

Sub-lethal ammonia toxicity in largemouth bass

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Abstract

Guidelines for ammonia toxicity in fish are often determined using static exposure tests with immature fish over a 96-h period. These results may not be relevant to aquaculture, hauling or angling tournament scenarios where mature fish can be exposed to ammonia for shorter durations, often following additional stressors such as handling. The current study sought to quantify (1) the impact of ambient ammonia on the ability of largemouth bass to recover from exercise, (2) the behavioural response of largemouth bass to elevated ambient ammonia and (3) the concentration of ammonia that can accumulate in a live-release vessel at an angling tournament. After approximately 3 h, total ammonia (T_{amm}) concentrations in a live-release vessel at an angling tournament were almost 200 μM . Exposure of fish to 1000 μM T_{amm} (a value approximately 80% below the criteria maximum concentration for largemouth bass) caused significant reductions in ventilation rates, and increases in erratic swimming and irregular ventilation. Exposure to 100 μM T_{amm} impaired the ability of largemouth bass to recover from exercise relative to fish recovering in fresh water. Therefore, sub-lethal ambient ammonia concentrations cause physiological disturbances that can impair the recovery of largemouth bass from exercise.

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1. Introduction

Ammonia is a highly toxic waste product produced by plants and animals, and also generated by the decomposition processes of microorganisms. In fish, ammonia is generated in white muscle when adenylates are broken down to inosine monophosphate (IMP) and ammonium (NH_4^+) during anaerobic energy generation associated with burst exercise (van Waarde, 1983; Mommsen and Hochachka, 1988). Ammonia toxicity for fishes, expressed as total ammonia (T_{amm} , $[\text{NH}_3] + [\text{NH}_4^+]$), has previously been shown to increase with ammonia concentration, water pH and water temperature (Emerson et al., 1975; Bergerhouse, 1992; US EPA, 1999; Ip et al., 2001). Symptoms of ammonia intoxication in fish may include hyperventilation, erratic swimming, gulping at the surface, increased ventilation

rate, loss of equilibrium, convulsions, and, finally, death (Smart, 1976; Hillaby and Randall, 1979; Knoph, 1996; Ip et al., 2001).

Guidelines for acceptable water ammonia concentrations for a range of aquatic organisms have been produced by the United States Environmental Protection Agency (US EPA) (Roseboom and Richey, 1977; US EPA, 1999). However, many of these guidelines are based on acute ammonia exposure of 96 h or greater, and often for small (early life stages) individuals. Current US EPA guidelines for ammonia toxicity in the genus *Micropterus* (black basses), for example, are based on two studies: one involving immature largemouth bass [*Micropterus salmoides* Lacépède, Roseboom and Richey (1977)] <6 g in weight, and one involving smallmouth bass (*M. dolomieu* Lacépède) at early life stages (Broderius et al., 1985; US EPA, 1999). These guidelines may not be useful in determining acceptable ammonia concentrations for additional scenarios of ammonia exposure for older (larger) basses, especially considering the results of Thurston and Russo (1985) who showed that susceptibility to ammonia toxicity in rainbow trout (*Oncorhynchus mykiss* Walbaum) increased with fish size. As well, Ip et al. (2001) stated that the methods used for standard

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toxicity tests require static conditions using unfed, resting, stress-free animals, potentially maximizing their tolerance to ammonia; adding additional stressors in combination with elevated ambient ammonia levels may therefore exacerbate the toxic effects of ambient ammonia. Finally, during situations such as transport/hauling (Carmichael, 1984; Carmichael et al., 1984) or live-release angling tournaments (Kwak and Henry, 1995), adult (large) or sub-adult fish may be exposed to high concentrations of ammonia for relatively short (<6 h) time periods. To date, however, the physiological impacts of this type of short-term ammonia exposure have not been adequately studied.

Live-release angling tournaments have become a popular use of fisheries resources in North America. During a live-release angling tournament, ammonia has the potential to accumulate in situations where high densities of fish are maintained in relatively low volumes of water (Current Study). These situations can occur in two main instances including livewells used to hold fish during the angling day (Kwak and Henry, 1995), and live-release boats used to release fish at the end of the tournament. Furthermore, exposure to these elevated ammonia environments in livewells and live-release vessels typically occurs following additional stressors such as angling (exhaustive exercise), air exposure, and/or the weigh-in procedures (Suski et al., 2004). Thus, placing fish in these high ammonia environments may impact their ability to recover from the physiological disturbances associated with exercise or the “weigh-in.” Because angling tournaments often target the largest and most reproductively valuable fish within a population (Carlander, 1977; Suski and Philipp, 2004), it is important that survival following angling tournaments be maximized to reduce the impact of tournaments on largemouth bass populations.

The objectives of this study were first to quantify ammonia concentrations that accumulated on board live-release vessels from a live-release angling tournament to ascertain ammonia concentrations that tournament-caught fish may experience. Second, we sought to determine the concentration of ambient ammonia that resulted in behavioural modifications (indicative of sub-lethal toxicity) for largemouth bass and to quantify the behavioural response(s). Finally, we examined the impact of elevated ambient ammonia concentrations on the ability of largemouth bass to recover from exercise. To accomplish these goals, field and laboratory experiments were performed that integrated behaviour and the quantification of physiological parameters.

2. Materials and methods

For each of the three study objectives, there were three separate series of experiments, each carried out over a 2-year period.

2.1. Angling tournament sampling

To quantify the concentrations of water ammonia that can accumulate in a live-release vessel at an angling tournament, a tournament held on Big Rideau Lake, Portland, Ontario, Canada (44° 40'N 76° 15'W), was visited. At the tournament, 20 ml

water samples from the holding tanks of the live-release boat (approximately 1350 l total volume) were collected regularly during the time that fish were held, and collected water samples were held on ice in individually labelled plastic tubes until returning to the laboratory at Queen's University, Kingston, Ontario ($N=8$ samples collected; mean interval between collections = 29 min \pm 13 min standard error, $N=7$ intervals). Water temperature and pH were also quantified at the time of collection using a portable dissolved oxygen meter (YSI 55, YSI Incorporated, Yellow Springs, Ohio, USA) and portable pH meter (Orion 250A+, Thermo Electron Corporation, Beverly, MA, USA). Mean dissolved oxygen concentration during the holding period was 7.0 mg/l (\pm 1 mg/l standard error, $N=7$ measurements). During the time that fish were held in the live-release vessel, water was not exchanged with the lake, and no water quality chemicals (i.e., salt, commercial water treatments etc.) were used. Upon returning to the laboratory, water samples were held at -20 °C until ammonia analyses were performed as described below.

2.2. Behavioural assessments

To determine ammonia concentrations that result in behavioural modifications in largemouth bass, a laboratory experiment was performed in the aquatic holding facility at Queen's University. Largemouth bass ranging in size from 54 to 147 g (mean weight = 97 g) were acquired from Pure Springs Trout Farm, Shannonville, Ontario, and transported to Queen's University. Fish were held for 5 weeks in a common holding tank continuously supplied with dechlorinated tap water and were fed commercially available trout pellets approximately once per week; feeding was terminated several days prior to the start of experiments. To simulate summer temperatures, holding water was gradually increased from 13 °C to 26 °C over this acclimation time with daily temperatures in the last week averaging 25.6 °C (\pm 0.4 standard error, $N=7$ measurements). Prior to the start of experiments, NH_4Cl was carefully added to individual 20 l containers of fresh, dechlorinated 25 °C water, to adjust the ammonia concentration to either 0, 1000, or 4000 μM total ammonia (T_{amm}). Solutions were then thoroughly mixed by hand, an airstone was added, and one largemouth bass was taken from the common holding tank and placed in each container ($N=6$ per concentration). Fish were transferred to the container of ammonia (rather than adding ammonia to a container with a fish) to ensure that added NH_4Cl could be adequately mixed creating a homogeneous solution. Largemouth bass were held in the different ammonia concentrations for 6 h, and were monitored every hour to visually quantify ventilation rate, occurrence of erratic swimming behaviour (twitching, quivering) and occurrence of irregular ventilation (episodic or intermittent ventilation as opposed to regular rhythmic ventilation). A twitch was defined as the occurrence of sporadic, intermittent, deep contractions of epaxial muscle, while quivering was defined as prolonged (approximately 1–4 s), repeated, shallow contractions of the epaxial musculature. Dissolved oxygen, temperature and pH were also monitored regularly throughout the 6-h experiment.

2.3. Recovery from exercise

To determine the impacts of ambient ammonia on the physiological recovery from exercise in largemouth bass, it was first necessary to describe the time course of plasma ammonia in largemouth bass exercised and recovered entirely in fresh water. For this, largemouth bass ranging in size from 290 to 460 mm TL (average TL = 331 mm) were collected, by angling, from the Lake Opinicon at the Queen's University Biological Station, Chaffey's Lock, Ontario (44° 31'N, 76° 20'W) (note that these fish were larger and heavier than fish used in the previous series of experiments). Fish were then held at approximately 25 °C for at least 48 h in tanks continuously supplied with fresh Lake Opinicon water to adjust to laboratory conditions and were not fed during this time. To obtain control (resting) values for blood and muscle parameters, largemouth bass were placed in darkened, aerated, Perspex boxes continuously supplied with fresh Lake Opinicon water at ambient temperature. After 24–48 h in the boxes, the flow of water to the fish was terminated and a lethal dose of anaesthetic [250 mg/l 3-aminobenzoic acid ethyl ester methanesulfonate (MS222) buffered with 500 mg NaHCO₃/l] was added. Following the cessation of ventilation, fish were sampled for blood and white muscle according to the methods of Suski et al. (2003).

To induce a physiological disturbance that replicated angling, individual largemouth bass were netted from a holding tank and transferred to an oval tank containing Lake Opinicon water at ambient temperature. Fish were then chased around the tank by tail grabbing for 1 min, after which they were transferred to a container of water with a lethal dose of anaesthetic and sampled for blood and white muscle. Several studies have shown that manual chasing of fish for 1-min results in significant physiological disturbances, and replicates the physiological disturbances induced by angling (Gustavson et al., 1991; Suski et al., 2003, 2004). Finally, to determine the time required for plasma ammonia to return to control (resting) values following exercise, largemouth bass were exercised for 1 min as described above, and then transferred to darkened Perspex boxes continuously supplied with fresh, aerated Lake Opinicon water. After 1, 2 or 4 h of recovery time, fish were anaesthetized using a lethal dose of anaesthetic and sampled for blood and white muscle. All blood and white muscle samples were collected according to the methods of Suski et al. (2003).

To quantify the impacts of ambient ammonia on the ability of largemouth bass to recover from exercise, largemouth bass were netted from a holding tank and exercised in ambient Lake Opinicon water by manual chasing for 1 min as described above. Following exercise, fish were transferred to darkened Perspex boxes and left to recover for 2 h in aerated water that contained T_{amm} concentrations of either 0 μM (fresh water), 100 μM , 200 μM , 400 μM or 1000 μM . After 2 h of recovery time, fish were anaesthetized and sampled for blood and white muscle according to the methods of Suski et al. (2003). Previous work on recovery from exercise in largemouth bass has shown that, after 2-h recovery in fresh water, most physiological variables have begun to recover from disturbances associated with exercise, but all physiological parameters have not

returned to control (resting) values (Suski et al., 2006). Allowing fish to recover from exercise for 2 h in different ammonia concentrations would allow us to determine if certain concentrations accelerated recovery relative to ambient water, or if recovery was impaired.

This study was conducted following guidelines established by the Canadian Council of Animal Care, and also within the Animal Care guidelines of Queen's University.

2.4. Analytical techniques

Analyses for plasma and white muscle variables are described in detail in Suski et al. (2003). Quantification of ammonia in both water and plasma samples followed procedures outlined in Ivanèè and Degobbis (1984). Plasma osmolality was quantified with a freezing-point depression osmometer (Advanced Instruments Incorporated, Model 3M0, Norwood, MA, USA) and plasma chloride was quantified using a chloride titrator (Radiometer Incorporated, Model CMT 10, Copenhagen, Denmark). Plasma lactate concentration was determined using a commercially available lactate assay (Sigma-Aldrich Co., St. Louis, MO, USA, Product #826-A) that followed the methods of Lowry and Passonneau (1972). Plasma cortisol concentration was measured by competitive protein binding using a commercially available kit (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, California, USA). Tissue lactate, adenosine triphosphate (ATP), and phosphocreatine (PCr) were measured following the enzymatic methods of Lowry and Passonneau (1972) after grinding tissue samples to a fine powder under liquid nitrogen using a mortar and pestle. An additional portion of muscle tissue was used to measure glycogen following the methods of Hassid and Abraham (1957). Water content in white muscle was determined by drying pre-weighed frozen tissue in an 80 °C oven for several days until a constant weight was obtained.

2.5. Data analysis

Changes in ammonia concentration on board the live-release vessel over time were assessed using a linear regression analysis (Sokal and Rohlf, 1995). Ventilation rates for largemouth bass sampled during the toxicity experiments were compared using a two-way repeated-measures analysis of variance (ANOVA; main effects: time and ammonia concentrations) followed by a Tukey–Kramer HSD *post-hoc* test to discern differences between groups (Sokal and Rohlf, 1995). For largemouth bass exercised and recovered entirely in fresh water, physiological variables quantified at the different sampling times were compared to the control treatment using a one-way ANOVA followed by a Dunnett's *post-hoc* test (Zar, 1999). For largemouth bass recovered in different concentrations of ambient ammonia, physiological variables were compared to two control groups using a Dunnett's *post-hoc* test: the 2-h recovery in fresh water (0 μM ammonia) as well as the control (resting) treatment. Statistical analyses were performed using JMPIN Version 4.0.4 (SAS Institute, Cary, NC, USA), and the level of significance (α) for all tests was 0.05. Results are shown as means \pm 1 standard error (SE) where appropriate.

3. Results

3.1. Angling tournament sampling

During the angling tournament examined on Big Rideau Lake, ammonia concentrations on board the live-release vessel increased significantly, and reached almost 170 μM T_{amm} after 3 h and 5 min (Fig. 1) ($[T_{\text{amm}}] = -232.7 + 0.02(\text{Time})$; $F_{1,6} = 79.5$, $r^2 = 0.93$, $P < 0.0001$).

3.2. Behavioural assessments

Significant reductions in ventilation rates occurred after 1 h for largemouth bass exposed to 4000 μM T_{amm} while significant reductions in ventilation rates occurred in the 1000 μM T_{amm} treatment after 2 h (repeated-measures ANOVA, $F_{35,83} = 25.2$, $P < 0.0001$; Tukey–Kramer HSD test, $P < 0.05$) (Fig. 2). Ventilation rates remained significantly lower than control treatments for both experimental groups, for the duration of the experiment (Tukey–Kramer HSD test, $P < 0.05$) (Fig. 2). Furthermore, 15–33% of the largemouth bass in the 1000 μM T_{amm} treatment exhibited erratic swimming throughout the study (Fig. 3A), while irregular, sporadic ventilation patterns appeared in fish in the 4000 μM T_{amm} and 1000 μM T_{amm} treatments after 1- and 5-h exposure, respectively (Fig. 3B). For all treatments during the experiment, pH averaged 7.86 ($N = 59$ measurements, range = 7.3–8.3).

3.3. Recovery from exercise

Following 1 min of exercise, largemouth bass exhibited a four-fold increase in plasma ammonia (ANOVA, $F_{4,31} = 8.0$, $P = 0.0001$; Dunnett’s test, $P < 0.05$) (Fig. 4). This increase returned to control levels after 1 h of recovery in fresh water, and then remained at control levels during the remainder of the recovery period (Dunnett’s test, $P > 0.05$) (Fig. 4). Following 2-h recovery from exercise in different quantities of ambient ammonia, however,

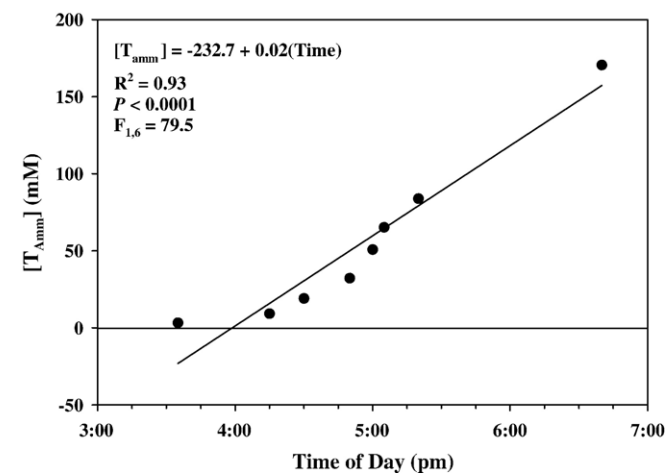


Fig. 1. Change in total ammonia concentration (T_{amm} in μM) on board a live-release vessel during an actual live-release angling tournament. The addition of fish to the vessel began at approximately 4:00 pm, and fish were released from the boat at approximately 6:30 pm.

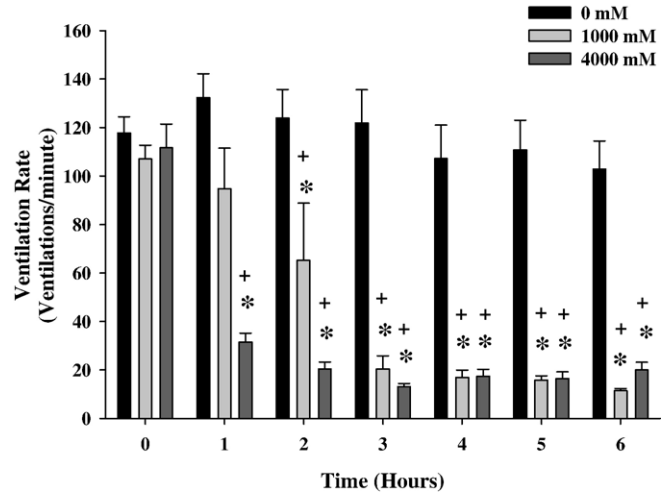


Fig. 2. Change in ventilation rate (ventilations/minute) for largemouth bass held 6 h in either 0 μM (fresh water), 1000 μM or 4000 μM total ammonia (T_{amm}). A plus sign (+) represents ventilation rates that differ significantly from the 0 μM treatment at a particular time point, and an asterisk (*) represents ventilation rates that differ significantly from the Time 0 sample within a treatment group (repeated-measures, two-way ANOVA; Tukey–Kramer HSD *post-hoc* test; $P < 0.05$). Error bars show ± 1 standard error (SE). For each group, $N = 6$, except for the 4000 μM treatment where $N = 5$ at 3 h and $N = 4$ at 4 h, 5 h and 6 h.

mean plasma ammonia concentrations did not differ significantly from control values until ambient ammonia reached 400 μM (Dunnett’s test, $P < 0.05$) (Table 1). When fish were exercised and recovered for 2 h in ambient ammonia concentrations of 1000 μM , mean plasma ammonia values were approximately four-fold greater than control values (Dunnett’s test, $P < 0.05$) (Table 1).

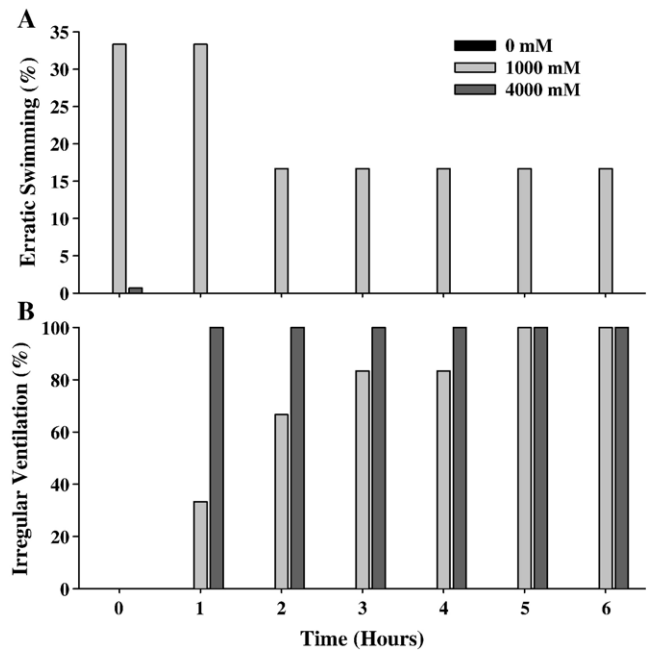


Fig. 3. Percentage of largemouth bass in either 0 μM (fresh water), 1000 μM or 4000 μM total ammonia (T_{amm}) that exhibited (A) erratic swimming (twitching, quivering) and (B) irregular ventilation (episodic or intermittent ventilation as opposed to regular rhythmic ventilation). See caption for Fig. 2 for sample sizes.

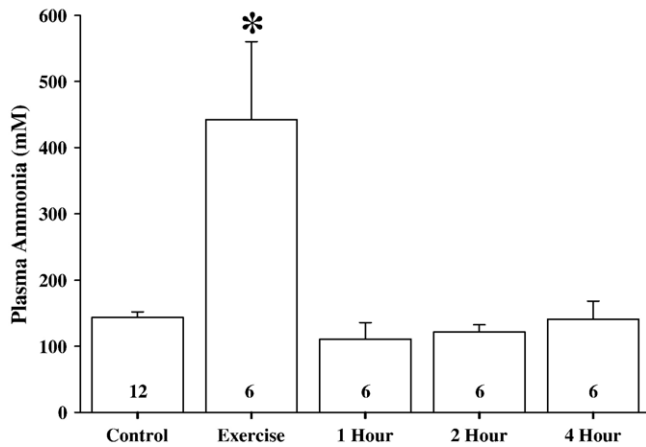


Fig. 4. Plasma ammonia concentrations for largemouth bass exercised 1 min in fresh water and allowed to recover in fresh water for either 1, 2, or 4 h. An asterisk (*) represents a significant difference from the control group for a treatment (ANOVA; Dunnett's test; $P < 0.05$). Error bars show ± 1 standard error (SE) and sample sizes are shown on individual bars.

Mean plasma cortisol concentrations of fish recovered 2 h in 100 μM ambient ammonia were approximately double control values, but the mean plasma cortisol concentrations of all recovery environments did not differ significantly from fish recovered in freshwater (Table 1) (ANOVA, $F_{5,39} = 2.4$, $P = 0.06$). Mean plasma osmolality was significantly elevated relative to the control treatment for fish recovered 2 h in 0, 100, 200 and 1000 μM ambient ammonia (ANOVA, $F_{5,38} = 8.3$, $P = 0.0001$; Dunnett's test, $P < 0.05$), while the control treatment and fish recovered in 400 μM ambient ammonia showed mean plasma osmolality levels that were significantly lower than fish recovered 2 h in freshwater (Table 1) (Dunnett's test, $P < 0.05$). Mean plasma glucose concentrations for largemouth bass recovered 2 h in 400 μM and 1000 μM ambient ammonia were approximately 80% greater than control values and than fish recovered for 2 h in 0 μM ammonia (Table 1) (ANOVA, $F_{5,40} = 7.3$, $P = 0.0001$; Dunnett's test, $P < 0.05$). Mean plasma chloride concentrations did not differ significantly from mean control values at any time during the experiment (ANOVA, $F_{5,38} = 0.9$, $P = 0.5$) and mean white muscle water content did not differ among any treatment groups (ANOVA, $F_{5,35} = 2.2$, $P = 0.07$) (Table 1).

Table 1

Mean concentrations of plasma and tissue parameters (\pm SE) for largemouth bass exercised 1 min in fresh water and allowed to recover for 2 h in various concentrations of ambient total ammonia (T_{amm})

Parameter	Control	0 μM	100 μM	200 μM	400 μM	1000 μM
Plasma ammonia ($\mu\text{M/l}$)	143.7 \pm 8.3 (12)	122.0 \pm 10.6	149.8 \pm 21.7	196.1 \pm 6.6	298.9 \pm 42.0*, ⁺	457.0 \pm 56.0*, ⁺
Plasma cortisol (ng/ml)	50.3 \pm 12.7 (15)	64.8 \pm 8.7	110.0 \pm 14.2	83.1 \pm 11.3	50.3 \pm 12.7	90.0 \pm 19.2
Plasma osmolality (mosM/l)	303.8 \pm 5.0 [†] (14)	334.0 \pm 3.2*	328.6 \pm 5.3*	325.9 \pm 4.8*	309.0 \pm 4.0 [†]	337.7 \pm 4.0*
Plasma chloride (mEq/l)	97.6 \pm 4.7 (14)	99.3 \pm 2.6	97.6 \pm 5.2	97.3 \pm 3.2	86.3 \pm 5.2	98.2 \pm 1.8
Plasma glucose (mM/l)	5.9 \pm 0.4 (16)	6.0 \pm 0.7	8.9 \pm 0.8*	8.4 \pm 1.1	10.7 \pm 1.5*, ⁺	10.9 \pm 1.1*, ⁺
Plasma lactate (mM/l)	1.1 \pm 0.1 (14)	5.8 \pm 0.8*	8.0 \pm 0.7*	8.3 \pm 0.7*, ⁺	5.7 \pm 0.8*	9.7 \pm 0.8*, ⁺
Muscle lactate (mM/kg)	2.2 \pm 0.3 (14)	4.5 \pm 1.4	10.0 \pm 1.0*, ⁺	10.5 \pm 1.5*, ⁺	9.5 \pm 2.9*	11.0 \pm 1.6*, ⁺
Tissue water content (%)	79.4 \pm 0.4 (11)	79.3 \pm 0.2	80.0 \pm 0.3	79.8 \pm 0.4	80.5 \pm 0.2	79.5 \pm 0.4

Control refers to undisturbed fish, and 0 μM refers to fish recovered for 2 h in fresh water. An asterisk (*) represents a significant difference for a treatment from the Control group and a plus sign ([†]) represents a significant difference from the 0 μM recovery group (ANOVA; Dunnett's test; $P < 0.05$). Sample sizes for each treatment is $n = 6$, except where indicated in parentheses.

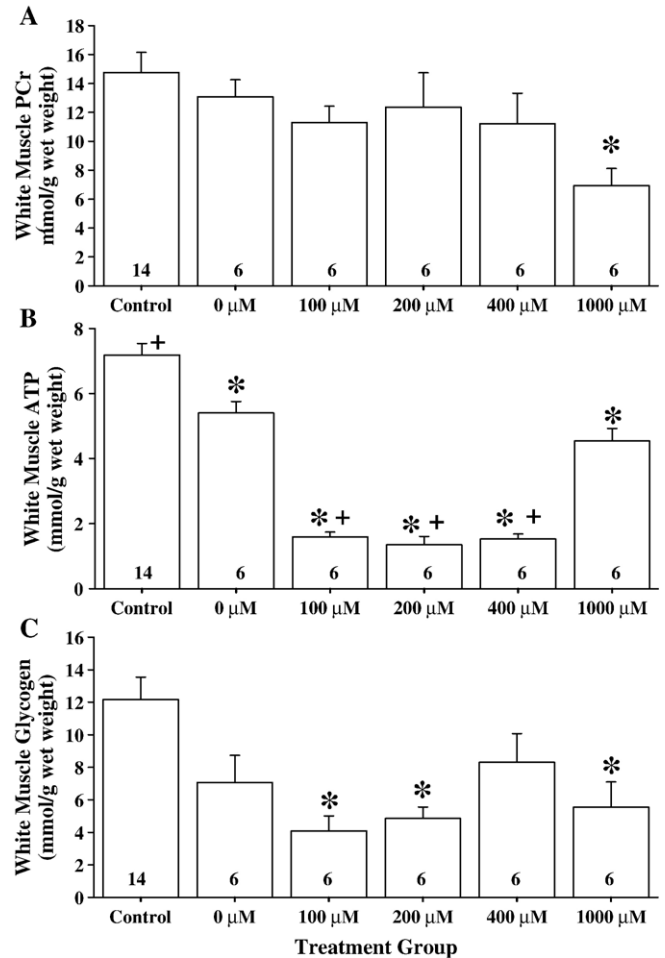


Fig. 5. Muscle (A) phosphocreatine (PCr), (B) adenosine triphosphate (ATP) and (C) glycogen concentration for largemouth bass exercised 1 min in fresh water and allowed to recover 2 h in various concentrations of ambient total ammonia (T_{amm}). An asterisk (*) represents a significant difference from the control group for a treatment, and a plus sign (+) represents a significant difference from the 0 μM recovery group for a treatment (ANOVA; Dunnett's test; $P < 0.05$). Error bars show ± 1 standard error (SE) and sample sizes are shown on individual bars.

After 2-h recovery in fresh water, mean plasma lactate concentrations were approximately six times greater than control (ANOVA, $F_{5,38} = 36.7$, $P < 0.0001$; Dunnett's test, $P < 0.05$) (Table 1). Largemouth bass recovered 2 h in 200 μM and

1000 μM ambient ammonia, however, showed mean plasma lactate concentrations that were 41% and 65% (respectively) greater than fish recovered in fresh water (Dunnett's test, $P < 0.05$). Two-hour recovery in 0 μM ammonia allowed mean muscle lactate levels to return to control values, but recovery in all ambient ammonia concentrations resulted in mean muscle lactate concentrations that were elevated approximately five times above control values (ANOVA, $F_{5,38} = 9.4$, $P < 0.0001$; Dunnett's test, $P < 0.05$) (Table 1). Recovery from exercise in 200 μM and 1000 μM ammonia resulted in mean white muscle lactate concentrations that were approximately 2.4-fold greater than fish recovered in 0 μM ammonia (Dunnett's test, $P < 0.05$) (Table 1).

Two hours of recovery allowed white muscle PCr to return to control values for all treatments except the 1000 μM ambient ammonia treatment where mean PCr concentrations were 47% below control values (ANOVA, $F_{5,38} = 2.7$, $P = 0.04$; Dunnett's test, $P < 0.05$) (Fig. 5A). Two hours of recovery, however, did not permit white muscle ATP concentrations to return to control values for any treatment, and fish recovered in 100 μM , 200 μM and 400 μM ambient ammonia showed mean white muscle ATP concentrations that were approximately one-quarter those of fish recovered in 0 μM ambient ammonia (ANOVA, $F_{5,38} = 58.7$, $P < 0.0001$) (Fig. 5B). Following recovery in 100 μM , 200 μM and 1000 μM ambient ammonia, mean white muscle glycogen concentrations were 33%, 40% and 45% (respectively) below control values, but ambient ammonia levels did not affect muscle glycogen concentrations relative to fish recovered in freshwater (ANOVA, $F_{5,38} = 5.2$, $P = 0.0009$) (Fig. 5C).

4. Discussion

Largemouth bass exhibited behavioural modifications following 2-h exposure to ambient ammonia concentrations of 1000 μM T_{amm} , a concentration approximately 80% below the criteria maximum concentration (CMC) for this species (i.e., the 1-h concentration of ammonia that should not be exceeded more than once every 3 years on average) (US EPA, 1999). These behavioural modifications included erratic swimming and irregular ventilation patterns, both of which have been reported for other freshwater fish species during ammonia exposure (Smart, 1976; Ip et al., 2001). In addition to these symptoms, largemouth bass exhibited reduced ventilation rates during ammonia exposure, primarily resulting from sporadic, intermittent ventilation patterns. Hillaby and Randall (1979) reported an increase, and then a subsequent decrease, in ventilation rates for rainbow trout exposed to elevated internal ammonia concentrations, similar to the results in our study. In contrast, previous static toxicity tests involving both Atlantic salmon (*Salmo salar* L.) (Knoph, 1996) and rainbow trout (Smart, 1978) reported that elevated external ammonia caused an increase in ventilation rates, likely due to the stimulatory actions of catecholamines that are released during ammonia exposure (Knoph, 1996), or from increased oxygen requirements associated with ammonia toxicity (Smart, 1978).

One minute of exercise for largemouth bass resulted in a host of metabolic, osmotic and ionic disturbances to plasma and white muscle that included consumption of energy stores, the production of lactate and a three-fold increase in plasma ammo-

nia. Changes of this nature have previously been reported for numerous fish species following similar exercise treatments (Wood, 1991; Milligan, 1996; Kieffer, 2000; Suski et al., 2006). The increase in plasma ammonia concentration likely occurred as adenylates were broken down to inosine monophosphate (IMP) and NH_4^+ as part of anaerobic energy generation (van Waarde, 1983; Mommsen and Hochachka, 1988) as has been documented in studies involving rainbow trout (Driedzic and Hochachka, 1976; Mommsen and Hochachka, 1988; Wang et al., 1994). The magnitude of increase in plasma ammonia for exercised largemouth bass in the current study is also similar in magnitude to that of exercised rainbow trout (Mommsen and Hochachka, 1988).

In the current study, elevated plasma ammonia was cleared following only 1-h recovery post-exercise, likely via passive excretion at the gills (Wilkie, 2002). In contrast, studies with rainbow trout revealed that recovery of elevated plasma ammonia following exercise is a relatively slow process that required at least 4 h to return to control values (Mommsen and Hochachka, 1988; Wang et al., 1994). Recovery from exhaustive exercise is a complicated process that requires an aerobic environment, and takes from 2 to 12 h for most physiological variables to fully return to resting levels (Mommsen and Hochachka, 1988; Milligan, 1996; Richards et al., 2002; Suski et al., 2006). The difference in ammonia clearance rates between species may suggest an increased proficiency for ammonia excretion in largemouth bass, an increased reliance on internally converting ammonia to urea (Kong et al., 1998), or perhaps a greater focus on excreting ammonia rather than attempting to reincorporate ammonia into metabolic precursors (Mommsen and Hochachka, 1988).

When largemouth bass recovering from exercise are placed in an environment with elevated ambient ammonia, the ammonia generated from exercise cannot be passively excreted down its concentration gradient and it remains circulating in the blood (Wilkie, 2002). As a result, following 2-h recovery in elevated ambient ammonia, plasma ammonia concentrations remain at values exhibited immediately following exercise. Previous work has noted that plasma ammonia levels in rainbow trout correlate positively with ambient ammonia concentrations, and it is believed that this increase results from endogenous sources rather than an inward flux of external ammonia (Fromm and Gilette, 1968; Cameron and Heisler, 1983; Randall and Tsui, 2002). As a result of prolonged ammonia retention within their circulation, the ability of largemouth bass to recover from exercise is impaired relative to fish in fresh water, and effects are mainly evident in white muscle parameters. Recovery of plasma chloride was not affected by ambient ammonia concentrations, and, with the exception of the 400 μM ambient ammonia treatment, both plasma cortisol and plasma osmolality of largemouth bass recovered in ambient ammonia did not differ from individuals recovered in fresh water. Because the water content of white muscle did not differ between treatment groups, we can likely conclude that ambient ammonia concentrations had little impact on the recovery of ionic/osmotic parameters in plasma. Elevated ambient ammonia, however, resulted in elevated lactate concentrations in white muscle and plasma, as well as an impaired ability to replenish muscle energy stores

relative to fish that were recovered in fresh water, highlighting the impact of ammonia concentrations on muscle parameters. One possible mechanism for this impairment may be related to the fact that ammonia can substitute for K^+ in Na^+ , K^+ -ATPase and/or $Na^+/K^+/2 Cl^-$ cotransport (Randall et al., 1999; Ip et al., 2001), as well as increasing urine production (Lloyd and Orr, 1969), thereby impacting the ionic/osmotic status of recovering muscle (Wilkie, 1997; Ip et al., 2001). A more probable explanation, however, relates to the fact that ammonia can substitute for H^+ in Na^+/H^+ exchangers (Randall et al., 1999; Ip et al., 2001) and also that ammonia is typically created in the unionized form (NH_3), but quickly converted to ionized ammonia (NH_4^+) effectively removing protons generated by lactate dissociation and ATP hydrolysis (Hochachka and Mommsen, 1983; Mommsen and Hochachka, 1988; Wood, 1991). Both of these potential mechanisms can disrupt the pH of either plasma or muscle (Cameron and Heisler, 1983), and possibly impact proton excretion/reabsorption into white muscle (Milligan and Wood, 1986), thereby impairing recovery from exercise. More importantly, however, impairments to recovery for largemouth bass occur at T_{amm} of only 100 μM , far below concentrations that result in behavioural modifications during static toxicity tests, and 97% lower than recommended acute concentrations (US EPA, 1999).

The range of ammonia concentrations used in our study appeared to exert varying impacts on recovery from exercise in muscle parameters. For example, muscle lactate concentrations in largemouth bass recovered in 200 μM T_{amm} and 1000 μM T_{amm} ambient ammonia were significantly greater than those of fish recovered in fresh water. However, muscle lactate concentration of fish recovered in 100 μM T_{amm} and 400 μM T_{amm} ambient ammonia was not different than fish recovered in fresh water. Wood (2004) showed that low ammonia concentrations (70 μM T_{amm}) resulted in a stimulation of body mass accumulation and protein production for rainbow trout, but this growth stimulation was significantly reduced for rainbow trout held at 225 μM T_{amm} . Thus, small changes in ambient ammonia concentrations can have pronounced internal consequences for fish.

Based on the results of our study, largemouth bass caught during angling tournaments may be exposed to elevated concentrations of ambient ammonia, but these ammonia concentrations likely are not lethal on their own. After remaining on board a live-release vessel with no freshwater exchange for about 2.5 h, T_{amm} concentrations in the water were approximately 150 μM T_{amm} . After approximately 1.5 h in the live-release vessel, however, T_{amm} concentrations were approaching 100 μM — a concentration shown in the current study to negatively impact the ability of largemouth bass to recover from exercise. Because fish in angling tournaments are added to live-release vessels following air exposure associated with the weigh-in, it is likely that elevated ammonia concentrations in the live-release vessel impair the ability of largemouth bass to recover from metabolic disturbances incurred during such processes (Suski et al., 2004).

5. Recommendations

The typical approach for determining toxicity criteria for a substance is the use of LC_{50} tests in which the suggested 'limit'

of a concentration is based on the mortality of 50% of test subjects over a period of time. These tests involve a prescribed range of toxin concentrations, times, animals, etc. and are typically carried out over 72–96 h (US EPA, 1999). For this study, we integrated behavioural observations and quantification of sub-cellular physiological parameters to assess the response of largemouth bass to ambient ammonia across several levels of organization; the result was a comprehensive and informative technique to determine toxicity guidelines. If, for example, we had exclusively used standard toxicity tests (i.e., mortality across a range of concentrations) to determine if ammonia levels observed at angling tournaments were 'safe' for largemouth bass, we would have determined that the threshold of ammonia toxicity for largemouth bass is likely far greater than concentrations observed at tournaments, thereby concluding that ammonia is not a problem for tournament-caught fish. However, by integrating sub-cellular physiological parameters along with behavioural observations, we were able to ascertain that concentrations of ammonia observed at angling tournaments are not likely lethal, but may have the potential to impact the ability of largemouth bass to recover from physiological disturbances; these potentially negative effects may be seen at values approximately 97% below recommended guidelines (US EPA, 1999). Thus, our integrative approach resulted in the identification of a potential stressor that would have gone undetected with conventional approaches. We therefore advocate the continued integration of physiological tests with conventional LC_{50} procedures during toxicity studies.

There are many different instances where fish can be subjected to exercise, air exposure, and/or handling, and then forced to recover from these disturbances in water with elevated ammonia concentrations. These scenarios can include hauling, aquaculture or angling tournaments (Carmichael et al., 1984; Wood, 2004; Current Study). While the exact mechanisms responsible for fish mortality following angling tournaments are not known, it is believed that mortality results either from metabolic disturbances resulting from hypoxic stressors experienced during the weigh-in (Suski et al., 2004) or from the cumulative impact of multiple sub-lethal stressors incurred throughout the tournament (Schramm et al., 1987; Kwak and Henry, 1995; Suski et al., 2006). Additionally, sub-lethal exposure to ammonia has been shown to reduce swimming performance (Shingles et al., 2001) and suppress feeding (Woltering et al., 1978) in fish. Therefore, based on the findings from this study, we recommend that angling tournament organizers strive to maintain adequate water quality on board live-release vessels to minimize the potential for tournament-caught fish to experience additional stress from elevated ambient ammonia. Water quality in live-release vessels can be maintained either by frequent (or continuous) water changes when holding fish, or by reducing the density of fish being held by using additional vessels. At this point it should be noted that while 2.5 h of containment in a live-release vessel represents a worst-case scenario for angling tournaments in our experience, Carmichael (1984) and Carmichael et al. (1984) performed hauling simulation studies with largemouth bass for 4–30 h, suggesting that 2.5 h of confinement times following handling is possible in hauling and aquaculture scenarios. While the

concentrations of ambient ammonia during recovery may not be toxic or lethal on their own, future studies should be performed at additional angling tournaments across a variety of water temperatures using several different fish species (to identify the impact of water temperature on the ammonia concentrations to which tournament-caught fish can be exposed), and additional work on the impact of ammonia exposure on swimming performance and spawning activities should also be carried out.

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References

- Bergerhouse, D.L., 1992. Lethal effects of elevated pH and ammonia on early life stages of walleye. *N. Am. J. Fish. Manage.* 12, 356–366.
- Broderius, S., Drummond, R., Fiant, J., Russom, C., 1985. Toxicity of ammonia to early life stages of the smallmouth bass at four pH values. *Environ. Toxicol. Chem.* 4, 87–96.
- Cameron, J.N., Heisler, N., 1983. Studies of ammonia in the rainbow trout: physico-chemical parameters, acid–base behaviour and respiratory clearance. *J. Exp. Biol.* 105, 107–125.
- Carlander, K.D., 1977. *Handbook of Freshwater Fishery Biology*, vol. 2. The Iowa State University Press, Ames, Iowa.
- Carmichael, G.J., 1984. Long distance truck transport of intensively reared largemouth bass. *Prog. Fish-Cult.* 46, 111–115.
- Carmichael, G.J., Tomasso, J.R., Simco, B.A., Davis, K.B., 1984. Characterization and alleviation of stress associated with hauling largemouth bass. *Trans. Am. Fish. Soc.* 113, 778–785.
- Driedzic, W.R., Hochachka, P.W., 1976. Control of energy metabolism in fish white muscle. *Am. J. Physiol.* 230, 579–582.
- Emerson, K., Russo, R.C., Lund, R.E., Thurston, R.V., 1975. Aqueous ammonia equilibrium calculations: effect of pH and temperature. *J. Fish. Res. Board Can.* 32, 2379–2383.
- Fromm, P.O., Gillette, J.G., 1968. Effect of ambient ammonia on blood ammonia and nitrogen excretion of rainbow trout (*Salmo gairdneri*). *Comp. Biochem. Physiol.* 26, 887–896.
- Gustavson, A.W., Wydoski, R.S., Wedemeyer, G.A., 1991. Physiological response of largemouth bass to angling stress. *Trans. Am. Fish. Soc.* 120, 629–636.
- Hassid, W.Z., Abraham, S., 1957. Chemical procedures for analysis of polysaccharides. In: Colowick, S.N., Kaplan, N.O. (Eds.), *Methods Enzymol.*, vol. 3, pp. 34–50.
- Hillaby, B.A., Randall, D.J., 1979. Acute ammonia toxicity and ammonia excretion in Rainbow trout (*Salmo gairdneri*). *J. Fish. Res. Board Can.* 36, 621–629.
- Hochachka, P.W., Mommsen, T.P., 1983. Protons and anaerobiosis. *Science* 219, 1391–1397.
- Ip, Y.K., Chew, S.F., Randall, D.J., 2001. Ammonia toxicity, tolerance and excretion. In: Wright, P.A., Anderson, P.M. (Eds.), *Nitrogen Excretion*. New York, Academic Press Inc., New York, pp. 109–148.
- Ivančić, I., Degobbi, D., 1984. An optimal manual procedure for ammonia analysis in natural waters by the indophenol blue method. *Water Res.* 18, 1143–1147.
- Kieffer, J.D., 2000. Limits to exhaustive exercise in fish. *Comp. Biochem. Physiol. A* 126, 161–179.
- Knoph, M.B., 1996. Gill ventilation frequency and mortality of Atlantic salmon (*Salmo salar* L.) exposed to high ammonia levels in seawater. *Water Res.* 30, 837–842.
- Kong, H., Edberg, D.D., Korte, J.J., Salo, W.L., Wright, P.A., Anderson, P.M., 1998. Nitrogen excretion and expression of carbamoyl-phosphate synthetase III activity and mRNA in extrahepatic tissues of largemouth bass (*Micropterus salmoides*). *Arch. Biochem. Biophys.* 350, 157–168.
- Kwak, T.J., Henry, M.G., 1995. Largemouth bass mortality and related causal factors during live-release fishing tournaments on a large Minnesota lake. *N. Am. J. Fish. Manage.* 15, 621–630.
- Lloyd, R., Orr, L.D., 1969. The diuretic response by rainbow trout to sub-lethal concentrations of ammonia. *Water Res.* 3, 335–344.
- Lowry, O.H., Passonneau, J.V., 1972. *A Flexible System of Enzymatic Analysis*. Academic Press Inc., New York.
- Milligan, C.L., 1996. Metabolic recovery from exhaustive exercise in rainbow trout. *Comp. Biochem. Physiol. A* 113, 51–60.
- Milligan, C.L., Wood, C.M., 1986. Intracellular and extracellular acid–base status and H⁺ exchange with the environment after exhaustive exercise in the rainbow trout. *J. Exp. Biol.* 123, 93–121.
- Mommsen, T.P., Hochachka, P.W., 1988. The purine nucleotide cycle as two temporally separated metabolic units: a study on trout muscle. *Metabolism* 37, 552–556.
- Randall, D.J., Tsui, T.K.N., 2002. Ammonia toxicity in fish. *Mar. Pollut. Bull.* 45, 17–23.
- Randall, D.J., Wilson, J.M., Peng, K.W., Kok, T.W.K., Kuah, S.S.L., Chew, S.F., Lam, T.J., Ip, Y.K., 1999. The mudskipper, *Periophthalmodon schlosseri*, actively transports NH₄⁺ against a concentration gradient. *Am. J. Physiol.* 46, R1562–R1567.
- Richards, J.G., Heigenhauser, G.J.F., Wood, C.M., 2002. Lipid oxidation fuels recovery from exhaustive exercise in white muscle of rainbow trout. *Am. J. Physiol.* 282, R89–R99.
- Roseboom, D.P., Richey, D.L., 1977. Acute toxicity of residual chlorine and ammonia to some native Illinois fishes. *Illinois State Water Survey, Urbana, Report of Investigation*, vol. 85, 42 pp.
- Schramm Jr., H.L., Haydt, P.J., Portier, K.M., 1987. Evaluation of pre-release, post-release, and total mortality of largemouth bass caught during tournaments in two Florida lakes. *N. Am. J. Fish. Manage.* 7, 394–402.
- Shingles, A., McKenzie, D.J., Taylor, E.W., Moretti, A., Butler, P.J., Ceradini, S., 2001. The effects of sublethal ammonia exposure on swimming performance in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* 204, 2691–2698.
- Smart, G.R., 1978. Investigations of the toxic mechanisms of ammonia to fish — gas exchange in rainbow trout (*Salmo gairdneri*) exposed to acutely lethal concentrations. *J. Fish Biol.* 12, 93–104.
- Smart, G., 1976. The effect of ammonia exposure on gill structure of the rainbow trout (*Salmo gairdneri*). *J. Fish Biol.* 8, 471–475.
- Sokal, R.R., Rohlf, F.J., 1995. *Biometry*, 3rd ed. W.H. Freeman and Company, New York.
- Suski, C.D., Philipp, D.P., 2004. Factors affecting the vulnerability to angling of nesting male largemouth bass and smallmouth bass. *Trans. Am. Fish. Soc.* 133, 1100–1106.
- Suski, C.D., Killen, S.S., Morrissey, M., Lund, S.G., Tufts, B.L., 2003. Physiological changes in largemouth bass caused by live-release angling tournaments in southeastern Ontario. *N. Am. J. Fish. Manage.* 23, 760–769.
- Suski, C.D., Killen, S.S., Cooke, S.J., Kieffer, J.D., Philipp, D.P., Tufts, B.L., 2004. Physiological significance of the weigh-in during live-release angling tournaments for largemouth bass. *Trans. Am. Fish. Soc.* 133, 1291–1303.
- Suski, C.D., Killen, S.S., Kieffer, J.D., Tufts, B.L., 2006. The influence of environmental temperature and oxygen concentration on the recovery of largemouth bass from exercise: implications for live-release angling tournaments. *J. Fish Biol.* 68, 120–136.
- Thurston, R.V., Russo, R.C., 1985. Acute toxicity of ammonia to rainbow trout. *Trans. Am. Fish. Soc.* 112, 696–704.

- US EPA, 1999. 1999 Update of Ambient Water Quality Criteria for Ammonia. Office of Water, US Environmental Protection Agency, Washington, DC.
- van Waarde, A., 1983. Aerobic and anaerobic ammonia production by fish. *Comp. Biochem. Physiol. B* 74, 675–684.
- Wang, Y., Heigenhauser, G.J.F., Wood, C.M., 1994. Integrated responses to exhaustive exercise and recovery in rainbow trout white muscle: acid–base, phosphogen, carbohydrate, lipid, ammonia, fluid volume and electrolyte metabolism. *J. Exp. Biol.* 195, 227–258.
- Wilkie, M.P., 1997. Mechanisms of ammonia excretion across fish gills. *Comp. Biochem. Physiol. A* 118, 39–50.
- Wilkie, M.P., 2002. Ammonia excretion and urea handling by fish gills: present understanding and future research challenges. *J. Exp. Zool.* 293, 284–301.
- Woltering, D.M., Hedtke, J.L., Weber, L.J., 1978. Predator–prey interactions of fishes under the influence of ammonia. *Trans. Am. Fish. Soc.* 107, 500–504.
- Wood, C.M., 1991. Acid–base and ion balance, metabolism, and their interactions, after exhaustive exercise in fish. *J. Exp. Biol.* 160, 285–308.
- Wood, C.M., 2004. Dogmas and controversies in the handling of nitrogenous wastes: is exogenous ammonia a growth stimulant in fish? *J. Exp. Biol.* 207, 2043–2054.
- Zar, J.H., 1999. *Biostatistical Analysis*, 4th ed. Prentice Hall Inc., New Jersey.