoxic (Sollid and Nilsson 2006). Studies by Matey et al. (2008) using Qinghai scale-less carp (*Gymnocypris przewalskii*) showed that when fish were exposed to 0.3 mg O$_2$/L there was a reduction of filament epithelial thickness (>50%), elongation of respiratory lamellae, and increase of lamellar respiratory surface area (>60%). The gills began to recover and returned to their previous state within 3-7 days when returned to normoxic conditions (Matey et al. 2008). However, these changes in gill morphology due to hypoxia were not observed in other cyprinids such as common carp (*Cyprinus carpio*) (Hughes et al. 1983).

Extended ammonia exposure causes histopathological change in fish gills resulting in most cases in diminished respiratory surface area and decreased endogenous ammonia excretion capacity (Perry et al. 2010). Major changes caused by ammonia consist of epithelial lifting (Benli et al. 2008; Frances et al. 2000; Lease et al. 2003; Smart 1976; Spencer et al. 2008), lamellar fusion (Benli et al. 2008; Miron et al. 2008; Spencer et al. 2008), and proliferation of chloride cells (Benli et al. 2008; Chezhian et al.)
Both ammonia and hypoxia cause changes in the gill structures, but the changes caused by simultaneous exposure to both conditions have not been studied.

The objectives of this current experiment were to 1) assess the effect of hypoxia on the toxicity of ammonia in common carp (Cyprinus carpio), 2) determine if hypoxia causes gill remodeling in koi carp (Cyprinus carpio), 3) examine histopathological changes of the gills of koi carp exposed to ammonia and hypoxia simultaneously, and 4) observe the recovery of gills in normoxia with no ammonia after fish being exposed to hypoxia and sub-lethal ammonia concentrations. We hypothesize that ammonia would be more toxic to common carp in hypoxic conditions. It was not expected that gill remodeling would occur when exposed to hypoxia as koi carp already have protruding lamellae. However, ammonia was expected to cause histopathological changes (i.e. lamellar fusion and epithelial lifting). Gills were expected to begin to recover in normoxic conditions, but the extent of the damage from ammonia would be too great for the gills to fully recover in 3 days.

3.3 Materials and methods

3.3.1 Experimental system design

Experiments were conducted in a semi-recirculating system consisting of twelve 4-L aquaria in the Aquaculture Lab at The Ohio State University. The system water was refreshed at a rate of 3.3 L/min to prevent accumulation of metabolic wastes, and to maintain temperatures. The source of the incoming water was de-chlorinated Columbus city water. De-chlorination was achieved by using activated charcoal filters and then
additionally treating the water with sodium thiosulfate (4 mg/L) to keep chlorine levels below 0.1 mg/L.

A total of four experimental trials were conducted with twelve aquaria allocated into three treatments (hypoxia, moderate-hypoxia, and normoxia). Each trial conducted had a different concentration of un-ionized ammonia (0.00 mg NH₃/L, 0.25, 0.60, and 0.85), but oxygen level treatments remained the same. Fish size, temperature, dissolved oxygen, pH, NH₄⁺-N, and NH₃-N for each treatment are reported in Table 4. Koi were exposed to 0.61±0.12 mg NH₃-N /L for histopathological examination. Dissolved oxygen was adjusted using a large flow through countercurrent column which injected nitrogen into the water while simultaneously degassing the water (Blom et al. 1993) before being diverted to each tank. Water exiting the column had a dissolved oxygen concentrations with the same concentration as the moderate hypoxia treatment. Dissolved oxygen in each tank was adjusted to the desired treatment levels using an in-tank oxygen stripping or aeration column. Water entering each tank flowed through a 12”x2” column at a rate of 0.3L/min. Gas was mixed with the water using countercurrent exchange. Water entered at the top of the column and gas (nitrogen or air) was injected at the bottom using an air-stone. The columns were packed full with 7 x 10 mm plastic bio-substrate to increase the contact surface area of the incoming water to improve gas exchange. The normoxic treatment columns used atmospheric air to increase oxygen, and hypoxia treatment used nitrogen to further decrease oxygen. The moderate-hypoxia treatment did not have any gas injected into the in-tank column because the water entering the tank was already at the desired oxygen level (≈4 mg O₂/L).
Four experimental trials were conducted using common carp to calculate \( \text{LC}_{50} \) values for the three oxygen levels. Koi carp were used for the histological experiment due to available fish at the time of the experiment. All fish used were from broodstock held in the OSU facilities for at least 2 generations. Fish were not fed throughout the experiments. Fish were randomly distributed into the 12 tanks \((n=12)\) and given three days to acclimate to the tanks prior to changing the dissolved oxygen level. Oxygen levels were slowly lowered over a period of 24h. Fish were given three days to acclimate to the hypoxic conditions before ammonia exposure. Tanks were cleaned daily to remove any solids throughout the experiments.

3.3.2 Water quality

Water quality parameters measured were dissolved oxygen, temperature, pH, \( \text{NH}_4\text{-N} \), and \( \text{NH}_3\text{-N} \). Measurements were taken using a Profession Plus Handheld Multiparameter Instrument (YSI Inc. Yellow Springs, Ohio) equipped with oxygen, pH, ammonia, and temperature probes. Water quality was monitored twice daily at 9:00 and 16:00 during the period when fish were being acclimated to the tanks. Water quality was measured three times daily at 9:00, 12:00 16:00 during hypoxia and ammonia exposure.

3.3.3 Ammonia exposure and recovery

Fish were exposed to ammonia after three days of system acclimation and three days of hypoxia acclimation. Ammonia used was in the form of ACS grade ammonium chloride (Sigma Aldrich, St. Louis, Missouri). Concentrations used for experiments were based on the toxicity test results of Abbas (2006). The concentrations were chosen to achieve a trial with no mortality, partial mortality, and total mortality. Ammonium
solution was added to the system using a peristaltic pump. The solution was added to the holding tank where incoming dechlorinated city water and recirculating water mixed. The peristaltic pump added solution at a rate to maintain ammonia levels based on the flow of incoming water. Fish were removed immediately when mortalities occurred. Ammonia concentrations were maintained for 24 hours and then the peristaltic pump was turned off. Hypoxia exposure was also stopped at this point in time. Surviving fish were then given three days to recover be monitored for the next three days to observe if they are able to recover from the stress and to measure post-test mortality.

3.3.4 Sampling

The experiment consisted of three phases: tank acclimation (duration of 72 h), hypoxia acclimation (72 h), ammonia exposure (24 h), and recovery (72 h) (Figure 1). Fish samples for gill structure histology were taken at the end of each of the phases. Tank acclimation samples consisted of four fish randomly distributed into the experimental system, which served as a baseline for gill morphology. Hypoxia acclimation, ammonia exposure, and recovery samples all consisted of one fish per tank (n=4 per treatment). Fish were sacrificed by a single sharp blow delivered to the head following the Association (2007) guidelines and decapitation. Samples of gills were dissected and transferred to 10% neutral buffered formalin (Thermo Fisher, Kalamazoo, MI) for 48 h, and then transferred to 70% ethyl alcohol.

3.3.5 Histology

All the gill arches from one side of the fish were removed. Gills were dehydrated in alcohol, transilluminated in xylene and fixed in Canadian balsam on microscope slides.
Two gill arches were examined in each case. Longitudinal histological cross sections (3 \( \mu \)m) of the gill filaments were obtained and stained with toluidine blue (1% alcoholic solution) followed by fuchsin (1% aqueous basic solution) for morphological observations.

3.3.6 Statistical analysis

LC\(_{50}\) values and 95% confidence intervals for ammonia toxicity in each level of oxygen for common carp was determined using the Spearman-Karber method (Agency 2002). Differences in the proportion of koi that died during 24 hours of ammonia exposure were tested using a one-way analysis of variance (ANOVA) (\( P=0.05 \)). Data was checked for normality, constant variance, independence, and linearity. Significant differences among means of each treatment were found using the Tukey–Kramer method. The interaction of ammonia and dissolved oxygen on fish mortality was testing using a two-way ANOVA (\( P=0.05 \)). Gill histology was compared using observational differences between the gills structures of each treatment group.

3.4 Results

3.4.1 Ammonia toxicity and dissolved oxygen

Water quality parameters (temperature, pH, and dissolved oxygen) during the ammonia exposure were kept stable with little variation among experiments (Figure 8). The proportion of koi that died during the 24 h exposure to 0.61±0.12 mg NH\(_3\)-N/L was significantly higher in hypoxia in comparison to moderate hypoxia and normoxia (Figure 9). No difference in the survival of koi was found between normoxic and moderate hypoxic treatments. Twenty-four hour-LC\(_{50}\) values and 95% confidence intervals for
common carp in normoxia, moderate hypoxia and hypoxia treatments were 0.66 (0.55-0.81) NH$_3$-N/L, 0.50 (0.38-0.66), 0.45 (0.35-0.52), respectively (Table 5). No mortalities were observed in any of the treatments in 0.00 mg and 0.25 NH$_3$-N/L. Partial mortalities were observed in all groups in 0.60 mg NH$_3$-N/L and 100% mortality in all groups at 0.85 mg NH$_3$-N/L. Twenty four hour LC$_{50}$ was highly influenced by dissolved oxygen. The lethal concentrations decreased with the dissolved oxygen concentration indicating that fish exposed to ammonia in hypoxic conditions are significantly more susceptible to mortality caused by ammonia. The interaction of dissolved oxygen and ammonia was significant, confirming that ammonia in hypoxia is more toxic than in higher dissolved oxygen concentrations (Table 6).

3.4.2 Gill histology

Gills from fish in normoxia prior to exposure appeared to have extensive hyperplasia of epithelial cells between secondary lamellae (Figure 10A). This characteristic includes proliferation of the mucous cells (Figure 10a; thin arrows) and conspicuous separation of lamellar epithelium (Figure 10a; thick arrows). Mucous cells are numerous at the tip of gill filaments (Figure 10b).

After exposure to hypoxia edematous expansion of lamellar thickness was evident only in the most severe conditions (Figure 11C), whereas the moderate hypoxia (Figure 11B; arrows) resulted in frequent lamellar fusion and disappearance of mucous cells present in control group (Figure 11A; arrows). The most characteristic feature of hypoxia was a high frequency of epithelial lifting, the severe damage to gill morphology, and likely detrimental to their respiratory and excretory functions.
Ammonia exposure results in a proliferation of mucous cells (Figure 12D; arrows) and complete disappearance of interlamellar cells in addition to previously observed lamellar fusion (Figure 12D; squares). In normoxic fish, ammonia caused severe edematous changes, epithelial lifting (Figure 12A, B), and mucous cell infiltrated interlamellar spaces (Figure 12A, thick arrows).

It was evident that the damage to gill structure persisted in all treatments following three days of recovery in normoxic condition and no ammonia (Figure 13 A-C). It appears that the interlamellar spaces were refilling with proliferating cell mass although high frequency of epithelial lifting was still present. Hypertrophy of interlamellar cells around the base of the lamellae appeared to be more advanced (faster recovery) in fish previously exposed to moderate hypoxia (Figure 13B) although cell fusion due to an accelerated rate of epithelial cell proliferation is very common (Figure 13B, arrows). Hypoxia and ammonia toxicity resulted in prolong period of slow recovery in carp gill morphology (Figure 13C). The swelling of epithelial cells and damaged integrity of those cells coincides with the proliferation process of interlamellar cell mass

3.5 Discussion

The 24-h LC50 in the normoxic treatment for common carp in our experiment was found to be 0.66 (0.55-0.81) NH3-N/L. This corresponds to results of Abbas (2006) who found the 24-h LC50 of 5 g common carp to be 0.58 and 2.02 mg NH3-N/L when reared at 24°C with a pH of 6.48 and 7.52, respectively. Ammonia toxicity increasing with hypoxia is well documented in other species fish species (Alabaster et al. 1979a; De Leão Serafini et al. 2009; Merkens and Downing 1957; Thurston et al. 1981). Ammonia
toxicity in our study was increased by 25% in moderate hypoxia and 32% in hypoxia. Likewise, Thurston et al. (1981) using rainbow trout found that the 96-h LC₅₀ for rainbow trout decreased from 0.812 mg NH₃/L at 8.61 to 0.316 at 2.64 mg O₂/L.

Gill pathologies in carp exposed to ammonia differ remarkably from those described in rainbow trout (Soderberg 1985). The authors noted frequent aneurysm and hematoma leading to gill filament destruction in rainbow trout exposed to variable levels of un-ionized ammonia (0.05-0.2 mg/L). Thurston et al. (1984) observed during chronic exposure of rainbow trout to 0.076 mg NH₃/L extensive hypertrophy of lamellar epithelium and lamellar fusion. Interestingly, histopathological changes caused by ammonia persisted for months but did not cause fish mortality (pH 7.4). Earlier data on common carp exposed to ammonia indicated that this species can withstand much higher concentrations than trout. However, Flis (1968) reported survival of carp in 13.5-18.4 mg/L total ammonia at 7-15°C water. At this dose and normoxic conditions (9.2 – 10.8 mg O₂/L) epithelial cells of the gills began swelling and destruction of integrity was observed within 6-9 days leading to cell disintegration and “wavy” capillaries.

In our experiment fish exposed to ammonia exhibited epithelial lifting, edema of lamella, and increased mucous cells in the normoxic treatment. Lamellar fusion, proliferation of mucous cells, and disappearance of interlamellar cells in the hypoxia treatment occurred. Ammonia has been shown to increase mucus production (Smart 1976), and mucus has been shown to cause a reduction in the efficiency of oxygen uptake from the water by the gills by increasing the diffusion distance and disturbing the flow of water along the lamellae (Ultsch and Gros 1979). Furthermore, lamellar epithelial
lifting has been observed in fish exposed to sub-lethal to lethal concentrations of ammonia (Frances et al. 2000; Lease et al. 2003; Smart 1976; Spencer et al. 2008). Smart (1976) was of the opinion that epithelial lifting in rainbow trout was unlikely to reduce the effectiveness of the gills to extract oxygen from the water, but Lease et al. (2003) concluded that the increased diffusion distance caused by epithelial lifting in the Lost River sucker (*Deltistes luxatus*) did not decrease respiratory efficiency. Lamellar fusion is a significant symptom due to it reducing the surface area of the gills which is the opposite of what is needed in order to obtain enough oxygen during hypoxic conditions. Although Perry et al. (2010) argued that hypoxia exposure did not limit ammonia excretion in goldfish, but excretion was decreased by 25% in comparison to normoxic control. Smart (1976) did not find evidence that ammonia toxicity is caused by the suffocation of fish due to the damage caused to the gills, it appears that this damage does reduce their ability to tolerate hypoxia and thus causing an additive effect on the toxicity of ammonia and hypoxia. Sinha et al. (2012) indicated that toxicogenomic analysis of potential biomarker genes in common carp may indicate much earlier and subtler concentration of ammonia. However, the authors provided only data on total ammonia concentration (1.08 ± 0.08 mg/L) in a wide range of pH (7.0-7.5) which makes these results difficult to relate to toxicological action of un-ionized ammonia.

In silver catfish (*Rhamdia quelen*), Miron et al. (2008) observed pH-dependent pathologies of gill structure where lamellar edema and fusion were frequent in fish exposed to 1.45 mg/L of un-ionized ammonia at a pH of 7.5. In the control group, presumably normoxic conditions, fish gill structure had no interlamellar cells and
matched closely the appearance of gill structure of carp exposed to moderate hypoxia and ammonia (Fig. 11 C). Therefore, interpretation of results from ammonia toxicity studies using fish has to be discussed in relation to past and current conditions (i.e. pH) as well as with respect to oxygen concentrations.

3.6 Conclusion

In common carp ammonia toxicity was increased with decreasing dissolved oxygen concentrations. Changes in gill structure after three days of moderate to severe decrease in dissolved oxygen have not resulted in gill remodeling as found in crucian carp by Sollid et al. (2003). Ammonia however, resulted in disappearance of interlamellar cells, and these changes were accompanied by lamellar fusion. After three days of recovery in normoxic conditions, gills began to recover, but still had numerous lesions. Interlamellar spaces experienced a rapid proliferation of cells. Rapid proliferation of the interlamellar cell mass resulted in cellular fusion of the adjacent secondary lamellae in the case of fish exposed to moderate hypoxia. Results from the gill histology indicate that common carp do not exhibit gill remodeling when exposed to hypoxia, although sublethal ammonia does cause rapid histopathological changes to gills that may affect respiration, excretion (CO$_2$ and NH$_3$) of metabolites and ion transfer.
3.7 Tables and Figures

Table 4. Mean ± Standard deviation (n=4) of daily water quality parameters for each treatment during the 24 hour ammonia exposure.

<table>
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<tr>
<th>Trial</th>
<th>Treatment</th>
<th>Species</th>
<th>Weight (g)</th>
<th>Temperature (°C)</th>
<th>DO (mg/L)</th>
<th>pH</th>
<th>NH₃-N (mg/L)</th>
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</thead>
<tbody>
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<td>Normoxia</td>
<td>Common Carp</td>
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<td>0.28 ± 0.04</td>
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<tr>
<td></td>
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<td></td>
<td>1.23 ± 0.26</td>
<td>2.69 ± 0.04</td>
<td>7.19 ± 0.07</td>
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</tr>
<tr>
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<td>Moderate</td>
<td>Common Carp</td>
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<td>1.57 ± 0.26</td>
<td>7.23 ± 0.22</td>
<td>0.26 ± 0.02</td>
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<tr>
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<td>Hypoxia</td>
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<td>9.90 ± 0.60</td>
<td>9.47 ± 0.06</td>
<td>7.13 ± 0.08</td>
<td>0.85 ± 0.04</td>
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<td>2</td>
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<td>0.47 ± 0.12</td>
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<td></td>
<td>2.78 ± 0.22</td>
<td>7.13 ± 0.12</td>
<td>0.85 ± 0.12</td>
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<td>Moderate</td>
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<td>1.59 ± 0.61</td>
<td>7.08 ± 0.43</td>
<td>0.86 ± 0.12</td>
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<td>Hypoxia</td>
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<td>10.23 ± 0.24</td>
<td>9.47 ± 0.19</td>
<td>7.05 ± 0.19</td>
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<td>8.62 ± 0.68</td>
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<tr>
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<td></td>
<td>2.78 ± 0.22</td>
<td>7.09 ± 0.29</td>
<td>0.56 ± 0.03</td>
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<td>1.59 ± 0.70</td>
<td>7.03 ± 0.58</td>
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<tr>
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<td>Hypoxia</td>
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<td>9.34 ± 0.84</td>
<td>0.66 ± 0.22</td>
<td>0.22 ± 0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8.15 ± 0.79</td>
<td>7.27 ± 0.73</td>
<td>0.06 ± 0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Normoxia</td>
<td>Koi carp</td>
<td>10.29 ± 0.26</td>
<td>1.84 ± 0.03</td>
<td>0.03 ± 0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.74 ± 1.05</td>
<td>3.10 ± 0.12</td>
<td>7.12 ± 0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td></td>
<td>11.46 ± 0.69</td>
<td>0.38 ± 0.02</td>
<td>0.02 ± 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.37 ± 0.79</td>
<td>7.19 ± 0.59</td>
<td>0.59 ± 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hypoxia</td>
<td></td>
<td>10.31 ± 0.21</td>
<td>0.41 ± 0.04</td>
<td>0.04 ± 0.06</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5. The 24-h LC$_{50}$ and of un-ionized ammonia to common carp juveniles (mg NH$_3$-N/L). 95% confidence intervals are reported in the parenthesis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>24-h LC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>0.66 (0.55-0.81)</td>
</tr>
<tr>
<td>Moderate hypoxia</td>
<td>0.50 (0.38-0.66)</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>0.45 (0.35-0.52)</td>
</tr>
</tbody>
</table>

Table 6. Results from a two-way ANOVA comparing mortality and ammonia toxicity in three treatment levels of oxygen (hypoxia, moderate hypoxia, and normoxia). Carp were exposed to ammonia for 24-h. Results are considered significant at P=0.05.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Sum of Squares</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>1</td>
<td>10.66</td>
<td>947.8</td>
<td>0.000</td>
</tr>
<tr>
<td>Dissolved oxygen</td>
<td>2</td>
<td>0.12</td>
<td>5.3</td>
<td>0.074</td>
</tr>
<tr>
<td>NH$_3$</td>
<td>37</td>
<td>7.33</td>
<td>17.6</td>
<td>0.006</td>
</tr>
<tr>
<td>Dissolved oxygen*NH$_3$</td>
<td>4</td>
<td>0.31</td>
<td>6.8</td>
<td>0.045</td>
</tr>
<tr>
<td>Error</td>
<td>4</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 8. Mean daily water quality conditions throughout the experiment. Fish were given three days for tank acclimation prior to hypoxia exposure. Dissolved oxygen was lowered over 24 hours and then maintained until the end of ammonia exposure. Following ammonia exposure, all fish were returned to normoxic conditions without ammonia to recover for three days.
Figure 9. Mean ± standard deviation (n=4) of the proportion of koi that died during 24 hours of exposure of 0.61±0.12 mg/ NH$_3$-N L. Significance was determined using 1-way ANOVA Tukey-Kramer test. Different lowercase letters beside bars indicate significant statistical differences between treatments (α= 0.05).
Figure 10. Gill histology prior to being exposed to hypoxia. Small arrows are pointing at mucous cells and large arrows are pointing at mucopolysaccharides stained secretory cells.
Figure 11. Stained gills of fish sampled after being exposed to treatments of different levels of oxygen for 3 days. Arrows in A indicated mucous cells and squares in B are emphasizing lamellar fusion. A) Normoxia B) Moderate Hypoxia C) Hypoxia.
Figure 12. Gills after 24 hours of exposure to ammonia. A & B) Normoxia, C) Moderate Hypoxia, D) Hypoxia.
Figure 13. Gills following 3 days of recovery from ammonia exposure A) Normoxia B) Moderate Hypoxia C) Hypoxia.
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