ENVIRONMENTAL EFFECTS OF NITRITE ON GOLDFISH (*CARASSIUS AURATUS*) COMMUNICATION

by

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ABSTRACT

Increased agriculture and industrial activity have elevated concentrations of nitrogenous compounds in aquatic ecosystems. Exposure to elevated nitrite concentrations disrupt physiological and endocrine processes such as ion and hormone regulation, respiratory, and cardiovascular activity. However, there is a lack of research in the effects of nitrite on sensitive organs such as the olfactory epithelium. Fish olfactory systems are highly sensitive and can detect odors in the picomolar concentration. Odorant signals are integrated in the brain and trigger vital physiological functions (e.g. reproduction and feeding) and behavior. Therefore, disruption of the olfactory system will have a cascade of effects, affecting the viability of species in the short or long term. To detect odors, this specialized sensitive organ is continuously exposed to the aquatic environment, making it highly susceptible to toxins like nitrite. We hypothesize olfactory tissue will be adversely affected by nitrite. Thus, nitrite will accumulate and structurally alter the olfactory organ, which will change behavioral responses to odors and therefore act as a neurotoxin. The goal, to determine the acute and chronic effects of nitrite and its accumulation in the olfactory system and vital organs in goldfish. To test our hypothesis, three experiments were conducted; acute nitrite exposure, chronic nitrite exposure, and behavior assessments on chronically exposed fish. Acute exposure fish were exposed to nitrite concentrations (0, 0.3, 1.0, and 10 mM) for 5 days. Chronic exposure fish were exposed to nitrite concentrations (0, 0.01, 0.1, and 1.0 mM) for 69 days. Behavioral assessments were conducted on fish chronically exposed to nitrite concentrations (0, 0.01,

0.3, and 1.0 mM) for 28 days. In both experiments gill, nose and brain was collected for biochemical and histological analysis and nitrite accumulate was significant in acute and chronic experiment. Histological analysis showed an inflammatory process and cellular alteration in acute and chronical exposure. Behavioral assessment showed a decrease in food odor preference after nitrite treatments. Results demonstrate that environmental nitrite concentrations are potentially acting as a neurotoxin that alters olfactory function.

1. INTRODUCTION

1.1. Nitrite in The Environment

The rapid growth of urban and industrial areas has caused an increased influx of nitrogenous compounds into aquatic ecosystems. Significant amounts of ammonium enter aquatic environments through sewage effluent (Eddy *et al.*, 1994). When nitrogenous compounds like ammonium enter an aquatic ecosystem, aquatic bacteria preform a three-step process in which nitrifying bacteria oxidize ammonium (NH_4^+) to nitrite (NO_2^-) which is further oxidized to nitrate (NO_3^-) (Figure 1) (Lewis & Morris, 1986; Eddy *et al.*, 1994; Philips *et al.*, 2002; Kroupova *et al.*, 2005; Kocour *et al.*, 2018). Nitrite is the intermediate compound and in high oxygen conditions it can naturally accumulate by nitrification from ammonium to nitrite; in low oxygen conditions it accumulates by denitrification from nitrate to nitrite (Figure 1) (Eddy *et al.*, 1994).

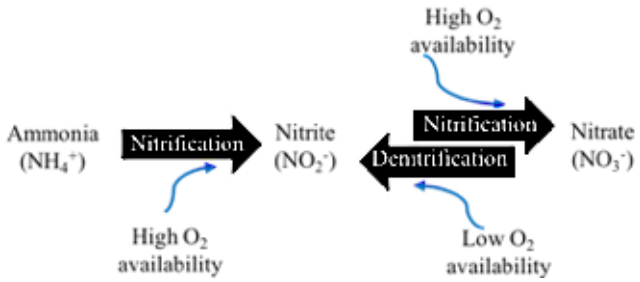

Figure 1. Diagram of the nitrite cycle in the aquatic environment. *Nitrite can accumulate in aquatic environments either through nitrification in high oxygen conditions or through denitrification in low oxygen conditions. The process of nitrification and denitrification is performed by aquatic, nitrifying bacteria.*

Nitrite can accumulate in aquatic ecosystems and may range from 30 nM to 1 mM (Table 1) (Kroupova *et al.*, 2005; Lam *et al.*, 2009; Paulmier & Ruiz-Pino, 2009; Lefevre *et al.*, 2011; Bristow *et al.*, 2015; Zeiger & Hubbart, 2016; Pottinger, 2017; Faust *et al.*,

2018). Nitrite accumulation occurs naturally and is closely associated with oxygen availability in the water column, meaning nitrate is found in oxygenated environments while anoxic environments have higher concentrations of ammonium and nitrite (Eddy *et al.*, 1994). A recent examination of the lower Seine river and estuary in France further demonstrated the correlation between anoxic environments and increasing nitrite concentrations (Garnier *et al.*, 2007). Oxygen-minimum zones can reach nitrite levels of 0.005 mM (Lam *et al.*, 2009; Lam *et al.*, 2011; Kalvelage *et al.*, 2013). Hypoxic zones occur when aquatic environments receive a nutrient overload of nitrogen, leading to algae blooms, in which bacteria overgrow and consume large quantities of oxygen (Eddy *et al.*, 1994). Hypoxic zones in the Gulf of Mexico experience an increase of nitrite due to massive nitrogen inputs from the Mississippi River (Garnier *et al.*, 2007; Bristow *et al.*, 2015). A recent review the Mississippi River indicated the Lower Mississippi Alluvial Valley experienced higher total nitrogen concentrations than previously reported (Faust *et al.*, 2018). The Mississippi River drains into the Gulf of Mexico; thus, a significant proportion of nitrogen input eventually drains into the gulf, causing swells of algae blooms, hypoxic zones and increased nitrite concentrations (Eddy *et al.*, 1994; Garnier *et al.*, 2007; Bristow *et al.*, 2015; Faust *et al.*, 2018). Increasing human populations consequently increases the demand on agriculture. Therefore, it is likely that both hypoxic zones and nitrite concentrations will increase in aquatic environments in the future.

Table 1. Nitrite concentrations in various aquatic ecosystems

Location	Nitrite concentration (mM)	Reference
Eastern Pacific	<0.002	Paulmier & Ruiz-Pino, 2009
Eastern Tropical South Pacific	≤0.0003	Lam et al., 2009
Hinkson Creek Watershed, Central Missouri	0.039	Zeiger & Hubbart, 2016
Lower Mississippi Alluvial Valley (various sites)	Overlying water: 0.140 Pore water: 0.004-0.182	Faust et al., 2018
Gulf of Mexico	≤0.003	Bristrow et al., 2015
Northwest England	≤0.20	Pottinger, 2017
Czech Republic	10-40	Kroupova et al., 2005
Arabian Sea	0.001	Lam et al., 2011

1.2. Nitrite and Vertebrates

Nitrite can be found in drinking water. Therefore, humans may inadvertently accumulate nitrate in plasma through daily water consumption.(Fan & Steinberg, 1996). Nitrite irreversibly binds to the heme group of hemoglobin in red blood cells (called then methemoglobinemia), which compromises the oxygen carrying capacity and thus decreases oxygen availability to vital organs (Lewis & Morris, 1986; Eddy *et al.*, 1994; Kroupova *et al.*, 2005; Lefevre *et al.*, 2011). In infants, the formation of methemoglobin in red blood cells results in “blue baby syndrome” and can reduce oxygen availability in vital organs (Fan & Steinberg, 1996).

Humans are readily exposed to nitrites; it can be found in cured meats, drinking water, and in farm-raised fish (Fan & Steinberg, 1996; Manthey-Karl & Schmidt, 2018). Pregnant women who consume these products are susceptible to stillbirths and infant deaths due to a defect of the central nervous system, known as anencephaly (Fan & Steinberg, 1996). Additional studies indicate that nitrite affects the central nervous system of adults. In two independent investigations, people who experience cluster headaches and migraines contained significantly higher nitrite concentrations (40-50 mM) in plasma than normal (Shimomura *et al.*, 1999; D'amico *et al.*, 2002).

1.3. Nitrite and Fish

In fish, nitrite is actively transported across the gill epithelium and can accumulate in aquatic organisms internal tissues and extracellular fluid (e.g. plasma) (Bath & Eddy, 1980; Margiocco *et al.*, 1983; Lewis & Morris, 1986; Eddy *et al.*, 1994; Huertas *et al.*, 2002; Jensen, 2003; Kroupova *et al.*, 2005; Kroupova *et al.*, 2008; Patrik Saoud *et al.*, 2014; Kocour *et al.*, 2018). The concentrations in tissues and plasma can reach environmental concentrations or higher (Bath & Eddy, 1980; Williams & Eddy, 1986; Eddy *et al.*, 1994; Jensen, 2003; Kroupova *et al.*, 2005). Carp exposed to 1 mM nitrite showed an increase in nitrite accumulation in plasma (Jensen *et al.*, 1987). Fish accumulated nitrite and concentrations reached treatment concentrations in less than 5 hours (h) and above treatment concentrations after 5 h of exposure (Jensen *et al.*, 1987).

Nitrite is actively transported by the chloride-carbonate (Cl^-/HCO_3^-) membrane exchanger protein in gills. There is a close linear relationship between chloride, nitrite, and carbonate concentrations; when chloride is at low concentrations, uptake will shift to

nitrite (Jensen, 2003), and when environmental chloride is high, there is a decrease in the uptake of nitrite (Bath & Eddy, 1980; Tomasso, 1986; Williams & Eddy, 1986; Jensen *et al.*, 1987; Eddy *et al.*, 1994; Jensen, 2003). This assertion is supported by an experiment conducted on rainbow trout (*Salmo gairdneri*): fish were exposed to low concentrations of chloride (<0.3 mM/L) and nitrite (~ 0.7 mM/L) for 24 h exhibited nitrite blood plasma that were elevated 5-10 times more than concentrations in the treatment (Bath & Eddy, 1980). However, when chloride was high (0.6 mM/L), there was a decrease in nitrite uptake, resulting in an increase in survival rate (50%) (Bath & Eddy, 1980). Moreover, research shows that fish have processes that allow them to remove nitrite. In a study conducted by Gisbert et al. (2004), Siberian sturgeon (*Acipenser baerii*) yearlings showed that they were capable of actively removing nitrite from the blood plasma and gills after exposure. Sturgeons were exposed to 9.3 mM nitrite, the median lethal concentration for this species, for 18 hours (h). Fish were able to recover 6 h after return to control levels. This result indicated that fish have a mechanism to reduce nitrite levels.

In addition to accumulating in internal tissues, nitrite in is known to affect several physiological processes. For instance, it is well known that high nitrite concentrations in the plasma of teleosts and mammals reduce the carrying capacity for oxygen in red blood cells (Bath & Eddy, 1980; Eddy *et al.*, 1994; Huertas *et al.*, 2002; Gisbert *et al.*, 2004; Wuertz *et al.*, 2013). This reduction is caused by an irreversible oxidation of hemoglobin, leading to methemoglobinemia, or brown blood disease (Jensen, 2003; Kroupova *et al.*, 2005). Methemoglobinemia makes oxygen unavailable for vital organs and, therefore, is detrimental to teleosts and mammalian health (Bath & Eddy, 1980; Jensen *et al.*, 1987; Huertas *et al.*, 2002; Jensen, 2003; Hvas *et al.*, 2016).

Extensive research shows that internal accumulation of nitrite can disrupt several physiological processes including the endocrine system (Xiao *et al.*, 2017), ion regulation (Bath & Eddy, 1980; Jensen *et al.*, 1987; Huertas *et al.*, 2002; Jensen, 2003; Gisbert *et al.*, 2004), the cardiovascular system (Jensen *et al.*, 1987; Jensen, 2003), and nitrite acts as teratogen (Keshari *et al.*, 2016).

Nitrite has been found to disrupt the endocrine system and specifically affect thyroid hormones and sex steroids (Ciji *et al.*, 2013; Xiao *et al.*, 2017). Experimental observations on rohu (*Labeo rohita*), a member of the carp family, showed a decrease to 84.5% and 94.1% in serum triiodothyronine (T3) and thyroxine (T4), respectively, upon exposure to sub-lethal concentrations (0.043 mM, $1/5^{th}$ LC_{50}) of nitrite. This study also showed that serum testosterone and estradiol levels decreased (97.3% and 92.9%, respectively) at sub-lethal concentrations of nitrite (Ciji *et al.*, 2013). A study conducted by Xiao et al. (2017) showed that nitrite has adverse effects on thyroid stimulating hormone (TSH) causing a 50% decrease after 12 hours of nitrite exposure. Grass carp were exposed to nitrite (up to 0.35 mM) which caused a decrease in T3, T4, free T3, free T4 and TSH levels across all treatments. Histological analysis showed a decrease in the number of colloid spaces (Xiao *et al.*, 2017), a region within the thyroid follicle filled with proteinaceous liquid that is vital for synthesizing T4 (Norris & Carr, 2013). An interference of T4 synthesis indicating disruption of the thyroid (Norris & Carr, 2013).

Presence of nitrite has been found to critically alter ion regulation by influencing potassium balance and significantly elevating plasma potassium concentrations (Jensen *et al.*, 1987; Beeson *et al.*; Huertas *et al.*, 2002; Jensen, 2003). Elevation of $[K^+]$ is believed to be due to loss of K^+ from skeletal muscle of fish (Jensen *et al.*, 1987). An increase in

[K^+] is unfavorable for the heart and other excitable tissues because it can lead to heart failure and nerve malfunction (Jensen *et al.*, 1987; Jensen, 2003). The increase of [K^+] coupled with red blood cell shrinkage, together with the formation of methemoglobin by nitrite, will reduce the oxygen carrying capacity and can damage the fish cardiovascular system (Jensen, 2003).

Nitrite has been found to have potential teratogenic effects, chemical agents that disrupt development of an embryo or fetus. Zebrafish embryos treated with nitrite (1,000 mg/L or 21.7 mM) for 94 h showed gross underdevelopment compared and zebrafish embryos treated with nitrate (1000 mg/L or 16 mM) (Keshari *et al.*, 2016). Nitrite treated zebrafish embryos had a reduction in eye size, swim bladder non inflation, yolk sac edema, and craniofacial defects (Keshari *et al.*, 2016). Furthermore, the study found that fish exposed to nitrite (8.6, 13, and 17.4 mM) had lower percent survival (53, 38, and 41%, respectively) than fish exposed to similar or lower nitrate concentrations (16 mM, 95%) (Keshari *et al.*, 2016). These findings demonstrate that nitrites can be lethal at low concentrations and have severe toxic effects to fish.

Another effect of nitrite exposure is irritation and damage in external organs like gills (Gisbert *et al.*, 2004). After acute or chronic treatment with nitrite, gills undergo structural changes (Kroupova *et al.*, 2008; Wuertz *et al.*, 2013; Patrik Saoud *et al.*, 2014). Gills exposed to 9.3 mM nitrite treatment sampled at 0 h, 6 h, 18 h, 96 h, 240 h exhibited various structural changes on the secondary lamella, including hyperplasia, lamellar clubbing, mucous cell hypertrophy, epithelial cell hypertrophy, increased mucus secretion and necrotic epithelia (Gisbert *et al.*, 2004). When fish were chronically exposed to 0.01 mM nitrite for 28 days, there was a noted increase in edema, swelling in the secondary

lamella, and hyperplasia (Kroupova *et al.*, 2008). Furthermore, the same study found that fish chronically exposed to 0.6, 1.0, and 3.0 mM nitrite for 28 days had an increase in edema, hyperplasia, and fusion of secondary lamella (Kroupova *et al.*, 2008). A second study found similar results in fish treated with 0.44 mM, 0.88 mM, 1.75 mM, and 3.5 mM nitrite for 32 days: an increase in hyperplasia and secondary lamella fusion as well as heavy degeneration (necrosis) of the gill. (Wuertz *et al.*, 2013).

Although, ample research has been conducted on nitrite effects on internal physiology and gill, very little is known about the effects of nitrite on external sensory systems, including the teleost olfactory system in teleost. External tissues are constantly in contact with the environment which may lead to an increase of toxin accumulation and tissue damage before internal tissues. Furthermore, their external position may cause them to accumulate more nitrite overall than internal tissues. Therefore, the functionality of sensitive external tissues, like the olfactory tissues, maybe reduced before internal tissues.

1.4. Fish Olfaction

Olfaction in fish is used to detect environmental cues which is key to regulating several physiological processes such as, coordinating reproduction, spawning, migration, feeding, avoidance of predation, parental care, and offspring-parent interactions. In order to detect environmental cues, aquatic vertebrates have developed a sensory system devoted to detecting and identifying substances. In order for this system to function it must constantly be exposed to the environment. The sensory tissue constantly exposed to the environment is called the olfactory tissue. The olfactory tissue is located in the head,

anterior to the eyes, and covered by a nasal flap. The olfactory tissues consist of lamellae that are arranged in a rose-like fashion that forms a rosette (Hansen *et al.*, 2004; Hara & Zielinski, 2006; Døving, 2010). The lamella are a series of folds and within each folds are cells embedded in an epithelium (Døving, 2010). Fish have evolved a highly specialized, extensive and diverse family of olfactory sensory neurons that are located within the epithelium (Hara, 1994; Hara & Zielinski, 2006; Stacey & Sorensen, 2009; Døving, 2010). The olfactory sensory neurons consist of four types of neurons: ciliated, microvillous, crypt, and Kappe (Muller & Marc, 1984; Hansen *et al.*, 2004; Døving, 2010; Ahuja *et al.*, 2014). These sensory neurons vary in dendrite length, and therefore their somas and nuclei lie at distinct depths forming a pseudostratified pattern (Døving, 2010). The sensory organ also contains basal cells, supporting cells, goblet cells and ciliated non-sensory cells (Hara, 1994; Hansen *et al.*, 1999).

Each olfactory sensory neuron (OSN), ciliate, microvilli, crypt, and Kappe, express only one type of receptor (Hansen *et al.*, 2004; Døving, 2010). The OSNs relay sensory information to the olfactory bulb, on which they terminate; the olfactory bulb then transmits the message to the telencephalon via the olfactory tract (Hansen *et al.*, 2004). Olfactory signals are then integrated in the brain and trigger physiological and behavioral responses (Døving, 2010). Fish OSNs are stimulated by four major classes of compounds: amino acids, sex steroids, bile acids/salts and prostaglandins (Hara, 1994). In electrophysiological experiments, recordings from goldfish OSNs suggest that neurons express odorant receptors for specific amino acids (Speca *et al.*, 1999). When amino acids are detected by the microvillous sensory neurons, an electrical signal is relayed to the olfactory bulb which then stimulates the lateral olfactory tract and the stimuli can

induce different feeding behaviors in fish (Døving, 2010). Therefore odors, such as amino acids, can be used to modulate behavioral response.

The olfactory system in vertebrates is highly specialized to detect and process odorants. Some olfactory receptors are specific to pheromones (Stacey & Sorensen, 2009). Pheromones are chemical odors used to communicate to conspecifics about current physiological status, mainly related to reproduction or migration. Teleosts produce prostaglandins, steroids, and metabolites that can be released and used as reproductive pheromones (Stacey & Sorensen, 2009). These reproductive pheromones are special sexual signals key to coordinating reproduction. In the event of chemoreception, detection of a chemical odor by the olfactory system occurs by binding to receptors located on the membrane of OSN (Hara, 1994).

1.5. Goldfish as a Study Organism

Goldfish (*Carassius auratus*) olfaction has been extensively studied and research has demonstrated the importance of olfaction in this species. Moreover, goldfish are an important model fish for cypriniforms. Goldfish have been shown to exhibit courtship behaviors and use sex pheromones to synchronize their spawning (Kobayashi *et al.*, 1986; Kobayashi *et al.*, 2002; Sorensen *et al.*, 2005; Hara & Zielinski, 2006; Stacey & Sorensen, 2009; Ghosal & Sorensen, 2016). Females release pre-ovulatory hormones (androstenediones and 17,20-dihydroxyprogesterone) that act as pheromones on male goldfish by inducing spawning behavior such as chasing, following, and nudging after olfactory detection of the signal (DeFraipont & Sorensen, 1993; Stacey & Sorensen, 2009; Hara, 2012; Ghosal & Sorensen, 2016). The release of these pheromones induces

an increase in lutinizing hormone (LH) in males, and this increase leads to an increase in milt or semen production (Hara, 1994; Kobayashi *et al.*, 2002; Sorensen *et al.*, 2005; Stacey & Sorensen, 2009). Courtship and spawning behavior finally occurs after release of prostaglandins PGF_{2a} and 15K-PGF_{2a} by the female goldfish (Hara, 1994; Kobayashi *et al.*, 2002; Stacey & Sorensen, 2009; Hara, 2012; Ghosal & Sorensen, 2016). Goldfish can detect odorants, including pheromones, at near picomolar concentrations (Sorensen *et al.*, 2005; Hara & Zielinski, 2006). Cumulatively, this research indicates that olfaction triggers an endocrine response which elicits and behavioral output in goldfish.

Goldfish have also been shown to use their olfactory system for food detection (Hara, 1994; Hara & Zielinski, 2006; Hara, 2012). Goldfish are able to detect various amino acids (L-cysteine, L-lysine, L-serine, L-arginine, and L-glutamic acid) (Hara, 1994; Hara & Zielinski, 2006). Furthermore, the detection of these amino acids elicits a feeding behavioral response such ask increased locomotion, pecking at the bottom, bottom searching (Hara & Zielinski, 2006). However, not all amino acids elicit the same feeding behavior response or the same magnitude of response. For example, fish stimulated by L-cystine and L-arginine will both increase locomotion (number of turns) (Hara & Zielinski, 2006). However, fish will elicit more pecking behavior when stimulated by L-arginine than L-cystine (Hara & Zielinski, 2006). Additionally, goldfish have demonstrated similar feeding behavior when stimulated by food odor. When compared to feeding behavior stimulated by amino acids, food odor elicits a greater behavioral response.

In order for goldfish to detect sex pheromones or odors, the olfactory tissue must constantly be exposed to the environment. Thus, this tissue is highly susceptible to

environmental pollutants like nitrite. External tissues like gills have been shown to accumulate nitrite and this causes structural changes at a level cellular in the tissue. Furthermore, the alteration of the tissue causes impairment of tissue function which leads to physiological impairments. Like gills, fish olfactory tissue could accumulate and be structurally altered by nitrite. Therefore, nitrite could be compromising the olfactory system in fish. Thus, impairing odor detection and change how fish respond to odors.

The purpose of this study is to determine how nitrite affects the olfactory system in fish. Furthermore, I aim to determine if nitrite at concentrations found in the environment, concentrations that do not cause mortality, affects the olfactory system in fish**. I hypothesize that acute and chronic exposure to nitrite affects primarily the external olfactory epithelia in goldfish.** As a result, olfactory function will be compromised, and goldfish will not be able to perform vital functions such as detecting food cues and associated swimming behavior. To test my hypothesis, I designed three experiments. In experiment 1 (acute nitrite exposure) and 2 (chronic nitrite exposure), goldfish tissues exposed to nitrite were analyzed for nitrite accumulation and histological alterations in nose and gill tissue. A third experiment determined the effects of nitrite exposure on food preference behavior.

Nitrite is accumulating in aquatic ecosystems and previous research demonstrates the toxic effects on fish at concentrations above the ones found in the environment. Furthermore, it has been established that nitrite changes the physiology in fish. However, studies have not explored the impacts of nitrite on the olfactory tissue, nor impact at concentration bellow the ones found in the environment. This olfactory sensitive tissue is highly vulnerable to environmental toxins. Thus, this research will provide insight on

toxicological effects of environmental nitrite and its effects on the olfactory system. The olfactory system is necessary to detect odors; these odors stimulate physiological changes which are necessary to elicit critical behavioral responses such as feeding, coordinate reproduction, and predator/prey interaction. These behavioral responses are necessary for the survival of any organism and propagation of any species. Thus, impairment of this system by nitrite could compromise the survival of fish in the long term. Therefore, understanding how environmental nitrite impacts the olfactory system may help us understand toxicological patterns in sensory neurons and devise plans in the management of fish species.

2. THESIS OBJECTIVES

The goal of this study was to determine the effect of environmental nitrite in the olfactory organ of goldfish (*C. auratus*). I hypothesize that sublethal concentrations of nitrite can affect the olfactory function which will dampen physiological functions and thus altering behavior. If nitrite affects the olfactory organ it will accumulate and structurally alter the tissue. As a result, the toxin will impair odor detection to preferred odors such as food odor. This impairment will alter fish behavior towards preferred odors. Thus, I predict nitrite is acting as a neurotoxin. The goal can be broken down into the following objectives:

2. 1 Establish if nitrite accumulates in the olfactory organ and how it compares to vital tissues at acute lethal concentrations

First, I aim to determine if nitrite will accumulate and structurally alter the olfactory organ by exposing fish to nitrite concentrations (0, 0.3, 1, and 10 mM) known to structurally gill and accumulate in tissues (Gisbert *et al.*, 2004). Second, I will determine the treatment concentration in which the olfactory organ will accumulation and be structurally altered. Lastly, I will compare the effects of the olfactory organs to other tissues known to be affected by nitrite. I hypothesize that acute nitrite treatments (0.3 mM, 1.0 mM, and 10 mM) will have adverse effects on goldfish tissues. I predict experimental nitrite treatments will accumulate in vital tissues (olfactory organ, gill, and brain) and tissue concentrations will increase as treatment concentrations increase and furthermore, nitrite accumulation will cause inflammation of the olfactory organ.

2. 2 Determine if chronic exposure to non-lethal environmental nitrite has similar effects in the olfactory organ as acute lethal concentrations

Nitrite concentrations in the aquatic environment are below lethal levels (Kroupova *et al.*, 2005; Lam *et al.*, 2009; Paulmier & Ruiz-Pino, 2009; Lam *et al.*, 2011; Bristow *et al.*, 2015; Zeiger & Hubbart, 2016; Pottinger, 2017; Faust *et al.*, 2018). I aim to determine if environmental nitrite exposure causes similar affects to the olfactory organs as acute nitrite exposure. I hypothesize fish exposed to environmental nitrite concentrations (0, 0.01, 0.1, 1 mM) will accumulate in tissues (olfactory organ, gill, and brain) and thus cause structural damage to tissues. Based on observations from the previous objectives, I predict nitrite will accumulate in tissues and concentrations will increase as treatment concentrations increase. Furthermore, I predict the accumulation will alter the olfactory organs cellular structure and the highest treatment will alter the cellular structure the most.

2. 3 Chronic concentrations of non-lethal nitrite exposure effects on behavior and odor preference

Previous research found that goldfish are able to detect amino acids and the chemoreception causes a physiological and/or behavioral response (Hara, 1986, 1994; Hara & Zielinski, 2006; Hara, 2012). When fish are stimulated by food odor and amino acids (cystine, lysin, arginine, and serine) fish will exhibit various behaviors such as increased locomotion activity and pecking activity (Hara, 1986; Hara & Zielinski, 2006). Fish exhibit decrease in locomotion activity and pecking activity when not stimulated by odors. I aim to determine if nitrite impairs odor detection by observing how nitrite alters

fish behavior. I hypothesize fish exposed to environmental nitrite concentrations (0, 0.01, 0.3, and 1mM) will have decrease odor perception and thus alter fish behavior. I predict fish exposed to nitrite will lose ability to smell food odor (anosmia) and therefore will have a decrease in locomotion and pecking activity.

3. MATERIALS AND METHODS

3.1. Fish

Goldfish for nitrite exposure and behavior experiments were purchased from PetSmart® and The Bait Bar, a family owned fishery (Bryan, Texas). All goldfish were sexually immature. PetSmart® goldfish were used to conduct the acute test and The Bait Bar fish were used to conduct the chronic and behavioral tests. Upon arrival at Texas State University Freeman Aquatic Laboratory, goldfish were treated with formalin (25 mg/ L) for 30 minutes in a 150 L tank. Afterwards, fish were moved and acclimated for two weeks in a multiple recirculating living stream with aerated water at 25 °C. Fish were fed Tetra® TetraFin Goldfish Flake food once a day.

3.2. Acute Nitrite Treatment

Goldfish (2.8 ± 0.09 g, n = 30/replicate, 3 replicates) were acclimated in 35 L tanks filled with 30 L water with aeration and carbon bio filter, at 20.5 °C for 7 days. After that, goldfish were exposed to the following nitrite concentrations: 0 mM (control), 0.3 mM, 1 mM, and 10 mM for 5-days in a renewal system. Acute experiments are short-term (hours to <14 days) exposure tests and organisms are exposed to lethal concentrations. A 5-day exposure period was chosen based on previous research, which showed that goldfish have 96hLC_{50} of ~4 mM (Tomasso, 1986) and can tolerate high nitrite concentrations compared to rainbow trout (*O. mykiss*) (Jensen, 2003) and tilapia (*Tilapia aurea*) (Tomasso, 1986). Furthermore, rainbow trout (*O. mykiss*) when exposed to 1 mM nitrite accumulate nitrite in extracellular fluid; accumulation will increases above treatment concentrations, and trout survive for 4 days under these conditions

(Jensen, 2003). Therefore, we predicted goldfish would survive treatments 0 mM, 0.3 mM, and 1 mM. Additionally, we predicted goldfish in the 10 mM treatment would exhibit severe physiological changes because it is well above the LC_{50}.

Nitrite treatment solutions were made by combining sodium nitrite (Sigma-Aldrich; ST. Louis, MO) and deionized water. At 10 mM sodium nitrite treatment, the salinity increase is only 0.02%. Thus, we do not expect an osmoregulatory effect due to our treatments. Aliquots of nitrite were made for treatment concentrations prior to treating fish. Fish were kept in aquariums, each treatment consisted of three replicates, and each replicate contained 10 goldfish. On day of exposure, carbon filters and bio-balls were removed from the 35 L tanks. Fish were not fed during nitrite exposure to reduce ammonium excretion from fish.

Daily water changes were conducted during exposure period. First, previously made sodium nitrite solutions were mixed with water in separate containers. Second, the water in the tank was removed using tubes. Simultaneously, with a second set of tubes, new treatment water was added as water was removed to maintain appropriate concentrations. To monitor nitrite and ammonia levels after daily water changes, API® 5 in 1 Aquarium Test Strips and API® Aquarium Ammonia Test Kit were used. Goldfish that died during exposure period were removed immediately upon discovery to maintain nitrite concentrations.

At the end of the 5-day period exposure, fish from were anesthetized using Tricaine mesylate (MS-222) at a concentration of 0.8 g/ L. The brain, gills, and olfactory epithelium were collected after sacrificing. To collect the olfactory epithelium, the nose cavity was kept intact, and all surrounding tissue was collected. Some of these tissues

were flash frozen at -20°C and stored at -80°C for long-term storage. These samples were used for biochemical analysis. Sub-samples of the tissues were placed in fixatives, either stored in Bouin or 4% paraformaldehyde (PFA) prepared in phosphate buffer saline solution (PBS). After 18 hours, Bouin samples were transferred to 70% ethanol and PFA samples were transferred to PBS and stored in the refrigerator (4 °C). Bouin samples were then shipped to the UTSA Histology and Immunohistochemistry Laboratory for paraffin.

3.3. Chronic Nitrite Treatment

Goldfish (n = 30/replicate, 3 replicates) were acclimated in 35 L aerated tanks at room temperature (20.5°C) with flow through aquaria without carbon filter or bio-balls for 14 days (Figure 2). Fish were fed daily with commercial brand food (see section 2.1 Fish). Following acclimation period, fish were exposed to nitrite concentrations: 0 mM, 0.01 mM, 0.1 mM, and 1 mM for 69-days in flow through aquaria. Chronic long-term tests generally use sub-lethal concentrations. The concentrations chosen are similar to environmental concentrations (Table 1). Additionally, previous research showed that rainbow trout (*O. mykiss*), when exposed to 0.01 mM and 0.1 mM for 29-days, exhibited physiological changes and, accumulation in muscle, plasma, and structural changes to gills (Kroupova *et al.*, 2008). Furthermore, findings from acute experiments showed that at 1.0 mM the olfactory organ begins to have adverse effects such as structural changes due to accumulation. Therefore, we predicted goldfish exposed to these concentrations (0.01 mM, 0.1 mM, and 1 mM) would survive treatment and display physiological and cellular changes. Fish were exposed to treatment conditions for 63-days because it is the

standard time for chronic exposure.

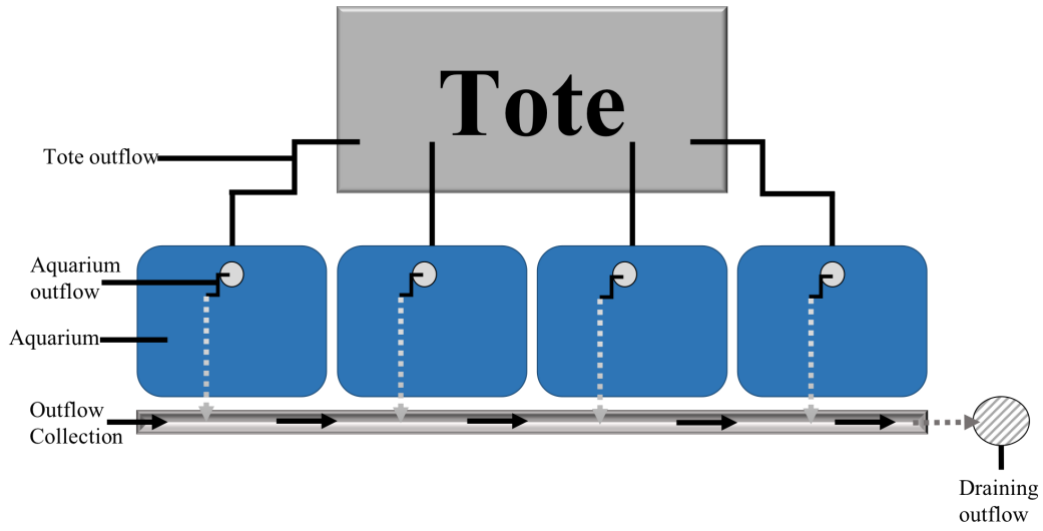

Figure 2. Continuous aquarium system for chronic nitrite exposure in goldfish. *Totes were filled with water with appropriate nitrite concentrations, aquariums were gravity feed with water from the tote. Valves were used to calibrate rate of tote outflow. Aquarium outflow drained into outflow collection which ultimately drained into Texas State University Freeman building sewer system.*

A similar procedure to the that used for acute treatment was used to make nitrite solutions for treatments. Like the acute treatment, aliquots of nitrite solutions were made ahead of time. Flow through aquaria were controlled by using a water release rate from a 132-L plastic water tank (tote, Figure 2) and the rate was set to ~ 35 mL/ min using valves (tote outflow, Figure 2). Plastic water tanks were refilled every other day. The plastic water tanks were emptied out before refilling them. Next, the valves (tote outflow, Figure 2) were closed and the water from the Freeman Aquatic Laboratory was used to refill the plastic water tank. Third, while plastic water tanks were being refilled, nitrite aliquots were added to the appropriate treatment tanks. Lastly, once the plastic water tanks were refilled, the valves (tote outflow, Figure 2) were reset to the appropriate rate. Daily procedures included feeding fish, checking valve rate, and recording any change in

fish behavior during the exposure period. This flow through system is best for chronic exposure experiments because it controls introduction of nitrite, easily maintains of nitrite concentrations at desired levels, and it decreases fish's stress by decreasing human interaction.

Daily water samples were taken and weekly water quality parameters (nitrite, nitrate, ammonia/ammonium, and pH) were measured and recorded in similar manner as during the acute experiment. Goldfish that died during experiment were removed immediately upon discovery to further control nitrite concentrations.

After the exposure period, similar protocols to the acute treatment were used to anesthetize fish, fix tissues, and store tissues for analysis. The same tissues (olfactory epithelium, gills, and brain) were collected as in the acute experiment. However, in this experiment the olfactory epithelium was dissected out from the nose cavity and all surrounding tissue was removed.

3.4. Nitrite Determination in Tissues

Flash frozen samples were analyzed for nitrite content using a colorimetric method (Hensley et al., 2003). Samples were weighed, homogenized in PBS, and centrifuged for 15 min at 15,000 relative centrifuge force (RCF) at 4°C. Supernatant was collected, cleaned with methanol (1:1) for protein precipitate, vortexed, incubated at room temperature for 10 min, and centrifuged for 30 min at 17,000 RCF at 4°C. Tissue samples from the acute experiment in treatments 1 and 10 mM were diluted 1/10 and 1/100, respectively. Tissues samples from the chronic experiment were not diluted. Samples were analyzed using the Griess colorimetric method (Hensley et al., 2003) in a

96-well microplate. After 3-5 minutes, optical density of samples was measured at 570 nm and compared to a standard curve.

3.5. Histological Analysis of Tissues

Bouin fixed samples were dehydrated with ethanol (70%) and shipped to UTSA Histology and Immunohistochemistry Laboratory to be embedded with paraffin. Paraffin embedded samples were stored in the refrigerator (4°C) and cut with a microtome into 7 µm thick sections and placed on slides. To stain tissues, paraffin was removed (100% xylene for 15-min) then hydrated in a graded series of decreasing ethanol concentrations (100%, 95%, and 70% for 15 min, 10 min, and 10-min, respectively) and finally stained with Alcian blue-PAS. Olfactory and gill tissue in the acute experiment were qualitatively analyzed. For each chronic treatment, gill tissue samples were cross-sectioned and analyzed using ten field areas in ten slides. Several parameters were used to examine samples for percent coverage of primary lamellas, clubbing (cb), total fusion (tf), hypertrophy (h), and cell count between the secondary lamella; as described in Mallatt (1985). Olfactory tissue from the chronic experiment was lost during the staining process and therefore there are no results for this tissue from the chronic experiment.

The olfactory tissue samples from the acute experiment preserved in PFA were examined for immunohistochemistry. These tissue samples were first rapidly frozen at -20°C (5-10 minutes) into blocks using a piezo. Immediately afterwards, samples were cut with the cryostat into 10 µm (thickness). Tissues were stained using a TUNEL IHC immunohistochemistry kit and counterstained with methyl-green which stains immune cells dark green.

3.6. Behavior Responses to Food Odor

Two-choice maze behavioral assays were used to evaluate goldfish responses to food odors pre and post nitrite exposure (Buchinger *et al.*, 2017). An experimental two-choice maze was constructed at Texas State University at the Freeman Aquatic Laboratory (Figure 3). Clear acrylic sheets purchased from GRANGER® were used to construct the maze. A submersible water pump (PondmasterTM POND MAG3) was used to create inflow and a multichannel peristaltic pump was used to release food odor (Cole-Parmer® CatalystTM Masterflex FH Series). A meshed barrier was added to create a laminar flow that would disperse the cue evenly through the odor channel (Jutfelt *et al.*, 2017). A series of dye tests were conducted to assess the laminar flow and distribution of dye.

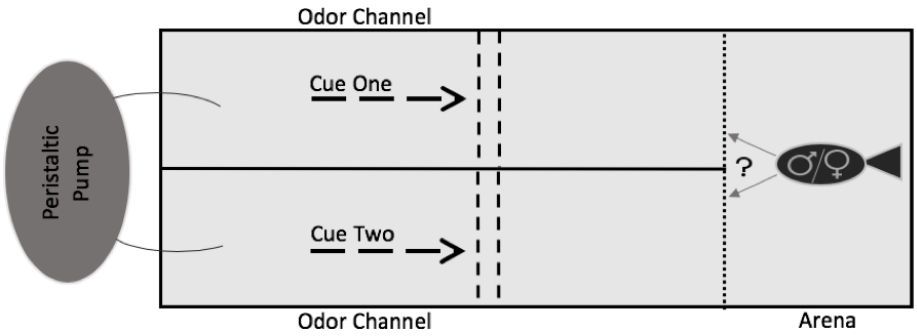

Figure 3. Acrylic two-choice maze was used to conduct behavioral assay on goldfish to evaluate responses to food cues. *Following an acclimated period in arena, fish were allowed to explore maze to establish side bias. Afterwards, fish were re-acclimated, and during this period food cue was placed on appropriate side and released using a peristaltic pump. A separate pump was used to create continuous water flow in the maze which aided in cue dispersal in the odor channel.*

Goldfish (n = 42) from the Freeman Aquatic Laboratory living stream population first had their fins clipped for identification, and then were placed in a 35 L flow-through tanks (Figure 2) to acclimate for a minimum of 7 days. A mixture of males and females

were kept in flow-through tanks. The experiments began in mid-June and goldfish were tested multiple times over a 28 day period. During this period, goldfish were exposed to nitrite treatments: 0 mM (control with fin clips), 0 mM (control without fin clips), 0.001 mM, 0.3 mM, and 1 mM. The control, 0 mM without fin clips, was used to control for the stress effects of clipping.

Fish were not fed one day prior the day of behavioral experiments to increase their appetite. Behavioral experiments consisted of an acclimation phase (5 minutes), exploring phase (5 minutes), re-acclimation phase (5 minutes), and exposure to cue phase (5 minutes). The time goldfish spent (seconds) in either channel was recorded. During the exploring phase, fish were let to explore the maze to establish channel preference bias. The cue side for each behavior experiment was based on the channel preference bias; cue was released on the opposite side of the preferred side.

Food odor was made using 5 g of Tetra® TetraFin Goldfish Flake food homogenized with 1-L of water. Odors were made in plastic jar containers 1-2 days prior to behavioral trials and stored in fridge (4°C) until needed. Food odors containers were allowed to reach room temperature before being used in behavior trial. Each behavior trial utilized 1-L of water (one plastic container). After behavior trials leftover plastic containers were discarded.

3.7. Statistical Analysis

Tissues that were analyzed for nitrite accumulation were compared to a standard curve. After nitrite concentrations were calculated concentrations were converted to µg nitrite /g tissue. Concentrations were values were then log transformed and analyzed using a two-way ANOVA ($P < 0.05$). A one-way ANOVA ($P < 0.05$) was used on chronic gill histological parameters (see section 2.1) to determine differences among treatments.

For behavioral trials, each goldfish in each treatment were tested (trials) once a week for 4 weeks. During trials the time spent in the control and experimental channels were recorded and an index of preference (i) was calculated for each test (i=[ae/(ae+be) – ac/(ac+bc)]). The time spent in the control channel before odor exposure was calculate (bc = before odor release control channel and be = before odor release experimental channel, respectively), and after odor exposure (ac = after odor release control channel and ae = after odor release experimental channel). The indices of preferences were evaluated using a Wilcoxon signed-rank test ($\alpha = 0.05$) (Li *et al.*, 2002).

4. RESULTS

4.1. Acute Nitrite Treatment

The goal of this thesis was to determine the effect of high nitrite concentrations in the olfactory tissue of goldfish. To do this, I first had to establish if nitrite accumulates in the olfactory tissue upon acute exposure, a short-term exposure where fish are exposed to concentrations above environmental conditions and/or lethal concentrations. Second, I wanted to establish how the accumulation in the olfactory tissue compared to other vital tissues, such as the gill that are known to accumulate nitrite. Lastly, I wanted to establish if nitrite accumulation altered the cellular structure of the olfactory tissue and how this compared to gill.

Mortality in treatments 0 to 1 mM nitrite was between 0 and 7.4% and was not statistically significantly different from 0 mM. Mortality at 10 mM nitrite was 22 ± 6.4 % and was statistically significantly different than control (Figure 4A).

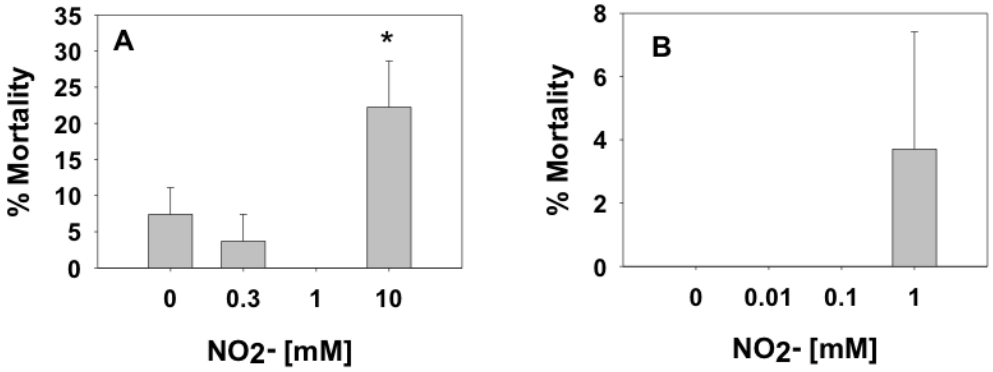

Figure 4. Percent mortality in fish exposed to nitrite in acute (A) and chronic (B) experiment.

Fish exposed to nitrite accumulated nitrite in all tissues (olfactory, gill, and brain) (Figure 5). Nitrite concentrations increase in parallel to the treatment concentrations.

Two-way ANOVA found the interaction between treatment *vs* tissue to be statistically significant ($P < 0.05$) at 1 mM and 10 mM nitrite, denoted by the numbers. Nitrite accumulated in tissue of fish treated 0.3 mM nitrite; however, tissue averages were not statistically significantly different from control, denoted by number.

Nitrite accumulated in the olfactory tissue in all nitrite treatments (0.3 mM, 1 mM, and 10 mM) (Table 2). Nitrite accumulation began in treatment 0.3 mM and compared to control, concentrations increased by a factor of 2.8. At 1 mM, nitrite concentrations in olfactory tissue doubled again and when compared to tissue concentration in treatment 0.3 mM. Treatment 10 mM had the highest nitrite concentration and concentration quadrupled, compared to 1 mM.

Nitrite accumulated in all tissues in all treatments, however tissue concentrations varied between tissues within a treatment (Figure 5). Post hoc analysis found statistical significance between tissues concentrations within a treatment, denoted by letters ($P < 0.05$). All tissues accumulated nitrite in treatment 0.3 mM and nitrite concentrations were only significantly different for the olfactory tissue, which had the lowers concentrations compared to other tissues. At 1 mM nitrite again accumulated in all tissues, concentrations in the olfactory tissue were similar to the gill but surpassed the brain. However, the olfactory tissue was not significantly different from the gill and brain. At 10 mM the same pattern emerged as previous treatment (1 mM). Yet, in this treatment the olfactory tissue was found to be significantly different from the other tissues.

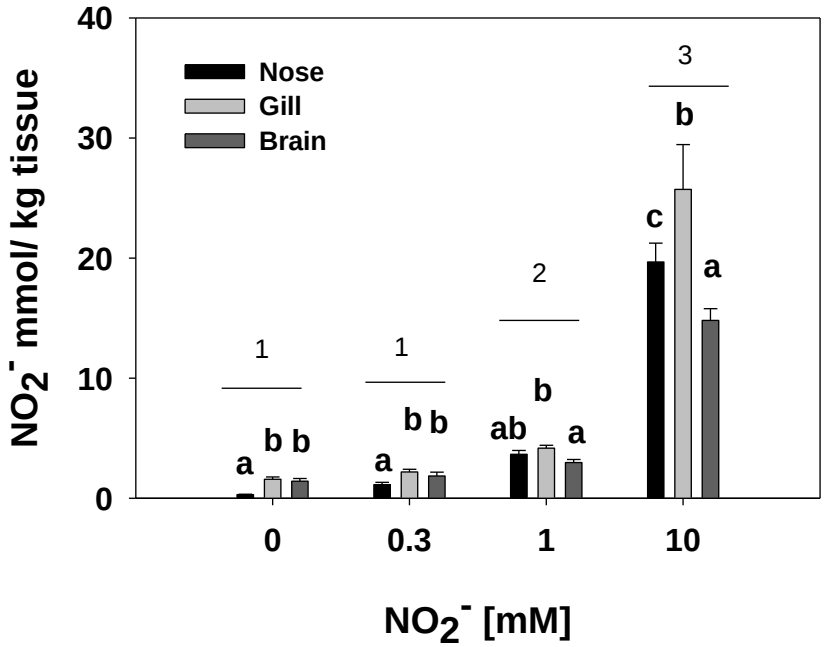

Figure 5. Nitrite accumulation in nose, gill and brain of goldfish exposed to several, acute nitrite concentrations. *Two-way ANOVA showed that the interaction between treatment and tissue was significant ($P < 0.05$). Numbers denote statistical differences between nitrite treatments. Letters denote statistical different between tissues within treatments.*

Table 2. Nitrite accumulation in tissues from acute and chronic experiment in millimole (mmol) of nitrite per kilogram (kg) of tissue.

		Gill				Nose				Brain			
Treatment (mM)		Mean	SEM	Max	Min	Mean	SEM	Max	Min	Mean	SEM	Max	Min
Acute Treatment	**0**	1.6	0.19	3.3	0.15	0.30	0.027	0.44	0.19	0.30	0.03	2.3	0.24
	0.3	2.2	0.22	5.1	0.48	1.1	0.18	3.4	0.36	1.72	0.33	4.06	0
	1	4.0	0.28	8.9	0.0	3.4	0.37	5.9	0.0	2.97	0.25	5.61	1.38
	10	23.01	3.8	66.5	0.0	19.7	1.57	25.8	13.6	14.8	0.98	19.8	12.29
Chronic Treatment	**0**	0.851	0.256	4.21	0.180	2.91	0.658	5.38	1.56	0.579	0.0762	1.22	0.180
	0.01	1.327	0.167	2.86	0.580	6.87	1.10	11.5	3.02	3.92	1.63	13.0	0.617
	0.1	0.994	0.053	196	56.4	2.69	0.759	171.5	43.3	0.535	0.186	644.2	64.0
	1	0.880	0.081	1.59	0.324	4.80	0.772	11.5	1.81	0.339	0.133	1.51	0.0734

Qualitative histological analysis of olfactory epithelia showed that nitrite treatments dramatically altered the cellular structure of the tissue (Figure 6-8). Control nose showed normal histology of the olfactory lamellae with a clear structural

organization of cell monolayer of the epithelia also known as the pseudostratified (Figure 6). Control also showed a continuous ciliated border (Figure 6A-B) and mucus cells (MC) in Alcian blue at the apical side of the lamella and wide interlamellar space (IL). At 0.3 mM and increase in cell size (hypertrophy) was observed in lamina propria and an increase of cell proliferation (hyperplasia) in connective tissues (stained by Alcian blue) (Figure 6C-D). At 1 mM and 10 mM lamellar congestion was evident and is characterized by hypertrophy and hyperplasia of the olfactory epithelia and lamina propia (Figure 6E-H). The increase in hypertrophy and hyperplasia in the lamina propia lead to a reduction of interlamellar (IL) surface area and fibrous mucus accumulation (M). Treatments 1 and 10 mM showed a dramatic loss of the cell monolayer of the epithelia.

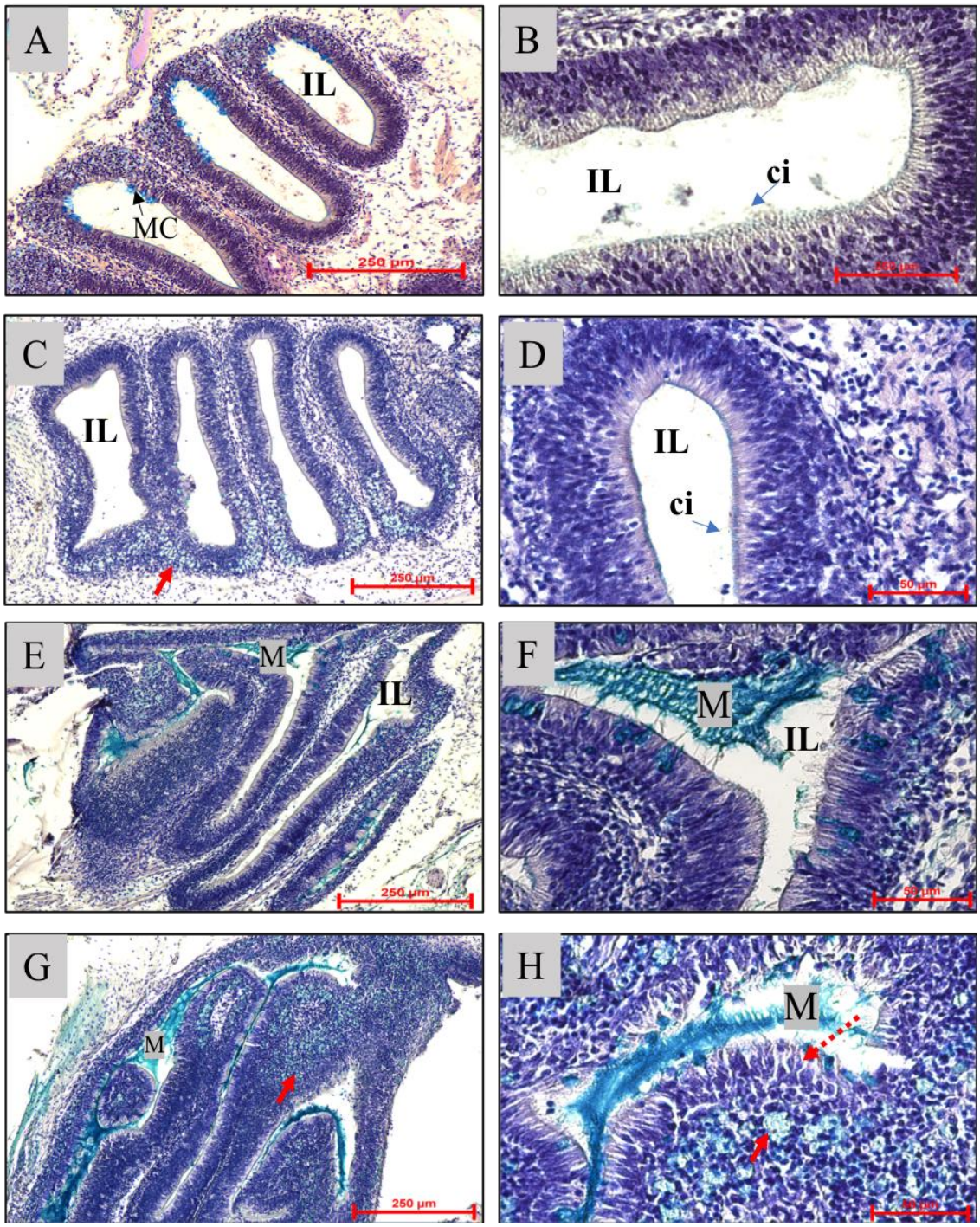

Figure 6. Goldfish nose morphology (Alcian blue PAS) after seven-day exposure to varying concentrations of nitrite: A) and B) control, C) and D) 0.3 mM, E) and F) 1 mM, G) and H) 10mM. *Normal olfactory lamellae histology in control include pseudostratified epithelia (note nuclei at several levels in the cell monolayer), a continuous ciliated border (ci), mucus cells in Alcian blue (MC), and wide interlamellar space (IL). All nitrite treated olfactory epithelia showed hypertrophy and hyperplasia (red arrow) in laminar propia and olfactory epithelia (stained by Alcian blue). At 1 mM and 10 mM a reduction of interlamellar (IL) surface area was observed due to a dramatic increase of hypertrophy and hyperplasia. Increase of mucus cells and fibrous mucus accumulation (M) was also observed in these treatments. Note the loss of integral structure in the olfactory sensory neurons (dashed arrow).*

Fish treated with 1 mM were analyzed for inflammatory response using TUNEL kit and methyl-blue counterstain. Samples were first examined using Alcian blue-PAS stain (Figure 7). Control samples showed clearly organized pseudostratified epithelium and a continuous layer of ciliated sensory neuron. Furthermore, the interlamellar (IL) space is clear of mucus and debris. Tissue samples from treatment 1 mM stained with DAB and methyl-blue counterstain revealed white blood cells infiltrated the olfactory epithelia at the somatic layer, were the nuclei of the sensory cells reside, and LP space (Figure 8).

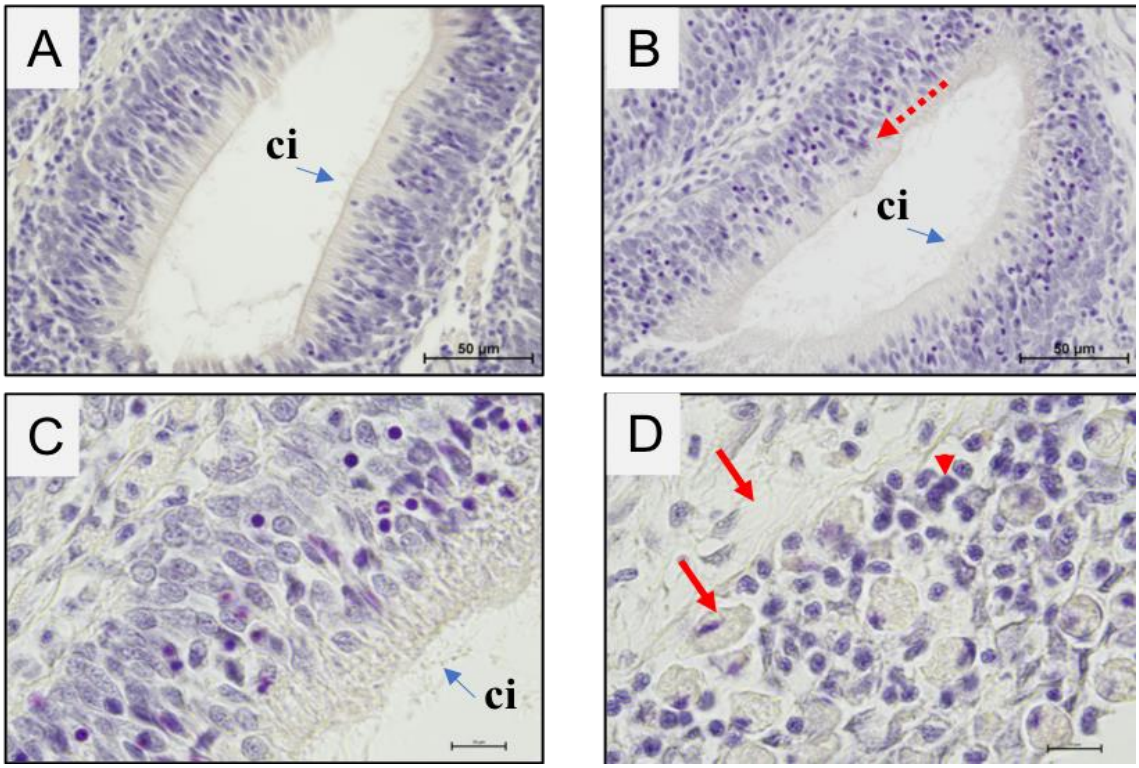

Figure 7. Goldfish nose morphology (Alcian blue-PAS) after seven-day exposure to nitrite: A and C control, B and D 1 mM. *Control nose showed similar histological morphology as in fig. 9. Cells in the epithelia of fish exposed to 1 mM nitrite displayed increase in size (hypertrophy) (red arrow), increase in number of cells (hyperplasia) (arrowhead), and a decline of pseudostratification (dashed arrow).*

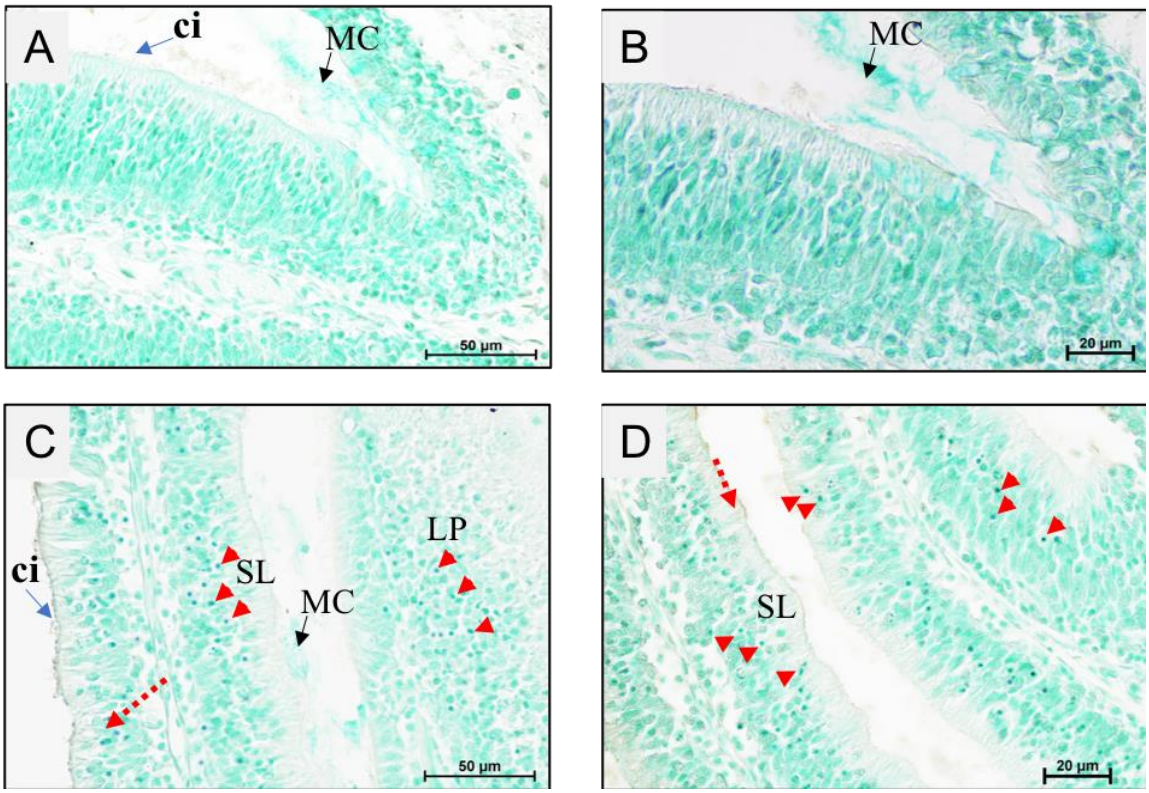

Figure 8. Leukocyte infiltration in goldfish olfactory lamella after seven-day exposure to nitrite A) and B) control, and C) and D) 1 mM*. Nitrite treated fish showed a decrease in pseudo stratification of cells (dashed arrow) and increase mucus production (MC). The nuclei of leucocytes (slightly more intensely stained) infiltrated the lamina propria (LP) and the somatic layer (SL) of epithelium.*

Gill tissue qualitatively analyzed revealed a hypoxic and irritative process in fish treated with nitrite (Figure 9). Control fish gill secondary lamella is characterized by the presence of pillar cells (pi, Figure 9) (Wilson & Laurent, 2002) and showed an interlamellar space fully covered by interstitial cells (Figure 9A-B). The interstitial cells including mucus and chloride cells. Red blood cells were present in the primary lamella of control gills but did not accumulated in the secondary lamella.

Gill tissue deterioration began at 0.3 mM and is characterized by a decrease in the interstitial cells number between the secondary lamellas described as half covered of the secondary lamellae (Figure 9C-D). The pillar cells, an hour-glass structural cell in the

secondary lamellas, in treatment 0.3 mM changed shape and become more oblong. Lastly, more red blood cells were observed in primary lamella and permeated the secondary lamella than in control. Decrease in secondary lamellas coverage and increase in red blood cells indicates a deprivation of oxygen supply.

At 1 mM secondary lamellas were fully covered by interstitial cells yet pillar cells were still oblong (Figure 9E-F). Epithelial cells between the secondary lamella distinctly grew in size, hypertrophy. Additionally, an increase of red blood cells filled the primary lamella and the secondary lamella, an indication of congestion and oxygen deprivation. Increase of red blood cells in the secondary lamella cause the secondary lamella to fold on itself known as clubbing.

At 10 mM the secondary lamellas were fully uncovered a characteristic of oxygen deprivation (Figure 9G-H). Like in previous treatments, pillar cells maintained were found to be oblong shape while others were clearly hypertrophic (pi blue arrow, Figure 9H). Some red blood cells were observed in the secondary lamella. However, red blood cells did not fully permeate the secondary lamellas as in 1 mM.

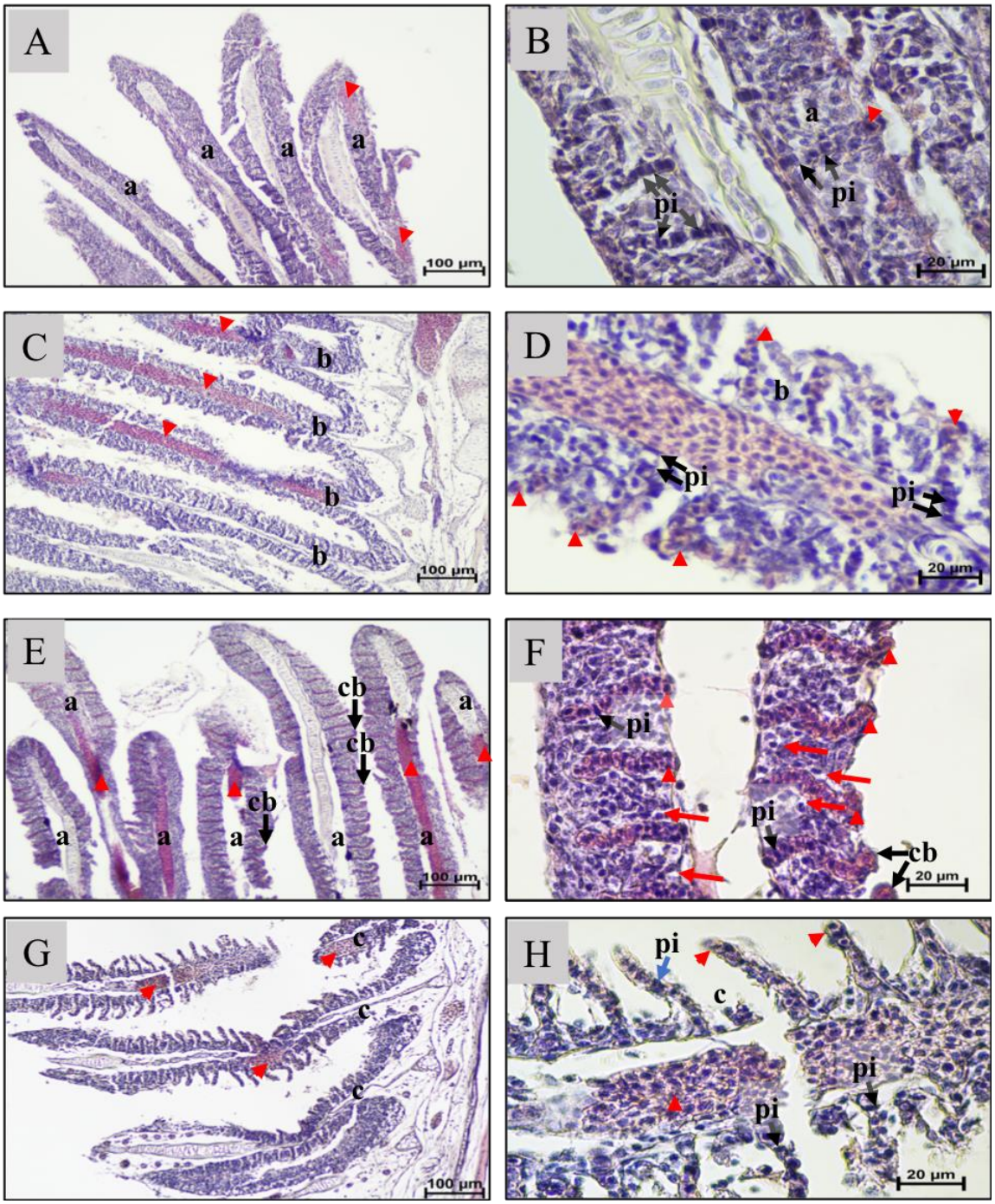

Figure 9. Goldfish gill morphology after seven-day exposure to varying concentrations of nitrite: A) and B) control, C) and D) 0.3 mM, E) and F) 1 mM, G) and H) 10 mM. *Control fish gill secondary lamella fully covered (a) by interstitial cells. Pillar cells (pi) present and hourglass shaped. Red blood cells (red arrowhead) are present in control gills and in primary lamella but not in the secondary lamella. At 3.0 mM secondary lamellas decreased and only half covered (b), pillar cells became oblong, and red blood cells permeated the secondary lamella. At 1 mM secondary lamellas were fully covered (a), red blood cells filled the primary lamella (red arrowhead) and causing clubbing (cb). Epithelial cells between the secondary lamella distinctly grew in size, hypertrophy (red arrow). At 10 mM the secondary lamellas were fully uncovered (c), red blood cells were observed in the secondary lamella (red arrowhead), and pillar cells were still oblong shaped while others were clearly hypertrophic (pi blue arrow).*

4.2. Chronic Nitrite Treatment

This central goal of this thesis was to determine how chronic exposure to environmental nitrite concentrations affects fish communication. Therefore, it is vital to first determine if nitrite physiologically affects and causes cellular alterations to the olfactory tissue. Therefore, to determine this I conducted a chronic experiment in which fish were exposed to nitrite concentrations that mimic common environmental concentrations. Environmental nitrite concentrations are typically found below lethal levels; they do not cause mortality. First, it was essential to establish if environmental nitrite concentrations will accumulate in vital tissues (olfactory tissue, gill, and brain). Second, to then verify if the accumulation causes structural changes to external tissues (gill), and lastly to compare results to acute experiment.

Mortality ranged between 0 and 3.7% and was not significantly different than those of control (Figure 4B).

Fish exposed to nitrite accumulated nitrite in all tissues (olfactory, gill, and brain) (Figure 10). Nitrite concentrations did not increase in parallel to the treatment concentrations. Instead, nitrite accumulation was in the lowest treatment (0.01 mM) and the highest treatment (1 mM). Two-way ANOVA found the interaction between treatment *vs* tissue to be statistically significant ($P < 0.05$) in treatment 0.01 mM and 1 mM, denoted by the numbers. Nitrite accumulated in treatment 0.1 mM however, tissue averages were not statistically different from control, denoted by number.

Nitrite accumulated in the olfactory tissue in all nitrite treatments (0.01 mM, 0.1 mM, and 1 mM) (Table 2). Nitrite accumulation began in treatment 0.01 mM and compared to control, concentration was significantly higher (denoted by numbers, Figure

10). At 0.1 mM, nitrite concentrations in olfactory tissue dropped and was not significantly different from control. The olfactory tissue in fish in treatment 1 mM had an increase in nitrite concentration and was significant however, were concentrations were lower than tissues in 0.01 mM. Gill tissues accumulated nitrite in all treatments however, mean concentrations in treatment was not significantly different from control treatment.

Nitrite accumulated in all tissues in treatments 0.01 mM and 1 mM, however tissue concentrations varied between tissues within a treatment (Figure 10). Post hoc ANOVA analysis found statistical significance between tissues concentrations within a treatment, denoted by letters ($P < 0.05$). All tissues accumulated nitrite in treatment 0.01 mM and nitrite concentrations were significantly different for all tissues. The olfactory tissue contained the highest concentration and the gill had the lowest. At 0.1 mM nitrite again accumulated in all tissues and all tissues were significantly different from each other. The olfactory tissue concentrations surpassed the gill and brain. However, this time gill tissue concentrations surpassed the brain and all tissues were significantly different from each other. At 1 mM the same pattern emerged as previous treatment (0.1 mM).

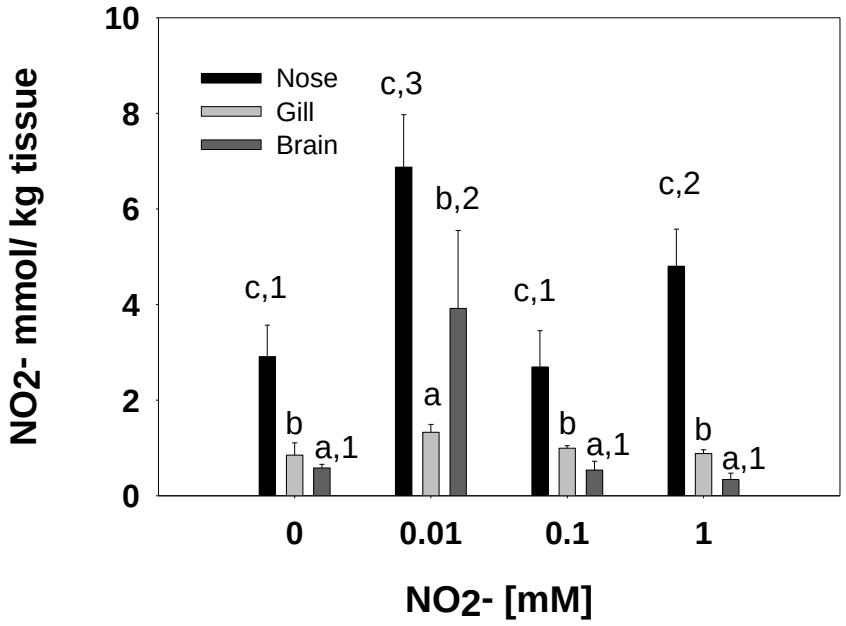

Figure 10. Nitrite content in nose, gill, and brain of goldfish chronically exposed to 0, 0.01, 0.1, and 1 mM NO_2^- for 69-days. *Two-way ANOVA showed that interaction between treatment and tissue was significant ($P < 0.05$). Numbers denote statistically significant differences among treatments in each tissue. Letters denote statistically significant differences among tissues in each treatment.*

Chronic nitrite treatments resulted in various adverse effects on the cell structure of primary and secondary lamellas of goldfish gills (Figure 11). Control fish were characterized by fully covered secondary lamellas; depicted as interlamellar surface fully cover by interstitial cells (Figure 11A-B). They also contained pillar cells but not blood present in the secondary lamella. At 0.01 mM secondary lamella covering decreased to fully uncovered and many interstitial cells between the secondary lamellas were dramatically reduced indicating necrosis (Figure 11C-D). Additionally, pillar cells maintained their shape however, there is an increase in cell size, hypotrophy.

At 0.1 mM, the primary lamella increased in covering to half covered compared to 0.01 mM, pillar cell shape changes to oblong or circular, and hypotrophy (Figure 11E-F). Furthermore, red blood cells in the secondary lamella are apparent and a reduction of

epithelial cells between secondary lamella. Thus, indicating congestion and decrease in oxygen availability in tissue.

At 1 mM secondary lamellas were half covered and hypotrophy of pillar cells were observed. Red blood cell infiltrated the secondary lamellas causing some clubbing. Additionally, there is an expansion of marginal blood channel (ma), were a majority of red blood cells pass through for gas exchange. Cumulatively, indicating vascular congestion and oxygen deprivation in fish (Figure 11G-H).

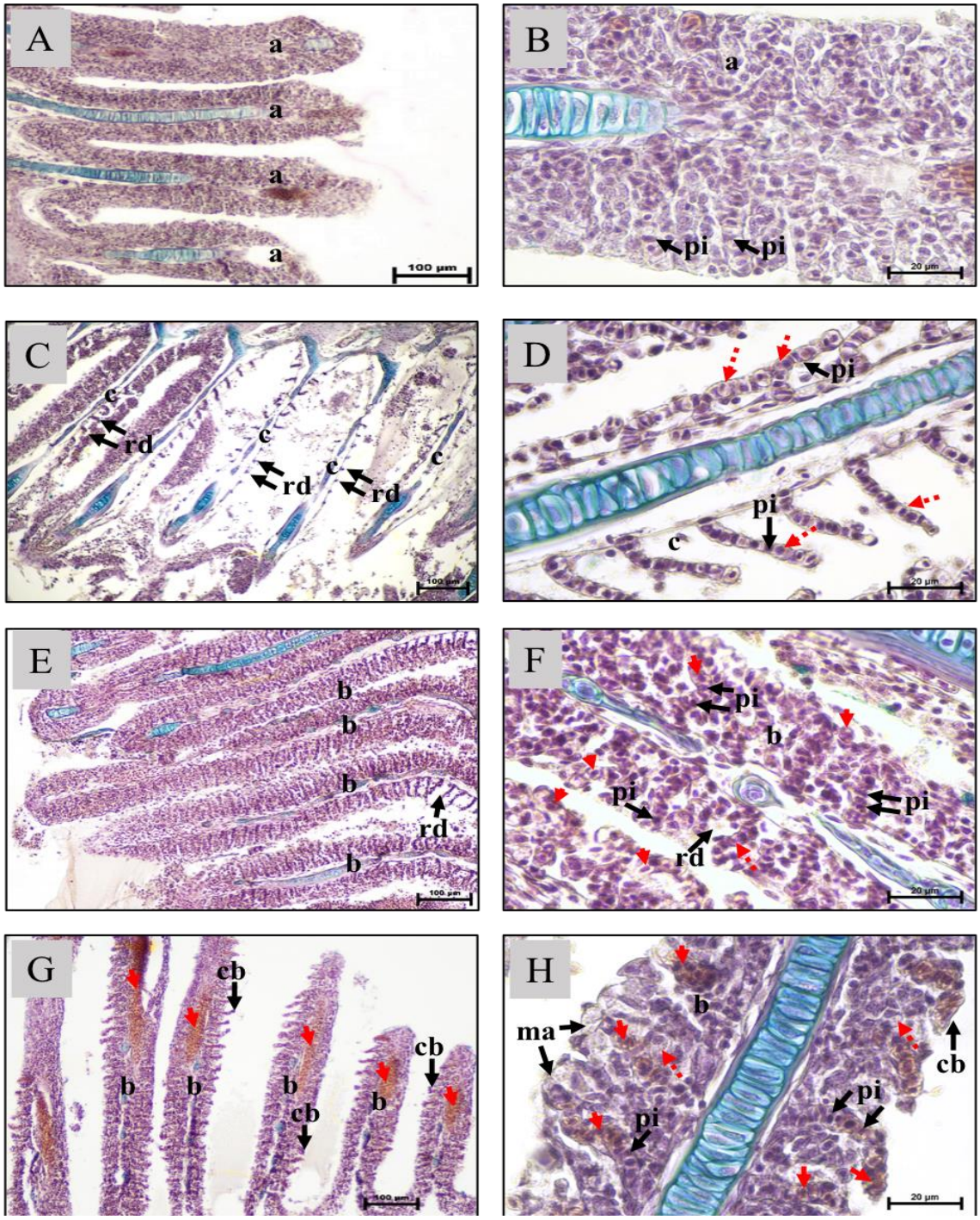

Figure 11. Goldfish gill morphology after 69-day exposure to varying concentrations of nitrite: A) and B) control, C) and D) 0.01 mM, E) and F) 0.1 mM, G) and H) 1 mM. *Control fish gills were fully covered (a), pillar (pi) cells were present, and no blood in secondary lamella was present. At 0.01 mM secondary lamella covering decreased to fully uncovered (c), interstitial cells between the secondary lamellas were dramatically reduced (rd) indicating necrosis. At 0.1 mM, the primary lamella increased in covering to half covered (b), pillar cell shape changes to oblong or circular, hypotrophy (dashed arrow), and red blood cells in the secondary lamella are apparent (arrowhead). At 1 mM secondary lamellas were half covered, hypotrophy of pillar cells, red blood cell infiltrated, and expansion of marginal blood channel (ma).*

These histological markers of irritation such as clubbing, hypertrophy, and reduction of secondary lamella covering were measure (Table 3). One-way ANOVA found significant differences ($P < 0.05$) these in percent clubbing, percent covered, and percent hypertrophy between treatments, denoted by the letters (Figure 12). Percent clubbing of the secondary lamella of gill significantly ($P < 0.05$) increased in all nitrite treatments. Percent coverage of the secondary lamella decreased in all nitrite treated fish, however, was only significantly different ($P < 0.05$) in treatment 1 mM. Lastly, percent hypotrophy increased in all treatments and all treatments were significant different from control ($P < 0.05$).

Table 3. Chronic gill histopathology. *Gill abnormalities measure for quantification. Letters denote significant differences between treatments (one-way ANOVA, P<0.05).*

Treatment [mM]	Total cell count Mean ± SE	Hypertrophy present Mean ± SE	% Hypertrophy Mean ± SE	% Clubbing Mean ± SE	% 2_{ry} Lamella covered Mean ± SE
Control (0)	103.8 ± 3.5	2.0 ± 2.0	7.0 ± 3.6$_{a}$	7.1 ± 1.0$_{a}$	83.9 ± 2.6$_{a,c}$
0.01	108.3 ± 4.7	83.2 ± 5.8	89.8 ± 8.2$_{b}$	13.2 ± 3.5$_{b,c}$	50.8 ± 5.2$_{a,c}$
0.1	88.1 ± 4.8	95 ± 3.2	59.4 ± 5.8$_{c}$	9.2 ± 1.9$_{c}$	46.0 ± 2.3$_{c}$
1	87.7 ± 7.6	97 ± 1.5	78.0 ± 3.8$_{b,c}$	45.9 ± 8.8$_{d}$	67.4 ± 2.2$_{b}$

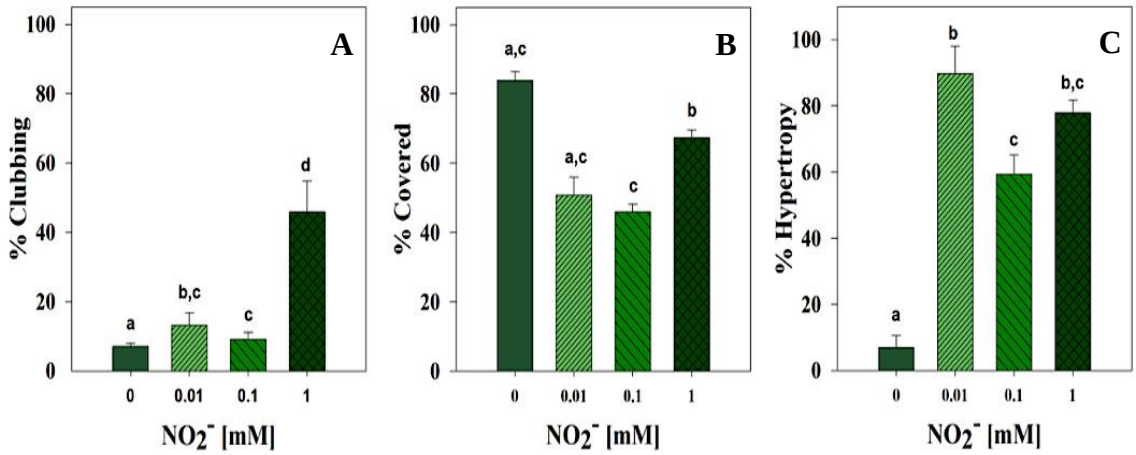

Figure 12. Goldfish gill abnormalities measured in chronic nitrite treatments. *(**A**) Quantitative analysis of gills show increase in clubbing as nitrite concentration increased. (**B**) Secondary lamella coverage decreased as in nitrite exposed fish. (**C**) Cell size increased in nitrite-exposed fish (hypertrophy). Letters denote significant differences between treatments (one-way ANOVA, P<0.05).*

4.3. Behavior

This thesis set out to assess nitrites effects on fish communication. In the acute and chronic experiments, we established that nitrite will accumulate in the olfactory tissue. Additionally, acute results show that accumulation will alter structural organization of the olfactory tissue. The chronic experiment demonstrated that some tissues may not accumulate nitrite however, tissue can still be structurally altered (gill). The goal of the behavior experiment was to determine if nitrite at concentrations that are known to accumulate and structural changes the olfactory tissue cause impairment to odor detection. To do this, first were exposed to the same nitrite concentrations from acute and chronic experiments. Then weekly behavioral trials were conducted to evaluate if odor detection weakened after nitrite exposure.

High index preference denotes fish that spent more time in experimental channel with food odor. Control fish showed a higher index preference to food odor than nitrite treated fish. The high index preference in control fish was found to be significantly

(Wilcoxon-signed rank test $P < 0.05$) indicating fish preferred to spend time in food odor channel than control channel (star, Figure 13). Goldfish exposed to non-lethal concentrations of nitrite (0.01, 0.3, and 1.0 mM) showed a decrease in index preference. Therefore, nitrite treated fish spend less time in the experimental channel with food odor. Nitrite treated fish showed a systematic decreased in index preference as treatment concentration increased: 37%, 43%, and 73% attraction index reduction in 0.01, 0.3 and 1 mM, respectively. Thus, fish exposed to the highest nitrite concentrations experienced the greatest decrease in index preference. Fish in all treatments were observed during behavior trials, and fish from all treatments displayed feeding behavior including surface water nibbling, searching, and nibbling at the bottom of the two-choice maze. Fish in treatment 1.0 mM exhibited similar feeding behavior during trials; however, fish also were less mobile in treatment 1.0 mM compared to control.

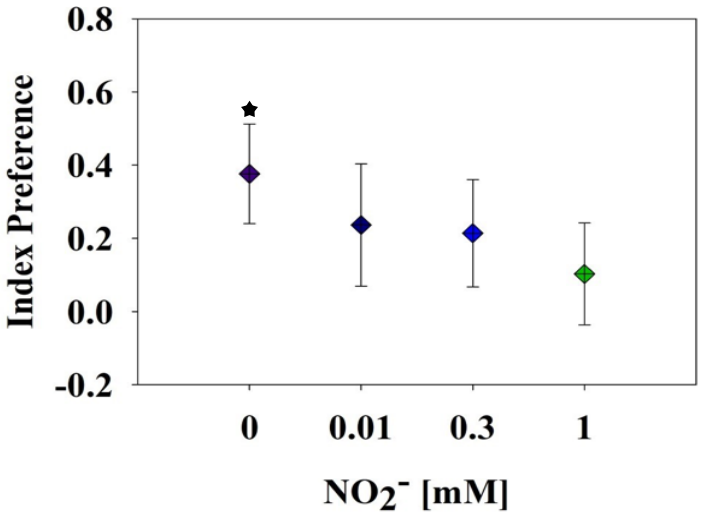

Figure 13. Two-choice maze behavioral responses of nitrite-exposed goldfish to food. *Goldfish were exposed to nitrite treatment for four weeks and tested weekly for responses to food odor. Index preference=[ae/(ae+be)-ac/(ac+bc)], bc is time spend in control channel before odor release, be is time spent in experimental channel before odor release, ac is time spent in control channel after odor released, and ae is time spend in experimental channel after odor release. A positive index indicates an attraction or preference for odor. A decrease in index preference for food odor was observed in nitrite-treated goldfish. Control fish index preference was significant ($P < 0.05$).*

5. DISCUSSION

5.1. Discussion

Aquatic organisms are constantly exposed to environmental toxins such as heavy metals, pesticides, and nitrite (Bristow et al., 2015; Garnier et al., 2007; Kalvelage et al., 2013; P. Lam et al., 2011; Phyllis Lam et al., 2009; Pottinger, 2017). A great deal of research has found that at acute exposure to nitrite concentrations can disrupt various physiological systems and diminish survival of aquatic organisms (Bath & Eddy, 1980; Margiocco *et al.*, 1983; Lewis & Morris, 1986; Eddy *et al.*, 1994; G. Scott & Crunkilton, 2000; Huertas *et al.*, 2002; Jensen, 2003; Gisbert *et al.*, 2004; Kroupova *et al.*, 2005; Wuertz *et al.*, 2013; Xiao *et al.*, 2017; Kocour *et al.*, 2018). Much of the research conducted on nitrite focuses on acute effects and lethality levels on various aquatic species (Tomasso, 1986; Huertas *et al.*, 2002; Gisbert *et al.*, 2004; Wuertz *et al.*, 2013; Patrik Saoud *et al.*, 2014; Xiao *et al.*, 2017). Acute toxicity tests are vital and are used to understand nitrites' impact on growth and survival of aquatic organisms, but neglect to inform about the impacts of chronic exposure to environmental nitrite. Environmental nitrite concentrations are lower than those found in acute toxicity tests, and do not cause mortality. Furthermore, aquatic organisms exposed to chronic concentrations of nitrite do not exhibit physiological impairments associated with those found in nitrite acute exposure. However, this does not indicate that chronic environmental nitrite concentrations do not harm or cause long term physiological effects on aquatic organisms. Thus, it is vital to determine if chronic exposure to environmental nitrite concentrations impair vital tissues.

For instance, constant exposure to low concentrations of nitrite toxins can damage

sensitive external sensory structures, such as olfactory epithelia, that are continuously exposed to the aquatic environment. Olfaction is a multi-network system: it integrates several steps such as sensory perception in the olfactory epithelium, central process in the brain, and concomitant physiological and behavioral responses, such as hormone production and feeding. A reduction of sensory perception changes a fishinteraction with environment and reduces long-term survival of species. Therefore, chronic nitrite exposure could impair a fish sensory perception, consequently altering the multi-network system and cause a cascade effect. Hence, histopathology, physiology and behavior modulation can be utilized as a link between toxin presence and physiological disruption. One sign of physiological disruption due to nitrite exposure is nitrite accumulation in tissues. Nitrite accumulation in tissues has been previously observed in various fishes such as rainbow trout (*Oncorhynchus mykiss*) (Bath & Eddy, 1980; Margiocco *et al.*, 1983; Eddy *et al.*, 1994; Kroupova *et al.*, 2008), Siberian sturgeon (Huertas *et al.*, 2002; Gisbert *et al.*, 2004), largemouth bass (*Micropterus salmoides*) (Tomasso, 1986; Jensen, 2003), striped bass (*Morone saxatilis*) (Jensen, 2003), pike-perch (*Sander lucioperca*) (Wuertz *et al.*, 2013), and goldfish (*Carassius auratus*) (Tomasso, 1986; Jensen & Hansen, 2011). Based on previous research, nitrite accumulation first occurs in plasma (Bath & Eddy, 1980; Eddy *et al.*, 1994; Kroupova *et al.*, 2008; Jensen & Hansen, 2011). Furthermore, plasma nitrite concentrations were often found to be up to 10-times higher than environmental concentrations. For instance, rainbow trout exposed to 0.2 mM nitrite for 24 hours were found to have elevated nitrite concentration in plasma (1– 3 mM), a 10-fold increase and well above environmental concentrations (Eddy *et al.*, 1994). A second study attained similar results: rainbow trout exposed to 7 mM nitrite for 24 hours were

found to have blood plasma nitrite concentrations 5- to10-times greater than environmental concentrations (Bath & Eddy, 1980). Goldfish exhibited a similar physiological response, having a 10-fold increase of nitrite concentration in blood plasma also well above ambient concentrations. Increase of nitrite concentrations in plasma leads to elevated nitrite concentrations in vital tissues. However, concentrations of nitrite in internal tissues typically do not reach environmental nor plasma concentrations. However, external tissues such gills have been found to have higher concentrations than plasma. For instance, rainbow trout exposed to 0.032 mM nitrite for 12 hours were found to have accumulated nitrite in gill (3.29 ± 0.7 µg/g), liver (1.97 ± 0.3 µg/g), brain (2.27 ± 0.6 µg/g), and muscle (1.12 ± 0.18 µg/g) (Margiocco *et al.*, 1983). In the same study, rainbow trout exposed to the same concentration for 24 hours were found to have accumulated nitrite in gill tissues (10.68 ± 0.8 µg/g), liver (7.13 ± 1.5 µg/g), brain (9.25 ± 1.7 µg/g), and muscle (0.8 ± 0.12 µg/g) and blood plasma (7.88 ± 1.2 µg/g) (Margiocco *et al.*, 1983). Thus, in this study, gill tissues (10.68 ± 0.8 µg/g) and brain (9.25 ± 1.7 µg/g) were found to have a higher concentration than plasma (7.88 ± 1.2 µg/g) (Margiocco *et al.*, 1983). The results of the study illustrated that gills can accumulate more nitrite than any other tissue. Gills are external tissues that are continuously exposed to the environment and play an important role in transport of ions and nitrogen balance (Hwang *et al.*, 2011). Thus, higher accumulation of nitrite in gills can be a consequence of direct exposure to the pollutant and a specific set of cellular transporters otherwise used for osmoregulation (Jensen & Hansen, 2011). Likewise, the olfactory epithelium, an external tissue, can be site of pollutant accumulation. Likewise, the olfactory epithelium, an external tissue, can be site of pollutant accumulation.

Thus, I hypothesized that fish acutely exposed to nitrite would accumulate nitrite in tissues, and this accumulation will be more dramatic in an external tissue. Our experiments showed that nitrite accumulates in nose, gill, and brain tissues as ambient nitrite concentrations increased. However, when comparing the two external tissues, the rate of nitrite accumulation within treatments was higher in nose than gill (i.e. nose accumulate one order of magnitude more nitrite than gill when comparing values between control and the highest treatment) , suggesting a mechanism of detoxification of nitrite in gill (Jensen, 2003) that may be absent in the nose, or be less efficient. Moreover, the fact that treatment with 1.0 mM and 10 mM nitrite yielded nitrite concentrations in gill tissues that were several orders of magnitude higher than the concentration in the environment was indicative of an uptake mechanism. This mechanism has been described in gill. Nitrite is actively pumped inside the gill using the Cl-/ HCO-3 transporter (Bath & Eddy, 1980; Margiocco *et al.*, 1983; Eddy *et al.*, 1994; Huertas *et al.*, 2002; Jensen, 2003; Gisbert *et al.*, 2004). A similar uptake mechanism may also exist in nose, although it has yet to be described. Our findings demonstrate that external tissues actively take up nitrite, since passive uptake would lead to a lower tissue concentration closer to the concentrations found in the environment.

Acute exposure to nitrites revealed that gill tissue accumulated the highest concentrations of the toxin at 10 mM. This is likely due to gills' constant exposure to environmental conditions and its physiological role as a site of nitrogenous excretion and gas exchange. Gill accumulated nitrite at treatment concentration as low as 1.0 mM which is a subacute concentration, and it has been commonly found in aquatic environments (Avnimelech *et al.*, 1986; Kamstra *et al.*, 1996). Based on the findings, 1.0

mM nitrite seems to be the lower limit at which accumulation overcomes detoxification of the compound, and this limit seems to be applicable to the nose, too. Nitrite accumulation in brain was lower than external tissues. This could be due to (1) the brain having a mechanism to convert nitrite to NO, a neurotransmitter (Esplugues, 2002) or (2) the toxin inability to cross the blood brain barrier.

The nitrite accumulation in goldfish gill and brain was consistent with those found in other experiments in other fish species (Jensen & Hansen, 2011). In those works, nitrite accumulation also varied among tissues, and tissue concentrations increased as ambient treatment concentrations increased. For instance, goldfish acutely exposed to 1 mM nitrite for 24 hours accumulated nitrite in plasma above environmental concentrations and in white skeletal muscle, but lower than plasma concentrations (Jensen & Hansen, 2011). Similar results were observed in Siberian sturgeon yearlings exposed to 9.3 mM nitrite for 18 hr. Sturgeons accumulated nitrite in gill, liver, muscle, and plasma (12.9 ±1.5, 8.8 ±2.5, and 7.2 ±0.1 mM, respectively) (Gisbert *et al.*, 2004). These findings parallel that of Siberian sturgeon yearlings exposed to 0.5, 2.8, 3.9, and 6.0 mM in which nitrite concentrated in plasma, muscle, liver, and gill.

Rate of accumulation of nitrite in tissues can vary among species (Tomasso, 1986; Eddy *et al.*, 1994; Jensen, 2003). Toxicity tests show that some species, like rainbow trout (Kroupova *et al.*, 2008) and channel catfish (Tomasso, 1986), are more efficient in the uptake of nitrite than other species like largemouth bass (Tomasso, 1986) and striped bass (Jensen & Hansen, 2011) that have unusually lower nitrite uptake. Fish that take up more nitrite are considered sensitive, and fish that have low nitrite uptake are considered resistant (Eddy *et al.*, 1994; Jensen, 2003). Fish that are sensitive to nitrite like channel

catfish (Tomasso, 1986) and goldfish (Jensen & Hansen, 2011) accumulate more nitrite in plasma versus resistant fish like Centrarchids (Tomasso, 1986) that have lower plasma nitrite concentrations. Therefore, sensitive fish accumulate higher concentrations of nitrite in tissues than resistant fish.

In the acute experiment we found nitrite accumulation in external tissues like gill and nose. Therefore, we predicted fish under chronic nitrite exposure would accumulate nitrite in external tissues as well. Indeed, chronic nitrite exposure also showed an increase of nitrite in tissues. Nitrite concentrations in nose were higher in chronic that those in acute experiment. This is because this time, the extractions were performed only in the lamella and not the full nasal cavity. Thus, olfactory lamella accumulates more nitrite than the surrounding epithelia in the nasal cavity. In the chronic experiment, tissue concentrations did not increase as ambient concentrations increased, but follow an intriguing pattern. It was found that the higher nitrite concentrations in olfactory epithelium and brain are at the lowest treatment concentration (0.01 mM). A possible explanation could be that nitrite at 0.01 mM is too low to activate mechanisms of detoxification in nose and brain. This is further supported by the results from treatment with 0.1 mM nitrite where nitrite concentrations in olfactory epithelia and brain dramatically decreased. This may indicate that tissues undergo a physiological shift, protecting tissues from accumulation at 0.1 mM nitrite concentrations but not at 0.01 mM nitrite. It is known that nitrite can be found in the aquatic environment at 0.01 mM and 0.1 mM concentrations. Thus, although 0.01 and 0.1 mM nitrite concentrations are well below lethal levels, they can be responsible for a build up of nitrite in internal tissues that could be causing a long-term physiological disruption in fish.

Very few chronic experiments have been conducted on nitrite, and none of them measure nitrite concentrations in external tissues. However, nitrite accumulation has been observed in internal organs in few chronic experiments. Juvenile pike-perch chronically exposed to nitrite (0.01, 0.02, 0.04, and 0.08 mM) for 32 days accumulated nitrite in blood plasma and muscle tissue (Wuertz *et al.*, 2013). Nitrite concentrations in plasma were higher than muscle tissue, and nitrite plasma concentration elevated dramatically (7.6 ± 6.3 µg/mL) in treatment with 0.08 mM nitrite (Wuertz *et al.*, 2013). Nitrite concentrations in muscle tissue did not increase as dramatically from 0.02 ± 0.01 µg/g in the control to 0.67 ± 0.05 µg/g in the lowest treatment (0.01 mM) and only increased slightly 0.67 ± 0.05 µg/g in the highest treatment (0.08 mM) (Wuertz *et al.*, 2013). These results resemble those from Kropupova (2008) in which rainbow trout were exposed to 0.02 mM and 0.06 mM nitrite for 32 days. Rainbow trout accumulated nitrite in plasma, and concentrations increased as environmental concentrations increased. Additionally, muscle tissue accumulated nitrite, but concentrations were below that of plasma. Thus, fish chronically expose to nitrite accumulate the toxin in internal tissues parallel to increase ambient treatment concentration. However, our results demonstrate that not only internal, but external tissues, are highly susceptible to environmental nitrite. Moreover, the mechanism of accumulation and detoxification may be different under different environmental concentrations.

Accumulation of nitrite in tissues is deleterious to the integrity of the cell and tissues. Previous studies show that, when fish gills accumulate nitrite, structural changes occur within the tissue such as hypertrophy, hyperplasia, clubbing of the lamellae, and necrosis (Gisbert *et al.*, 2004; Kroupova *et al.*, 2005; Patrik Saoud *et al.*, 2014). In our

experiments we found that both acute and chronic exposure induced histological changes. Fish acutely exposed to nitrite had dramatic changes in external organs, the gill and olfactory epithelia. In the olfactory epithelia acutely exposed to 1.0 and 10 mM nitrite, the interlamellar (IL) surface area was dramatically reduced and filled with mucus, restricting water passage from the environment into the olfactory epithelium; hence, it reduces the sensory neurons ability to detect environmental stimuli. Additionally, the olfactory epithelium in fish exposed to 1.0 and 10 mM lost its pseudostratified structure; the arrangement of sensory neurons was no longer organized in a single cell layer. A loss of organization in the pseudo stratification denotes deterioration of the olfactory epithelium. These findings suggest further impairment of the olfactory epithelium and its ability to detect environmental stimuli.

Parallel to the loss of cell structure in the olfactory epithelium, an inflammatory process was observed. This process was characterized by an infiltration of leucocytes (white blood cells) in the lamina propria olfactory epithelia. Thus, the results suggest an immune response occurs when fish are acutely exposed to 1.0 mM. Therefore, inflammation can also be considered as a symptom of nitrite exposure which can further impede detection of environmental stimuli.

The inflammatory process has been observed in fish with damaged olfactory processes in experiments in which the olfactory nerve axons was severed (axotomy). Goldfish were axotomized and samples were periodically taken. Three days after axotomy the olfactory epithelium was severely degraded, the pseudo stratification structure was compromised, and leucocyte infiltration was also observed (Hansen *et al.*, 1999). Thirteen days after axotomy, an increase in mucus cell production was observed

in fish (Hansen *et al.*, 1999). These results are in line with the results of our experiment, indicating that nitrite exposure is as destructive to the olfactory epithelium as an axotomy. The olfactory epithelium is vital for odor detection in fish (Hara, 1986, 1994; Døving, 2010). When environment stimuli are detected by the olfactory epithelium, the signal is relayed to various parts of the brain which integrates the information in order to respond with a physiological and/or behavioral change (Hara, 1986, 1994; Døving, 2010). Thus, a disruption of the olfactory transduction signal will affect integration of information in brain.

Structure dictates function; therefore, we hypothesized that goldfish under chronic exposed of nitrite concentrations would experience a structural change in the olfactory epithelium that would impair odor detection from environmental cues. Consequently, the damage to the olfactory epithelium would alter the behavior in fish to environmental stimuli. Goldfish exposed to 0, 0.01, 0.3, and 1.0 mM nitrite showed a significant drop in odor detection and associated behavior compared to control. Thus, nitrite alters fish behavioral response to environmental stimuli (Tierney *et al.*, 2010).

Disruptive effects of toxicants on olfaction and its subsequent physiological changes have previously been recorded. In a thorough review of the effects of contaminants on olfaction found that toxins such as pesticides, heavy metals, and acid disrupt responsiveness to odorants (Tierney *et al.*, 2010). Eelectro-olfactogram (EOG) experiments in which fish (*Oncorhynchus kisutch*) were exposed to copper (5 µg/L for 30 min) found a reduction in odorant responsiveness up to 75% (Tierney *et al.*, 2010). EOG performed on *Salmo salar* exposed to atrazine (1µg/L for 30 min) revealed a 100% decrease in responsiveness to odorants, and similar results were found in other species.

Chum salmon (*Oncorhynchus keta*) exposed to copper (24 and 58 µg/L) for 4 hours showed a decrease in membrane function (Tierney *et al.*, 2010). Similar histological changes were observed in rainbow trout exposed to 20 µg/L copper for 15 days. Tierney et al. (2010) found apoptotic responses in olfactory sensory neurons. Altered cell growth and death have been observed in rainbow trout chronically exposed to copper. For example, fish exposed to 20 and 40 µg/L copper for 40 weeks showed an increase in goblet cells (mucus cells) and degenerated cells in the olfactory epithelium (Tierney *et al.*, 2010).

Behavioral responses to contaminant have been previously reported. Fish have been found to avoid many metals, specifically arsenic, cadmium, chromium, cobalt, copper, iron, mercury, and nickel to name a few (Tierney *et al.*, 2010). For example, it has been well established the olfactory sensory neurons Atlantic salmon use for priming (i.e. milt and hormone production) are impaired after being exposed to pollutants (Tierney *et al.*, 2010). Interestingly, when fish are exposed to pesticides, EOG experiments showed a reduction in odorant response and a decrease in plasma testosterone 11-ketotestosterone (an androgenic hormone in teleosts), 17,2ßP (hormone that increases gonadotropin II), and expressible milt (Tierney *et al.*, 2010).

Additionally, observable behavioral changes in rainbow trout response to alarm cues, a response associated with stress and stress hormones, were altered when trout were exposed to cadmium. Unexposed fish responded to the alarm cue with freezing or avoidance and a four-fold increase in plasma cortisol (Tierney *et al.*, 2010). However, fish exposed to 2 µg/L cadmium had a 2-fold increase in plasma cortisol and an impaired alarm response. The alarm cue is released by fish when the fish interacts with a predator

and is thus vital for survival (G. R. Scott & Sloman, 2004). Many experiments on trace metals have revealed an increased susceptibility to predation (G. R. Scott & Sloman, 2004).

Similarly, to heavy metals and pesticides, chronic exposure to nitrite causes goldfish to experience a change in behavioral response to odorant. Food odor preference decreased as nitrite concentrations increased. Thus, nitrite exposure may decrease survival rate of fish chronically exposed to nitrite. Additionally, nitrite may impair odor preference or perception of other cues such as alarm and hormones and therefore alter behavioral responses. Thus, nitrite is potentially as damaging to the olfactory epithelium as heavy metals and pesticides with the aggravation that nitrite is more commonly found in our aquatic environments.

5.2. Future Research

In this study nitrite concentrations were measure in the olfactory tissue. The results indicate a possible detoxification mechanism, a nitrite removal process. Future studies could explore this possibility by exposing fish to low concentrations of nitrite and allow fish to recover after a certain time point. Experiments such as these have been conducted on gill tissue (Gisbert *et al.*, 2004).

This is the first record of environmental nitrite concentrations accumulating in the olfactory tissue and it is prudent to understand this mechanism. Therefore, future studies should consider examining accumulation rate of the olfactory tissue. This can be conducted by measuring nitrite accumulation in olfactory tissue at various time points. Additionally, the accumulation rate could be compared to other tissues like gill to

determine if nitrite impacts external tissues similarly. Furthermore, future studies should determine if accumulation rates vary between acute and chronic exposure.

This study histologically examined the olfactory tissue structure and I was able to determine that nitrite can structurally alter this tissue. Furthermore, nitrite was found to have an inflammatory response in the form of leucocyte infiltration in the olfactory tissue. Further histological examination is required to determine other possible immune responses such as apoptosis. Additionally, histology could be used to reveal the sensory neuron(s) that are interacting with nitrite. Using florescent tagging, researchers could tag specific odors (amino acids) and stimulate nitrite treated fish. The olfactory system in fish is highly specified and specific sensory neurons interact with specific odors. Thus, revealing which sensory neurons are impacted by nitrite.

Electro-olfactogram (EOG) have long been utilized in olfaction research in fish. It measures the electrical response in the olfactory tissue when stimulated by specific odors and cues (Hara, 2012). If nitrite is interacting with sensory neurons EOGs could be used to determine how the electrical activity is being disrupted by nitrite.

If nitrite is disrupting the electrical activity in the olfactory tissue, fish behavior will be altered. The behavior experiment in this study demonstrated how nitrite alters fish behavior to preferred odors such as food, however, olfaction is also utilized for reproduction. This requires of the integration of the endocrine system (Hara, 1986; Kobayashi *et al.*, 1986; DeFraipont & Sorensen, 1993; Hara, 1994; Kobayashi *et al.*, 2002; Sorensen *et al.*, 2005; Hara & Zielinski, 2006; Stacey & Sorensen, 2009; Døving, 2010; Hara, 2012; Ghosal & Sorensen, 2016). Fish use sex steroids as pheromones to coordinate reproduction and elicit courtship and spawning behavior. Females goldfish

release pre-ovulatory pheromones that are then detected by males. Male goldfish then release pheromones that stimulate females and ultimately leads to oocyte maturation (Hara, 1986; Kobayashi *et al.*, 1986; DeFraipont & Sorensen, 1993; Kobayashi *et al.*, 2002; Sorensen *et al.*, 2005; Hara & Zielinski, 2006; Stacey & Sorensen, 2009; Døving, 2010; Hara, 2012; Ghosal & Sorensen, 2016). Future studies should consider nitrites effects on reproductive behaviors and sex steroid production.

5.3. Conclusions

The present research aimed to examine the effects of nitrite on goldfish olfactory epithelium and odor detection. This study has demonstrated that nitrite accumulates in the olfactory epithelium and other tissues of goldfish, and its concentrations increase as ambient concentrations increase. In addition, the olfactory epithelium was found to have higher concentrations than gill in acute exposures and higher concentrations than any tissue in chronic exposures. Furthermore, the results demonstrate that the olfactory epithelium undergoes structural changes. These structural changes were linked to a loss of olfaction manifested in behavioral experiments. Thus, nitrite is acting as a neuro disruptor at acute and environmental concentrations. This study provides a deeper understanding of nitrite, its physiological effects on fish, and its effects on the olfactory system in fish. Furthermore, the study demonstrates the importance of experiments based on chronic exposure at concentrations found in the environment and the potential impacts of chronic exposure on the olfactory system. This study provided insight on fish olfaction, its sensitivity to nitrite at environmental concentrations and thus will be useful to aquaculture facilities and legislators that make guidelines for aquatic ecosystems

management.

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