CERTIFICATE

This is to certify that the thesis entitled “Acute Ammonia Toxicity and Chloride Inhibition of Nitrite Uptake in Non-Teleost Actinopterygiian Fishes” submitted for the award of Master of Science to the Nicholls State University is a record of authentic, original research conducted by Mr. Perry J. Boudreaux under our supervision and guidance and that no part of this thesis has been submitted for the award of any other degree, diploma, fellowship, or other similar titles.

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Abstract

Ammonia and nitrite toxicity varies among fish species. Ammonia exists in an ionized (NH^+_4) and un-ionized (NH_3) form and the proportion of total ammonia-N that is in the un-ionized form increases with temperature and pH. Un-ionized ammonia is generally associated with ammonia toxicity because it freely diffuses across gill membranes into plasma. The mechanism of ammonia toxicity is not well understood, but is probably associated with the central nervous system. For fish that transport environmental chloride with a gill uptake mechanism (chloride cell), environmental nitrite can be transported into plasma through the gill uptake mechanism. Thus, plasma-N concentrations can reach levels greater than environmental levels and have detrimental effects on the fish. The mechanism of nitrite toxicity is the oxidation of the hemoglobin iron molecule, which converts hemoglobin to methemoglobin faster than it can be enzymatically reduced back to hemoglobin. Methemoglobin can not reversibly bind with oxygen molecules and fish that are affected by nitrite toxicity suffer from hypoxia.

Because of the relationship between nitrite uptake and the gill chloride uptake mechanism, nitrite uptake can provide insight regarding the method of chloride uptake for fish. It is believed that the gill chloride uptake mechanism is a teleost ancestral characteristic. However, with the exception of shortnose sturgeon, nitrite uptake has only been determined for teleost. Because there is a lack of published studies on ammonia and nitrite toxicity studies for non-teleost fish, this study was designed to measure acute ammonia toxicity, to determine if non-teleost concentrate nitrite in their plasma, and to determine if chloride inhibits nitrite uptake for non-teleost fish.

The 96 hour LC50 for total ammonia-N for spotted gar *Lepisosteus oculatus*, alligator gar *Atractosteus spatula*, and paddlefish *Polyodon spathula* was determined in 37 L aerated glass aquaria, each containing one fish. Spotted gar and alligator gar were exposed to total ammonia-N (TAN) concentrations of 0, 50, 100, 200, or 400 mg/L for 96 hrs. Paddlefish were exposed to 0, 6, 12, 24, or 48 mg/L TAN for 96 hrs. The 96 hour TAN LC50 for spotted gar, alligator gar, and paddlefish was 35 ± 6.12, 135 ± 15, and 15.7 ± 2.25 mg/L, respectively. Alligator gar have the highest recorded ammonia tolerance published to date.

To determine if bowfin *Amia calva*, spotted gar, alligator gar, and paddlefish concentrate environmental nitrite in their plasma, individuals were exposed to concentrations of 0, 1, 10, or 100 mg/L nitrite-N. Bowfin, spotted gar and alligator gar were exposed for 72 hr and paddlefish were exposed for 24 hr. After exposure, all species had plasma nitrite-N concentrations greater than environmental levels. To determine if chloride inhibits nitrite uptake for spotted gar, alligator gar, and paddlefish, fish were exposed to 1 mg/L nitrite-N and 20 mg/L chloride as calcium chloride, or just 1 mg/L nitrite-N. Bowfin, spotted gar and alligator gar were exposed for 72 hr and paddlefish were exposed for 24 hr. Chloride effectively prevented nitrite from being concentrated in the plasma of all species. It appears that non-teleost fish concentrate nitrite in their plasma via their chloride uptake mechanism and this is an ancestral characteristic for teleost.

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Introduction

Ammonia is produced by deamination of ingested proteins and is the main nitrogenous waste product of fish. Ammonia can also be introduced into aquatic systems by industrial, municipal, and agricultural sources. Ammonia is typically oxidized to nitrite and then to nitrate by microbial metabolism. Both ammonia and nitrite can be toxic to fish, but toxic concentrations vary among species (Tomasso 1994).

Ammonia is important to aquaculture because fish are generally cultured at high densities and fed high protein diets. Ammonia is known to reach lethal levels under normal aquaculture conditions (Tomasso 1994). Ammonia exists in an ionized (NH^+_4) and un-ionized (NH_3) form. Un-ionized ammonia is generally associated with ammonia toxicity because it can freely diffuse across gill membranes into the plasma (Russo and Thurston 1991). The proportion of total ammonia-N (TAN) in the un-ionized (UIAN) form increases with temperature and pH (Colt and Tchobanoglous 1976; Russo and Thurston 1991; Randall 1991; Tomasso 1994). Therefore, TAN levels should be maintained at lower concentrations for higher temperature and pH levels (Tomasso 1994). The 96 hour median-lethal (96 hr LC50) ammonia concentrations for shortnose sturgeon *Acipenser brevirostrum* was determined to be 149.8 mg/L for TAN and 0.58 for UIAN (Fontenot et al. 1998). Tilapia have a 96 hr LC50 concentration of 2.40 mg/L for UIAN (Redner and Stickney 1979). According to Wise et al. (1989), the 96 hr LC50 for UIAN concentration for red drum *Sciaenops ocellatus* was 0.9 mg/L after 24 hr and 0.8 after 48 hr. The 96 hr LC50 for ammonia in white bass *Morone chrysops* fingerling was determined to be 0.63 mg/L (Ashe et al. 1996).

Because ammonia is oxidized to nitrite, nitrite can reach toxic concentrations in waters that have elevated ammonia levels. The mechanism of nitrite toxicity is the oxidation of the iron molecule in hemoglobin. This process converts hemoglobin to methemoglobin faster than it can be converted back to hemoglobin (Kiese 1974). Because methemoglobin can not reversibly bind with oxygen, molecules fish that are affected by nitrite toxicity have blood with a brown color and suffer from hypoxia. Additionally, sub-lethal concentrations of nitrite can inhibit growth (Kiese 1974; Williams and Eddy 1986). According to Colt et al. (1981) nitrite caused a reduction in growth of channel catfish *Ictaluris punctatus* when exposed to environmental nitrite-N concentrations of 1.60 mg/L and caused significant mortality at concentrations above 3.71 mg/L. The 96 hour median lethal concentration (96 hr LC50) of nitrite-N for shortnose sturgeon fingerlings was determined to be 11.3 mg/L (Fontenot et al. 1998). The 96 hr LC50 of nitrite-N for channel catfish is 7 mg/L (Russo and Thurston 1977) and is 22 mg/L for rainbow trout *Onchorynchus mykiss* (Palacheck and Tomasso 1984).

Plasma-N concentrations of some fish increase with exposure time and exposure dose (Eddy et al. 1983; Palacheck and Tomasso 1984; Fontenot et al. 1999). There is strong evidence that fish able to concentrate nitrite in their blood plasma uptake environmental nitrite by the gill chloride uptake mechanism (chloride cell; Tomasso and Grosell 2005). A molecular ratio of 9 chloride ions to 1 nitrite ion significantly reduces plasma nitrite-N concentrations of shortnose sturgeon (Fontenot et al. 1999) and a ratio of 16:1 completely inhibited an increase in methemoglobin levels of channel catfish (Palacheck and Tomasso 1984). The 24 hr LC50 of nitrite-N for rainbow trout can be significantly reduced by the addition of chloride ions (Eddy et al. 1983). Not all fishes concentrate environmental nitrite in their blood, which has been demonstrated for members of the teleost families Bothidae, Moronidae, Centrarchidae, Fundulidae, and Anguillidae (reviewed in Tomasso and Grosell 2005). There is a lack of published studies on ammonia and nitrite toxicity studies for non-telost fish such as bowfin *Amia calva*, gars *Atractosteus* spp. and *Lepisosteus* spp., and paddlefish *Polyodon spathula*.

Bowfin belong to the family Amiidae, which consists of one living species. Bowfin are ancient fish that evolved during the Jurassic period. Bowfin are characterized by a long, cylindrical body with a large head and a dorsal fin that extends more than half the length of the body. Bowfin retain ancestral traits such as an abbreviated heterocercal tail, lung-like gas bladder, and vestiges of a spiral valve in the digestive tract. Bowfin can be found from Canada to the Gulf of Mexico north and south and from Texas to southeast Pennsylvania west to east. Bowfin usually inhabit the vegetation of swamps, pools, and the backwaters of lowland streams (Page and Burr 1991).

Gars belong to the family Lepisosteidae which consist of seven species that are found in North America, Central America, Cuba, and the Isle of Pines. Gars are easily recognized by their long snout lined with sharp teeth and retain ancestral characteristics such as ganoid scales, a rounded heterocercal tail, vestiges of a spiral valve, and a lung-like air bladder that facilitates air breathing. Alligator gar *Atractosteus spatula*, longnose gar *Lepisosteus osseus*, shortnose gar *L. platostomus*, and spotted gar *L. oculatus* are the four species native to Louisiana. These gars can be found in rivers, lakes, swamps, and occasionally in brackish marshes (Page and Burr 1991). Because gars are top predators, it has historically been believed that these fish negatively impact commercial and recreational species. Thus, most research has focused on the feeding habits of gar rather than on their biology (Scarnecchia 1992). Gar diets in coastal areas typically consist of small fish and crustaceans such as menhaden *Brevooria patronus*, bay anchovy *Anchoa mitchilli*, sea catfish *Galeichthys felis*, fiddler crabs *Uca pugilator* and blue crabs *Callinectes sapidus* (Suttkus 1963).

Paddlefishes belong to the family Polyodontidae, which consists of two extant species of fishes. Paddlefish are characterized by a cartilaginous skeleton, a heterocercal tail, and a long paddle shaped snout (Page and Burr 1991). The snout of the paddlefish is covered by thousands of passive electroreceptors which may assist in finding food sources by detecting electrical fields emitted by zooplankton (Moyle and Cech 2004). Paddlefish inhabit the Mississippi River and Gulf of Mexico drainage basins in North America. They are commonly found in slow moving waters of large rivers (Page and Burr 1991). Paddlefish are currently being raised in aquaculture ponds along with catfish in Kentucky, Alabama, Illinois, and Oklahoma for their roe and meat, and to help reduce fishing pressure. Some of these aquaculture programs are devoted to restocking areas with threatened populations (Mims 2001).

Of all nitrite uptake studies currently published, the only non-teleost representative is the shortnose sturgeon (Fontenot et al. 1999). Within the teleosts, nitrite uptake vary at the order taxonomic level. For example members of Pleuronectiformes, Perciformes, Cyprinodontiformes, and Anguilliformes do not concentrate nitrite in their plasma (Figure 1). The mechanism of nitrite uptake is through the gill chloride uptake mechanism (Tomasso and Grosell 2005). Based on current literature, it apperars that an evolutionary change in the chloride uptake mechanism, as evidenced by nitrite uptake, has arisen more than once in the teleost. According to Fontenot et al. (1999) shortnose sturgeon member of Chondrostei concentrate nitrite in their plasma, but no other non-teleost actinopterygiian has been tested.

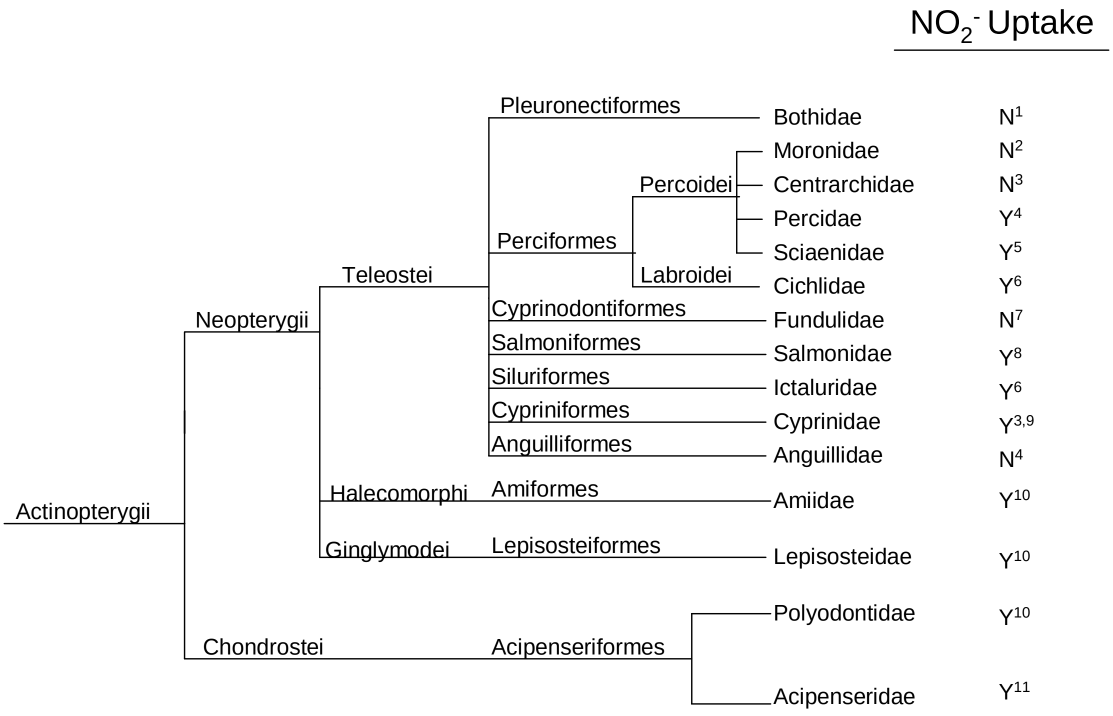

Figure 1. Phylogeny of fishes that have been tested for the ability to concentrate environmental nitrite in their plasma. Families that uptake nitrite at the gills are represented by a Y and those families that do not uptake nitrite at the gills are represented by an N (Modified from Tomasso and Grosell 2005). [1]Atwood et al. 2001, [2]Mazik et al. 1991, [3]Tomasso 1986, [4]Williams and Eddy 1986, [5]Wise and Tomasso 1989, [6] Palachek and Tomasso 1984, [7]Tomasso and Grosell 2005, [8]Bath and Eddy 1980, [9]Alcaraz and Rangel 2004, [10]This study, [11]Fontenot et al. 1999.

To further explore the phylogeny of ammonia toxicity and nitrite uptake, we conducted experiments with spotted gar (Neopterygii: Lepisosteiformes), alligator gar (Neopterygii: Lepisosteiformes), bowfin (Neopterygii: Amiiformes), and paddlefish (Chondrostei: Acipenseriformes).

Objectives

1. Determine the 96-hour ammonia median-lethal concentration for spotted gar, alligator gar, and paddlefish.
2. Determine if bowfin, spotted gar, alligator gar, and paddlefish concentrate environmental nitrite in their plasma.
3. Determine if chloride can inhibit environmental nitrite uptake for spotted gar, alligator gar, and paddlefish.

Methods

Bowfin were collected from Grand Bayou, LA, (29°53’24.3”N and 90°47’03.4”W) using 38 mm bar mesh monofilament gillnets, transported back to Nicholls State University, and maintained in a recirculating plastic 2,113 L circular tank (temperature 21.3 ± 1.7 °C, pH 8.08 ± 0.23, nitrite-N 0.80 ± 0.50 mg/L). Juvenile spotted gar were obtained from the spawning of broodstock at Nicholls State University and were maintained in static fiberglass tanks (temperature 21.8 ± 0.81 °C, pH 7.45 ± 0.17, nitrite-N 0.547 ± 0.61 mg/L). Larval alligator gar were obtained from Private John Allen National Fish Hatchery in Tupelo, MS, and Tishomingo National Fish Hatchery in Tishomingo, OK, and were reared under similar conditions as spotted gar. Juvenile paddlefish were obtained from Booker Fowler Fish Hatchery in Forest Hill, LA, and transported to Nicholls State University. Fish were maintained in a 2,113 L recirculating plastic circular tank (temperature 21.1 ± 0.45 °C, pH 7.73 ± 0.09, nitrite-N 0.279 ± 0.33 mg/L).

To produce larval spotted gar, adult spotted gar were collected with monofilament gillnets (38mm bar mesh) from a small lake (30°19’23.8”N, 91°32’0.25”W) in the Atchafalaya River Basin and transferred to Nicholls State University. The spotted gar were maintained under a natural temperature and photoperiod regime in three aerated 588 L fiberglass tanks connected to a common recirculation filter system. Spotted gar (12 females and 8 males, 572.4 ± 35.1 mm TL; 0.7 ± 0.1 g) were injected intramuscularly with 0.5 ml of Ovaprim® per kg of body weight on 23 April 2005 and transferred to a round, plastic, 6,645 L, aerated, re-circulating spawning tank. The spawning tank was supplied artificial lighting that mimicked a natural photoperiod of 14:10 hours. Artificial substrate was added to mimic submerged vegetation. Spawning was first observed on 25 April 2005. During spawning, eggs adhered to the bottom and sides of the spawning tank and to the artificial substrate. Hatching began on 30 April 2005, and newly hatched yolk-sac larvae attached to the substrate and sides of the spawning tank with an anterior suctoral disc. Larvae became free swimming five days after hatching.

Free swimming larvae were transferred to 19 L glass aquaria or fiberglass tanks and were fed three times daily with newly hatched *Artemia salina* nauplii. Fish were switched to a commercially available floating feed (44% protein, 15% lipid) 4 June 2005. Excess feed and feces were removed and a 50% water change was performed three times daily. Temperature (°C), salinity (ppt), dissolved oxygen (mg/L), pH, and total ammonia-N (mg/L) values were recorded daily. Fish that exhibited signs of cannibalism, such as very fast growth or observed eating others, were removed and placed into separate aquaria. Dead fish were removed daily and recorded.

The ninety-six hour median-lethal (96 hr LC50) ammonia-N concentration was determined for spotted gar (127.5 ± 5.31 mm TL; 4.98 ± 0.66 g), alligator gar (114.8 ± 6.85 mm TL; 6.31 ± 1.25 g), and paddlefish (179.9 ± 24.5 mm TL;16.3 ± 5.0 g) in 37 L aerated glass aquaria, each containing 30 L of test water and one fish. Total ammonia-N concentrations were measured by direct nesslerization (APHA 1989) at the beginning and end of each experiment. Temperature (^{o}C), pH and conductivity (µS) were measured daily, and were used to calculate the un-ionized portion of the total ammonia-N (Westers 2001). Fish were added to aquariums 24 hours prior to adding ammonia as ammonium chloride. Fish were not fed during experimentation and dead fish were removed daily. No control fish died during experimentation. Ninety-six hour LC50 values were determined by probit analysis (SAS 2003).

To determine the 96 hr LC50 ammonia concentration for paddlefish, fish were exposed to nominal concentrations of 50, 100, 200, and 400 mg/L TAN as ammonia chloride. After 24 hours all fish died except for control fish so the experiment was conducted again with nominal concentrations of 0, 6, 12, 24, and 48 mg/L TAN. Mean temperature (20.6 ± 1.3^{o}C), pH (7.85 ± 0.1), and conductivity (501.7 ± 124.1 µS) of all tanks were used to calculate the UIAN concentrations of 0, 0.153, 0.304, 0.603, and 1.188 mg/L. Spotted gar and alligator gar were exposed to nominal concentrations of 0, 50, 100, 200, and 400 mg/L TAN. For spotted gar mean temperature (22.1 ± 0.25 ^{o}C), pH (8.14 ± 0.3), and conductivity (1366 ± 1161 µS) of all tanks were used to calculate the UIAN concentrations of (0, 2.594, 5.188, 10.376, and 20.751). For alligator gar mean temperature (22.8 ± 0.8 ^{o}C), pH (7.90 ±0.2), and conductivity (1409 ± 1056 µS) of all tanks were used to calculate the UIAN concentrations of 0, 1.590, 3.180, 6.359, 12.719 mg/L.

Dose-response to nitrite uptake for bowfin (377.3 ± 37.1 mm TL, 395.6 ± 144 g), spotted gar (129.3 ± 3.2 mm TL, 4.9 ± 0.3 g), alligator gar (112.6 ± 6.8 mm TL, 5.3 ± 0.9 g), and paddlefish (191.6 ± 12.7 mm TL, 18.9 ± 4.6 g) were performed in 37 L glass aquaria, each containing 30 L of aerated water (<1 mg/L Cl^{-}) and one fish. Fish were added to experimental chambers 24 hours before adding nitrite-N in the form of sodium nitrite. Fish were exposed to nominal concentrations of 0, 1, 10, and 100 mg/L nitrite-N. Bowfin, spotted gar and alligator gar were exposed for 72 hours and paddlefish were exposed for 24 hours. For bowfin four replicates were used per treatment and for spotted gar, alligator gar, and paddlefish five replicates were used per treatment. Nitrite-N concentrations in experimental chambers were determined by the azo-dye method (USEPA 1974) at the beginning and end of each experiment, and temperature (^{o}C) and pH were measured daily. No feed was provided during the experiment and dead fish were removed daily. No control fish died during experimentation.

To determine if chloride can inhibit nitrite uptake, spotted gar (133.5 ± 16.2 mm TL, 5.2 ± 1.8 g), alligator gar (114.1 ± 5.0 mm TL, 5.58 ± 0.98 g), and paddlefish (167.4 ± 17.1 mm TL, 13.0 ± 3.12 g) were exposed either to 1 mg/L nitrite-N and 20 mg/L chloride as calcium chloride or only 1 mg/L nitrite-N. All experiments were conducted in 37 L aerated glass aquaria, each containing 30 L of test water (<1 mg/L Cl^{-}) and one fish. Fish were transferred to experimental chambers 24 hours prior to adding nitrite-N. Spotted gar and alligator gar were exposed for 72 hours and paddlefish were exposed for

24 hours. For alligator gar and paddlefish, seven replicates were used per treatment. Five replicates per treatment were used for spotted gar experiments. Nitrite-N concentrations in experimental chambers were measured at the beginning and end of each experiment, and temperature (^{o}C) and pH were measured daily. No feed was provided during experimentation and dead fish were removed daily.

To determine plasma-N concentrations, bowfin blood was obtained through a cardiac puncture and placed into sodium heparin coated capillary tubes. Spotted gar, alligator gar and paddlefish blood was obtained with sodium heparin coated capillary tubes from the hemal arch by severing the tail at the caudal peduncle. The capillary tubes were immediately spun in a hematocrit centrifuge for three minutes with an Adams Micro-Hematocrit Centrifuge (Clay-Adams Inc. New York). Plasma was collected by breaking the capillary tubes at the plasma-cell interface. Plasma nitrite-N concentrations were measured with a modification of the azo-dye method (USEPA 1974; Fontenot et al. 1999). Fifty µL of azo-dye and 10 µL of plasma were added to a spectrophotomer tube containing 3 mL of deionized water. After 10 minutes, the spectrophotomer tubes were transferred to a spectrophotometer (Gensys 20 Thermo Spec) and the absorbance at 540 nm was compared to the absorbance of a 5 mg/L standard. Recoveries of standard additions of nitrite-N to plasma were 99.9 ± 3.6. Analysis of variance (ANOVA) followed by a Tukey range test ($P \leq 0.05$) was used to determine treatment effects.

Results

All spotted gar exposed to 100, 200, and 400 mg/L TAN died. All spotted gar exposed to 0 mg/L and two out of five exposed to 50 mg/L TAN survived. The 96 hour LC50 for TAN for spotted gar was 35 ± 6.12, and 1.82 ± 0.32 for UIAN (Table 1).

All alligator gar exposed to 0 and 50 mg/L TAN survived and all fish exposed to 200 and 400 mg/L TAN died. Four out of 5 fish exposed to 100 mg/L TAN survived. The 96-hour LC50 of TAN for alligator gar was 135 ± 15 mg/L and the 96-hour LC50 of UIAN was 4.30 ± 0.47 (Table 1).

All paddlefish exposed to 0 and 6 mg/L TAN survived and all fish exposed to 24 and 48 mg/L TAN died. Three out of 4 fish exposed to 12 mg/L TAN survived. The 96-hour LC50 of TAN for paddlefish was 15.7 ± 2.25 mg/L, and the 96-hour LC50 of UIAN was 0.40 ± 0.06 mg/L (Table 1).

All bowfin exposed to 0, 1, and 10 mg/L nitrite-N survived and two out of four bowfin exposed to 100 mg/L nitrite-N died. All spotted gar exposed to 0 mg/L nitrite-N survived, two spotted gar exposed to 1 and 10 mg/L nitrite-N survived, and all spotted gar exposed to 100 mg/L nitrite-N died. All alligator gar exposed to 0 and 1 mg/L nitrite-N survived. Three of the five alligator gar exposed to 10 mg/L nitrite-N died and all alligator gar exposed to 100 mg/L nitrite-N died. All paddlefish exposed to 0 mg/L nitrite-N survived, but all other concentrations resulted in mortalities. Three paddlefish exposed to 1 mg/L nitrite-N survived and two paddlefish exposed to 10 mg/L nitrite-N survived. No paddlefish exposed to 100 mg/L survived. The plasma nitrite-N concentrations of all surviving bowfin, spotted gar, alligator gar, and paddlefish were elevated due to uptake and concentration of nitrite-N (Table 2).

Two spotted gar exposed to 1 mg/L nitrite-N survived and all spotted gar simultaneously exposed to 1 mg/L nitrite- N and 20 mg/L chloride survived. Three alligator gar exposed to 1 mg/L nitrite-N survived and all alligator gar simultaneously exposed to 1 mg/L nitrite-N and 20 mg/L chloride survived. Six out of seven

Table 1. Ninety-six hour median-lethal concentration (LC50, mean ± SE) for total and unionized ammonia for spotted gar, alligator gar, and paddlefish. N represents the number of replicates for each species.

Species	**N**	**Total Ammonia-N**	**Un-ionized Ammonia-N**
Spotted Gar	5	35 ± 6.12	1.82 ± 0.32
Alligator Gar	5	135 ± 15	4.30 ± 0.47
Paddlefish	4	15.7 ± 2.25	0.40 ± 0.06

Table 2. Mean (± SE) plasma nitrite-N concentrations (mg/L) for each species after exposure to different concentrations of nitrite-N (mg/L). Bowfin and alligator gar were exposed for 72 hours and paddlefish were exposed for 24 hours. No spotted gar, alligator gar or paddlefish exposed to 100 mg/L nitrite-N survived the duration of the experiment. N represents the number of individuals that plasma levels of nitrite-N was determined for each nitrite-N concentration One sample for spotted gar and one sample for alligator gar exposed to 0 mg/L could not be processed.

Species	**Nitrite-N Concentrations**	**N**	**Plasma-N**
Bowfin	0	4	0.9 ± 0.6
	1	4	10 ± 1.0
	10	4	30 ± 8.9
	100	2	156 ± 43.5
Spotted Gar	0	4	2.9 ± 1.2
	1	2	63.5 ± 7.7
	10	2	86.8 ± 14.0
	100	-	-
Alligator Gar	0	4	0.5 ± 0.3
	1	5	55 ± 10.6
	10	2	32 ± 17.3
	100	-	-
Paddlefish	0	5	0.5 ± 0.1
	1	3	75 ± 18.6
	10	2	61 ± 25.5
	100	-	-

paddlefish exposed to 1 mg/L nitrite-N died and all paddlefish exposed to simultaneously 1 mg/L nitrite-N and 20 mg/L chloride survived. All fish exposed to 1 mg/L nitrite-N and 20 mg/L chloride simultaneously showed reduced plasma-N levels compared to those exposed to just nitrite (Table 3).

Table 3. Mean (± SE) plasma nitrite-N concentration for spotted gar, alligator gar, and paddlefish exposed simultaneously to 1 mg/L nitrite-N and 20 mg/L chloride as calcium chloride or just to 1 mg/L nitrite-N. The gars were exposed for 72 hours and the paddlefish were exposed for 24 hours. Only one paddlefish exposed to 1 mg/L nitrite-N survived. One alligator gar sample was not obtained for the 0 mg/L treatment. Asterisks represent a significant (α = 0.05) difference between treatment means within each species.

Species	**Treatment**	**N**	**Plasma-N**
Spotted Gar	Nitrite Only	2	63.5 ± 7.7
	Nitrite and Chloride	5	1.8 ± 0.4*
Alligator Gar	Nitrite Only	3	61 ± 24.4
	Nitrite and Chloride	6	2.3 ± 0.4*
Paddlefish	Nitrite Only	1	78
	Nitrite and Chloride	7	2.5 ± 0.6*

Discussion

Ammonia, the main nitrogenous waste produced by fish exists in two forms, ionized and un-ionized. The un-ionized form easily diffuses across biological membranes, and can be lethal to fish. Although ammonia is a urinary waste product, it is also excreted through the gill membrane by a $Na^+/NH4^+$ exchange system (Fuller et al. 2003; Tomasso 1994; Wise et al. 1989; Redner and Stickney 1979). Ammonia excretion through gill membranes is reduced by environmental ammonia; therefore, high levels of environmental ammonia cause high levels of plasma ammonia (Tomasso 1994). Lethal concentrations of total ammonia decrease as the proportion of total ammonia in the un-ionized form increases with increased pH (Russo and Thurston 1991). According to Tomasso et al. (1980), toxicity of total ammonia to channel catfish increased as the environmental pH increased.

Acute toxicity (96 hr un-ionized ammonia LC50) values for a variety of fish species have been determined and range from 0.08 mg/L for pink salmon *Oncorhynchus gorbuscha* to 3.8 mg/L for channel catfish (reviewed in Russo and Thurston 1991). Alligator gar were able to survive ammonia concentrations nearly ten times the tolerance of paddlefish for 96 hr. Spotted gar were able to tolerate ammonia concentrations nearly five times the tolerance of paddlefish for 96 hr, but were unable to tolerate as much as alligator gar. Alligator gar have the highest tolerance to ammonia than any published fish to date. Paddlefish tolerance to ammonia is similar to that of shortnose sturgeon (Fontenot et al. 1998), and the gila trout *Oncorhynchus gilae* (Fuller et al. 2003)

Nitrite is produced by the aerobic nitrification of ammonia (Eddy and Williams 1987; Tomasso 1994). For some fish, environmental nitrite enters the blood plasma through the branchial chloride/bicarbonate exchange mechanism. This uptake makes nitrite more toxic to fish that uptake large amounts of chloride through the gill uptake mechanism when compared to fish that uptake chloride through another mechanism (Eddy and Williams 1987). Because the process of nitrite uptake is active, plasma concentrations become much greater than environmental concentrations. The addition of chloride can inhibit the uptake of nitrite by fish (Bartlett and Neuman 1998; Perrone and Meade 1977; Wedemeyer and Yasutake 1978).

Freshwater fish are in a hypoosmotic environment and small ions such as Na^+ and Cl^- are constantly being lost by diffusion through thin gill epithelium. Internal ionic homeostasis is maintained by active transport mechanisms in the gills (Moyle and Cech 2004). The chloride cell is actively involved in the uptake of environmental chloride ions. Na^+, K^+ ATPase pumps actively pump Na^+ into the blood creating a Na^+ deficency in the chloride cell which allows environmental Na^+ to enter the gill membrane passively. The active transport of Na^+ into the chloride cell compensates for the large amounts of salts lost through urination. The chloride cell is also responsible for exchanging some Na^+ for NH_4^+ and the exchange of HCO_3^-, a product of respiration, for Cl^- (Moyle and Cech 2004; Evans 1993). Not all fish uptake chloride by this mechanism (Tomasso and Grosell 2005). It has been suggested by Perrone and Meade (1977) that chloride and nitrite compete for uptake at the HCO_3^-/Cl^- exchange system. Dietary chloride may provide some protection from nitrite uptake in coho salmon because of longer retention of chloride ions in the plasma. This dietary chloride as well as environmental chlorides may interact to prevent environmental nitrite from entering the plasma (Perrone and Meade 1977). Some fish have the ability to differentiate between chloride ions and nitrite ions, which allows for the uptake of chloride instead of nitrite ions as seen in largemouth bass and green sunfish (Tomasso 1986).

Nitrite toxicity varies greatly among fish species due to the ability of some fish to exclude nitrite from blood plasma (Tomasso 1986). Russo and Thurston (1977) determined the 96 hr LC50 for rainbow trout to be 22 mg/L where as Palachek and Tomasso (1984) found that the 96 hr LC50 of largemouth bass to be 453 mg/L. Striped bass nitrite tolerance is also in the upper range at 163 mg/L using a 24 hr LC50 (Mazik et al. 1991).

This wide range in toxicity has been attributed to the ability of some species to prevent nitrite from entering the blood. Channel catfish are unable to prevent nitrite from entering the blood and therefore nitrite concentrations can become elevated in the blood (Palachek and Tomasso 1984). I have shown that bowfin, spotted gar, alligator gar, and paddlefish are unable to prevent nitrite from entering the blood resulting in elevated nitrite levels in blood plasma compared to exposure concentrations. Bowfin concentrate nitrite in their plasma 30 times the environmental concentration, spotted gar concentrate more than 60 times, alligator gar concentrate nitrite more than 50 times environmental concentrations, and paddlefish concentrate nitrite in their plasma more than 75 times the environmental concentration.

Chloride inhibits the uptake of environmental nitrite in some fish. Tomasso et al. (1979) determined that in channel catfish the addition of chloride at a ratio of 16 to 1 with nitrite completely suppressed nitrite-induced methemoglobinemia. It has also been determined that acclimating channel catfish in water containing sodium chloride did not enhance its ablility to inhibit nitrite uptake (Tomasso et al. 1979). Striped bass tolerated greater concentrations of nitrite when chloride was added as either calcium chloride or sodium chloride (Mazik et al. 1991). Chloride inhibited the uptake of nitrite when shortnose sturgeon were exposed to chloride at a ratio of 9 to 1 (Fontenot et al. 1999). I showed that chloride inhibits the uptake of nitrite for spotted gar, alligator gar, and paddlefish. There was a significant difference in plasma nitrite concentrations for fish exposed simultaneously to nitrite and chloride when compared to fish exposed only to nitrite. Plasma nitrite concentrations of fish exposed to nitrite only were more than 30 times greater than fish exposed to chloride and nitrite simultaneously at a ratio of 20:1.

Hemoglobin binds with oxygen and transports oxygen to cells. Hemoglobin contains divalent iron in the functional state, but when oxidized by autooxidation or by nitrite the iron is converted to the trivalent form resulting in the formation of methemoglobin (Kiese 1974). Methemoglobin is unable to transport oxygen to cells (Tomasso et al 1979), which causes hypoxia and death (Kiese 1974; Rodriguez-Moreno; Tarazona 1994). There is always a percentage of methemoglobin in blood because of autooxidation of hemoglobin. Reduction of methemoglobin to hemoglobin is mediated by NADH-dependent methemoglobin reductase (Figure 2). When nitrite is elevated plasma, oxidation of hemoglobin is accelerated to a point where oxidation is occurring faster than the reduction of methemoglobin to hemoglobin (Figure 2); (Kiese 1974). The rate at which hemoglobin is oxidized to methemoglobin depends on plasma nitrite concentrations, chloride concentrations, the species of fish, and the exposure time (Eddy and Williams 1987).

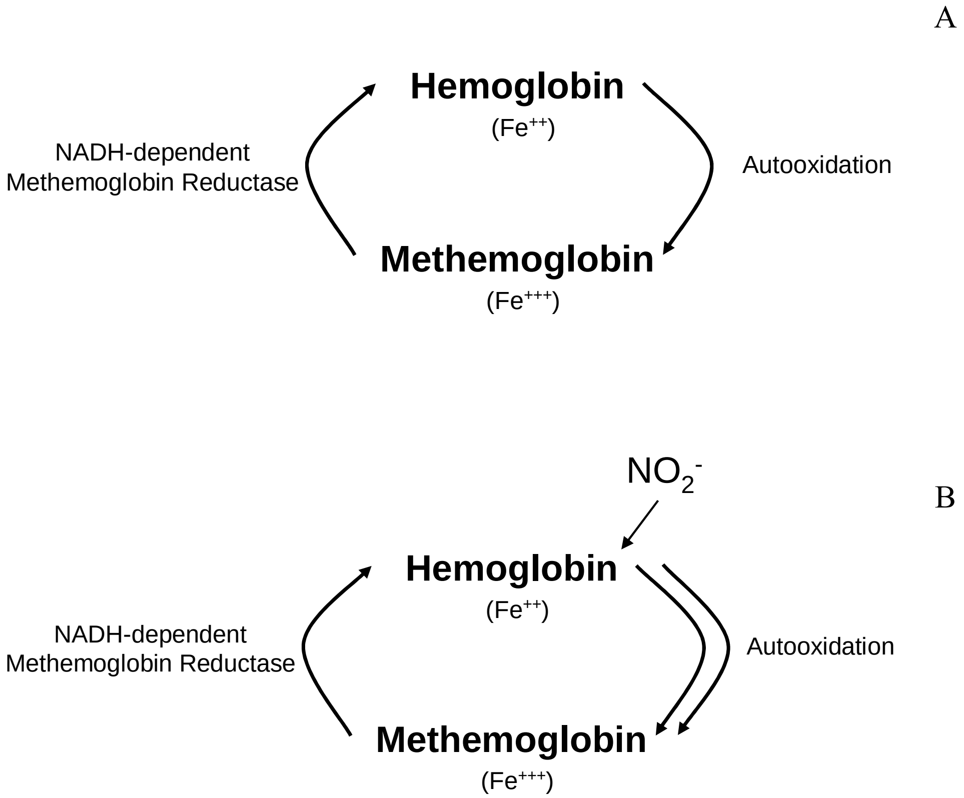

Figure 2. (A) The autooxidation of hemoglobin to methemoglobin and the enzyme mediated reduction back to hemoglobin by NADH-dependent methemoglobin reductase. (B) The oxidation of hemoglobin is increased with the addition of nitrite. Oxidation of hemoglobin to methemoglobin is occurring faster than the NADH-dependent methemoglobin reductase is able to reduce the methemoglobin back to hemoglobin.

Nitrite uptake has mostly been studied for teleosts (Figure 1). Because nitrite uptake is directly related to chloride uptake, nitrite uptake can provide insight into the evolution of chloride uptake in freshwater fish (Tomasso and Grosell 2005). Marshall (2002) suggests that because there is inconsistency of chloride uptake among different species of fish at the gills, the theory that osmoregulation has derived from multiple origins is supported. Another theory is that some groups of fish have adapted to cope with environmental conditions (Tomasso and Grosell 2005). According to Tomasso and Grosell (2005) fish that uptake chloride through a gill uptake mechanism also concentrate environmental nitrite in their plasma and those that do not uptake chloride through a gill uptake mechanism do not concentrate environmental nitrite in their plasma. There is strong evidence that supports the relationship between chloride uptake by a gill uptake mechanism and the ability to concentrate environmental nitrite in plasma (Tomasso and Grosell 2005).

I studied nitrite uptake in non-teleost actinopterygiian fish of the families Amiidae, Lepisosteidae, and Polyodontidae and determined that these fish concentrate nitrite in their blood plasma and that chloride can inhibit the uptake of nitrite. Based on the relationship between chloride and nitrite uptake by the gill uptake mechanism (Tomasso and Grosell 2005), I conclude that the gill chloride uptake mechanism is an ancestral characteristic of teleosts.

Recommendations

Based on growth studies of several species, nine percent or less of the 96 hr median-lethal ammonia concentration should not inhibit fish growth (Tomasso 1994). I determined the un-ionized ammonia LC50 to be 1.82 ± 0.32 mg/L for spotted gar, 4.30 ±

0.47 mg/L for alligator gar, and 0.40 ± 0.06 mg/L for paddlefish. I estimate that based on these LC50 values spotted gar, alligator gar, and paddlefish growth should not be inhibited if exposed to un-ionized ammonia concentrations of 0.16, 0.38, and 0.04 mg/L respectively. Finally, at least a 20:1 chloride to nitrogen (for nitrite-N) ratio should be maintained to prevent plasma-N concentrations from becoming elevated.

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APPENDIX I: AMMONIA TOXICITY

Total length (mm) and weight (g) of individuals of each species exposed to different nominal ammonia-N concentrations.

Species	Treatment	Repetition	Length	Weight
Spotted Gar	0	1	129	4.4036
Spotted Gar	0	2	120	3.1669
Spotted Gar	0	3	124	3.8818
Spotted Gar	0	4	136	4.7577
Spotted Gar	0	5	131	4.8355
Spotted Gar	50	1	134	4.6035
Spotted Gar	50	2	139	5.5408
Spotted Gar	50	3	125	4.724
Spotted Gar	50	4	124	4.1812
Spotted Gar	50	5	131	4.9327
Spotted Gar	100	1	126	5.3615
Spotted Gar	100	2	129	5.6068
Spotted Gar	100	3	121	5.2522
Spotted Gar	100	4	117	4.5535
Spotted Gar	100	5	130	5.2981
Spotted Gar	200	1	127	5.8058
Spotted Gar	200	2	126	4.8318
Spotted Gar	200	3	129	5.4002
Spotted Gar	200	4	129	5.5461
Spotted Gar	200	5	123	5.2441
Spotted Gar	400	1	128	5.7229
Spotted Gar	400	2	121	4.6165
Spotted Gar	400	3	135	6.0219
Spotted Gar	400	4	123	4.6505
Spotted Gar	400	5	130	5.6227
Alligator Gar	0	1	122	6.445
Alligator Gar	0	2	121	6.9209
Alligator Gar	0	3	109	4.6044
Alligator Gar	0	4	98	3.5067
Alligator Gar	0	5	108	4.6208
Alligator Gar	50	1	102	3.8859
Alligator Gar	50	2	126	6.8152
Alligator Gar	50	3	112	5.6871
Alligator Gar	50	4	111	5.9808
Alligator Gar	50	5	117	6.1639
Alligator Gar	100	1	117	5.5567
Alligator Gar	100	2	126	8.7283
Alligator Gar	100	3	120	6.5597
Alligator Gar	100	4	121	7.1231
Alligator Gar	100	5	118	7.0654
Alligator Gar	200	1	118	7.2949
Alligator Gar	200	2	115	7.5369
Alligator Gar	200	3	108	5.8317

Species	Treatment	Repetition	Length	Weight
Alligator Gar	200	4	114	6.6336
Alligator Gar	200	5	116	7.6081
Alligator Gar	400	1	116	6.2595
Alligator Gar	400	2	108	5.2508
Alligator Gar	400	3	112	6.7669
Alligator Gar	400	4	115	6.8243
Alligator Gar	400	5	121	8.1121
Paddlefish	0	1	219	25.2
Paddlefish	0	2	194	19.9
Paddlefish	0	3	192	18.4
Paddlefish	0	4	120	5.1
Paddlefish	6	1	172	14.1
Paddlefish	6	2	180	15
Paddlefish	6	3	191	17.2
Paddlefish	6	4	160	12.5
Paddlefish	12	1	191	16.2
Paddlefish	12	2	166	12.9
Paddlefish	12	3	179	18
Paddlefish	12	4	195	20.9
Paddlefish	24	1	157	11.6
Paddlefish	24	2	193	23.4
Paddlefish	24	3	160	13.4
Paddlefish	24	4	192	22.2
Paddlefish	48	1	174	16.3
Paddlefish	48	2	169	17.5
Paddlefish	48	3	183	20.8
Paddlefish	48	4	141	9.5

APPENDIX II: NITRITE UPTAKE

Plasma-N concentrations (mg/L), total length (mm) and weight (g) for individuals of each species exposed to different concentrations of nitrite-N.

Species	**Treatment**	**Repetition**	**Length**	**Weight**	**Plasma-N Concentration**
Alligator Gar	0	1	108	4.4	0.03
Alligator Gar	0	2	123	5.7	.
Alligator Gar	0	3	119	5.4	0
Alligator Gar	0	4	112	4.4	1.3
Alligator Gar	0	5	109	4.5	0.65
Alligator Gar	1	1	129	6.5	66.75
Alligator Gar	1	2	108	4.2	49.2
Alligator Gar	1	3	110	4.6	61.3
Alligator Gar	1	4	121	5.7	79.8
Alligator Gar	1	5	111	4.7	17.35
Alligator Gar	10	1	109	6.1	.
Alligator Gar	10	2	104	4.9	.
Alligator Gar	10	3	110	4	14.3
Alligator Gar	10	4	116	5.1	49.5
Alligator Gar	10	5	116	5.9	.
Alligator Gar	100	1	108	6.2	.
Alligator Gar	100	2	110	6.4	.
Alligator Gar	100	3	103	5.4	.
Alligator Gar	100	4	106	5.1	.
Alligator Gar	100	5	120	7.4	.
Bowfin	0	1	364	332.5	0.3
Bowfin	0	2	412	546	0.3
Bowfin	0	3	378	379.5	2.65
Bowfin	0	4	363	338	0.15
Bowfin	1	1	386	410	7.8
Bowfin	1	2	332	280	11.75
Bowfin	1	3	362	280.5	9.75
Bowfin	1	4	352	342	12
Bowfin	10	1	349	310.5	14.2
Bowfin	10	2	356	319.5	34.7
Bowfin	10	3	404	438.5	18
Bowfin	10	4	483	834.5	53.05
Bowfin	100	1	372	370	.
Bowfin	100	2	131	399.5	.
Bowfin	100	3	378	364.5	112.15
Bowfin	100	4	363	362.5	199.1
Paddlefish	0	1	206	23.4	0.3
Paddlefish	0	2	412	546	0.5
Paddlefish	0	3	378	379.5	0.6
Paddlefish	0	4	363	338	0.6
Paddlefish	0	5	207	19.4	0.45
Paddlefish	1	1	187	20.9	.

Species	Treatment	Repetition	Length	Weight	Plasma-N Concentration
Paddlefish	1	2	177	10.8	70.15
Paddlefish	1	3	184	15.6	45.55
Paddlefish	1	4	191	18.2	109.3
Paddlefish	1	5	210	27.7	.
Paddlefish	10	1	203	22.5	.
Paddlefish	10	2	169	13	86.5
Paddlefish	10	3	182	14.2	35.5
Paddlefish	10	4	186	20.5	.
Paddlefish	10	5	185	17.7	.
Paddlefish	100	1	169	15.4	.
Paddlefish	100	2	214	29.9	.
Paddlefish	100	3	188	19.7	.
Paddlefish	100	4	192	19.1	.
Paddlefish	100	5	202	21.2	.
Spotted Gar	0	1	131	4	4.8
Spotted Gar	0	2	99	1.6	.
Spotted Gar	0	3	125	3.9	1.9
Spotted Gar	0	4	127	4.1	4.9
Spotted Gar	0	5	132	4.2	0
Spotted Gar	1	1	159	7	71.1
Spotted Gar	1	2	136	6	.
Spotted Gar	1	3	129	5	.
Spotted Gar	1	4	139	7	.
Spotted Gar	1	5	162	8	55.8
Spotted Gar	10	1	121	5	.
Spotted Gar	10	2	144	6	100.8
Spotted Gar	10	3	125	5	.
Spotted Gar	10	4	128	6	.
Spotted Gar	10	5	124	4	72.8
Spotted Gar	100	1	123	4	.
Spotted Gar	100	2	127	4	.
Spotted Gar	100	3	124	5	.
Spotted Gar	100	4	121	5	.
Spotted Gar	100	5	108	4	.

APPENDIX III: CHLORIDE INHIBITION

Plasma-N concentrations (mg/L), total length (mm) and weight (g) for individuals of each species exposed to different concentrations of nitrite-N.

Species	Treatment	Repetition	Length	Weight	Plasma-N Concentration
Alligator Gar	Without Chloride	1	.	.	.
Alligator Gar	Without Chloride	2	109	4.6	21.15
Alligator Gar	Without Chloride	3	.	.	.
Alligator Gar	Without Chloride	4	.	.	.
Alligator Gar	Without Chloride	5	.	.	.
Alligator Gar	Without Chloride	6	112	5.2	56.95
Alligator Gar	Without Chloride	7	110	5.8	105.5
Alligator Gar	With Chloride	1	111	4.9	1.4
Alligator Gar	With Chloride	2	117	6	.
Alligator Gar	With Chloride	3	116	4.9	1.6
Alligator Gar	With Chloride	4	111	5	2.45
Alligator Gar	With Chloride	5	104	4.2	2.2
Alligator Gar	With Chloride	6	118	5.4	1.9
Alligator Gar	With Chloride	7	119	5.2	4.15
Paddlefish	Without Chloride	1	124	7.4	.
Paddlefish	Without Chloride	2	164	13.9	.
Paddlefish	Without Chloride	3	148	11.6	.
Paddlefish	Without Chloride	4	167	13.5	.
Paddlefish	Without Chloride	5	197	19.5	78.3
Paddlefish	Without Chloride	6	177	16.3	.
Paddlefish	Without Chloride	7	164	16.9	.
Paddlefish	With Chloride	1	183	13.3	4.95
Paddlefish	With Chloride	2	172	12.1	1.65
Paddlefish	With Chloride	3	175	11.4	4.8
Paddlefish	With Chloride	4	166	10.2	1.65
Paddlefish	With Chloride	5	179	13.3	1.3
Paddlefish	With Chloride	6	170	13.3	0.7
Paddlefish	With Chloride	7	158	9.5	2.1
Spotted Gar	Without Chloride	1	159	7.4	71.1
Spotted Gar	Without Chloride	2	136	5.9	.
Spotted Gar	Without Chloride	3	129	5.2	.
Spotted Gar	Without Chloride	4	139	6.9	.
Spotted Gar	Without Chloride	5	162	7.9	55.8
Spotted Gar	With Chloride	1	127	3.7	2.6
Spotted Gar	With Chloride	2	128	4.1	1.9
Spotted Gar	With Chloride	3	111	3.1	0.4
Spotted Gar	With Chloride	4	120	3.2	1.9
Spotted Gar	With Chloride	5	124	4.3	2.2

Biographical Sketch

Perry Boudreaux was born on May 7, 1981 in Houma, Louisiana and was raised in Dulac, Louisiana. After graduating from Allen J. Ellender Memorial High School in Houma, Louisiana, Perry attended Nicholls State University where he majored in General Biology and graduated in the Fall of 2003. Perry was unsure what he was going to do after graduating, so he decided to continue his education and enrolled in the graduate program at Nicholls State University. Perry conducted research on non-teleost actinopterygiian fishes under the guidance of Dr. Quenton Fontenot. Perry is scheduled to graduate in the Fall of 2005. Perry is currently seeking employment as a biologist.

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EDUCATION

M.S. Marine and Environmental Biology, December 2005, Nicholls State University, Thibodaux, Louisiana, 70310. Acute Ammonia Toxicity and Chloride Inhibition of Nitrite Uptake in Non-Teleost Actinopterygiian Fishes.

B.S. General Biology, December 2003, Nicholls State University, Thibodaux, Louisiana, 70310.

TEACHING EXPERIENCE

January 2004 - December 2005: Teaching Assistant, Nicholls State University, Department of Biological Sciences. Duties included teaching one biology lab per week, preparing quizzes, grading assignments.

RESEARCH EXPERIENCE:

1. Acute Ammonia Toxicity and Chloride Inhibition of Nitrite Uptake in Non-Teleost Actinopterygiian Fish.
2. Presence of Pathogens in Processed and Unprocessed Meat.

FIELD EXPERIENCE:

Small boat operation, pirogue operation, gill net sampling, seine sampling, water quality monitoring (dissolved oxygen, pH, salinity, specific conductance, ammonia, nitrite, and Secchi disk depth), four wheeler operation, GPS, transect estimation, soil analysis, local plant identification.

LABORATORY EXPERIENCE:

Care and maintenance of live fish, induce spawning, larvae rearing, water quality monitoring and maintenance, spectrophotometry, manage a team of laboratory workers.

MEMBERSHIP AND SERVICES

Parent Society of the American Fisheries Society
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Boudreaux, P. A. Ferrara, and Q. Fontenot. (In Prep) Acute Ammonia Toxicity and Chloride Inhibition of Nitrite Uptake in Non-Teleost Actinopterygiian Fishes. Journal of the World Aquaculture Society.

Ferrara, A., **Boudreaux, P**., and Q. Fontenot. (In Prep) Induced Spawning of Wild-Caught Spotted Gar *Lepisosteus Oculatus* in a Laboratory. Journal of the World Aquaculture Society.

SCIENTIFIC PRESENTATIONS:

2005. Bonvillain, C., Q. Fontenot, A. Ferrara, and **P. Boudreaux**. The Use of a Low-Water Refuge in the Atchafalaya River Basin by Adult Spotted Gar *Lepisosteus occulatus*. Annual Meeting of the Louisiana Chapter of the American Fisheries Society, Baton Rouge, Louisiana

2005. **Boudreaux, P**., Q. Fontenot, A. Ferrara, C. Bonvillain, J. Reulet, and M. Acosta. Induced Spawning of Wild-Caught Spotted Gar *Lepisosteus oculatus* in a Laboratory. Annual Meeting of the Louisiana Chapter of the American Fisheries Society, Baton Rouge, Louisiana

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