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[Article]

Un-ionized Ammonia Exposure in Nile Tilapia: Toxicity, Stress Response, and Susceptibility to *Streptococcus agalactiae*

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Abstract.—A series of experiments were conducted to determine the toxicity, behavior, blood glucose stress response, and disease susceptibility in Nile tilapia *Oreochromis niloticus* following un-ionized ammonia (UIA) exposure. The acute toxicity of un-ionized ammonia to Nile tilapia was measured in a 96-h static test. The median lethal concentration (LC50) was 1.46 mg/L UIA at 24 and 48 h postexposure, 1.33 mg/L at 72 h postexposure, and 0.98 mg/L at 96 h postexposure. No mortalities were noted in unexposed (0 mg/L) control fish or fish exposed to 0.5 mg/L UIA. However, 93–100% mortalities were observed within 24 h among fish exposed to 2.0, 3.0, or 4.0 mg/L UIA. In additional UIA exposure experiments, Nile tilapia were exposed to sublethal concentrations (0.32–0.37 mg/L UIA) for 24 h and then administered an intraperitoneal injection with 750 colony-forming units (CFU) of *Streptococcus agalactiae* per fish. Mortalities of UIA-exposed and control fish were not significantly different 21 d postchallenge. Blood glucose levels were not significantly different between exposed and control fish 24 h after the beginning of UIA exposure or between preexposure fish and 24-h postexposure fish. Glucose levels in both groups increased significantly after UIA exposure and subsequent bacterial challenge, suggesting that Nile tilapia experienced handling or infection stress and not necessarily UIA exposure stress alone. During a time course study with 24-h UIA exposure, sequential blood glucose samples indicated acute stress responses 1–4 h postexposure that decreased by 24 h postexposure. The results of this study indicate that exposure to increased UIA concentrations alone had acute, transient effects on stress responses in Nile tilapia and that 24-h exposure to sublethal UIA concentrations up to 0.37 mg/L did not increase susceptibility to *S. agalactiae*.

Ammonia is a well-known aquatic pollutant and toxin of fish, and it is produced as an end product of nitrogenous metabolism. Most teleost fish are ammoniotelic and excrete ammonia as their principle waste product. Fish primarily produce ammonia as a result of hepatic deamination of dietary amino acids, enzyme activity of gastrointestinal flora, and nerve and muscle tissue metabolic activity (Fromm and Gillette 1968; Redner and Stickney 1979). Environmental ammonia concentrations increase due to ammonia excretion or because of the breakdown of organic matter in the water (Tomasso 1994). The two forms of ammonia in the environment are un-ionized and ionized ammonia, and un-ionized ammonia (UIA) is toxic to fish because it can easily diffuse across gill membranes (Russo 1985; Tomasso 1994). The concentration of UIA increases with increasing pH and temperature (Emerson et al. 1975; Randall and Tsui 2002), thereby accentuating the effects of increased UIA concentrations.

Sublethal UIA concentrations are known to cause behavioral, physiological, and histologic changes in fish and have several possible mechanisms of toxicity. These mechanisms include causing water and mineral imbalances, decreasing blood pH, altering cardiac function, and affecting ATP levels (Tomasso 1994). Histologic changes include gill hyperplasia, hemorrhage, and telangiectasia, as well as degenerative changes in the kidneys and liver (Thurston et al. 1978; Daud et al. 1988). Exposure to sublethal UIA concentrations can also increase susceptibility to bacterial, fungal, and parasitic diseases such as columnaris (Amin et al. 1988), saprolegniosis (Carballo et al. 1995), and trichodiniasis (Hassan 1999). Ammonia exposure can cause increased susceptibility due to postexposure stress responses and to immune

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mechanism impairment (Mazeaud et al. 1977; Wedemeyer and McLeay 1981; Tomasso 1994). High UIA concentrations reduced the protection conferred by vaccination against *Streptococcus iniae*, an effect presumptively attributed to suppression of nonspecific or cellular immune mechanisms (Hurvitz et al. 1997).

High concentrations or increased durations of exposure to UIA may also directly result in mortalities. Because of the possible effects on fish health and survival, ammonia accumulation is of particular concern in aquaculture. Though the effects of UIA have been studied in hybrid tilapia (Mozambique tilapia *Oreochromis mossambicus* × Nile tilapia *O. niloticus* [Daud et al. 1988]) and blue tilapia *O. aureus* (Redner and Stickney 1979), the LC50, behavioral responses, stress responses, and disease resistance following UIA exposure have not been examined in Nile tilapia *O. niloticus*. The purpose of this study was to determine the effects of UIA on stress, disease resistance, and mortality in this economically important aquaculture species. Special emphasis was placed on the effect of UIA on fish challenged with *S. agalactiae*, an important emerging pathogen of wild and cultured fish (Plumb et al. 1974; Eldar et al. 1995; Evans et al. 2002; Glibert et al. 2002).

Methods

Fish.—Juvenile Nile tilapia were housed at the U.S. Department of Agriculture, Agricultural Research Service (USDA–ARS) Aquatic Animal Health Laboratory in Chestertown, Maryland. The fish were kept in glass aquaria supplied by dechlorinated tap water and maintained on a 12 h light : 12 h dark period. The fish were fed daily to satiation with Aquamax Grower 400 (Brentwood, Missouri). Daily evaluations of dissolved oxygen (DO) and temperature were measured using a YSI 85 oxygen conductivity, salinity, and temperature meter (Yellow Spring Meter, Yellow Springs, Ohio). Total ammonia nitrogen (TAN) and pH were determined daily using a Fresh Water Aquaculture Kit Model AG-2 (LaMotte, Chestertown, Maryland) or HACH test kit (Hach Company, Loveland, Colorado). The amount of UIA was calculated by multiplying the TAN by the appropriate conversion factor according to the measured water temperature and pH (Emerson et al. 1975). Fish were maintained and handled according to Institutional Animal Care and Use Committee (IACUC) approved guidelines.

Median lethal concentration (LC50) of tilapia to un-ionized ammonia.—Nile tilapia (mean weight = 12.6 ± 2.9 g) were acclimated in static 9-L plastic aquaria at 30°C 1 d prior to the experiment. Fish were fed 1 d prior to the experiment, with no feeding during the experiment. Three replicate tanks of five fish each were used for each group exposed to one of six UIA concentrations (0, 0.5, 1.0, 2.0, 3.0, or 4.0 mg/L). At the beginning of the experiment, an appropriate amount of ammonium chloride (NH_4Cl; Fisher Scientific, Fair Lawn, New Jersey) stock solution was added to each tank to achieve the desired UIA concentration. Tanks were observed at 0, 3, 4, 21, 23, 24, 31, 48, 72, 96, and 120 h postexposure to assess mortality and behavior. Water samples were also obtained at these time points to determine UIA concentrations. The LC50 was calculated using the trimmed Spearman–Karber (TSK) method, version 1.5 (U.S. Environmental Protection Agency 1991). Mortalities were removed immediately, and behavioral abnormalities were assessed at these regular intervals using a modified behavioral protocol checklist (Klesius et al. 2000). Scores were assigned daily to individual fish in the experiment and were based on the following scoring system: 0, no observed changes in behavior; 1, swimming abnormally, lethargic or unresponsive, changes in skin coloration; 2, hyperactive or excitable, rapid operculation; 3, death. Mean behavior scores were calculated per replicate treatment. Mean scores were divided equally to represent four ranges as follows: 0.0–0.75 (normal behavior), 0.76–1.50 (abnormal behavior), 1.51–2.25 (stressed behavior), and 2.26–3 (morbid behavior). The mean water quality conditions throughout the duration of the experiment were as follows: temperature, 29.84 ± 0.03°C; DO, 5.61 ± 0.08 mg/L; pH, 7.58 ± 0.02.

Responses to sublethal un-ionized ammonia and experimental S. agalactiae challenge.—Nile tilapia (mean weight = 16.2 ± 4.3 g in the first 24-h UIA challenge experiment; mean weight = 16.3 ± 2.5 g in the second 24-h UIA challenge experiment) were placed in static water with approximately 0.32–0.37 mg/L UIA or no UIA for 24 h. These concentrations of UIA were chosen for the 24-h UIA challenge experiments because they are above the no-observed-effect level (NOEL) calculated based on the LC50 from the first experiment. A stock solution of ammonium chloride (4 mg/L) was dripped into the experimental tanks over a 2-h period to obtain a final concentration of 0.32–0.37 mg/L UIA. A phosphate-buffered saline (PBS) sham stock solution without ammonium chloride was dripped into the four control tanks. A HACH test kit (Hach) was used to provide accurate TAN readings, and water samples were taken four times within each exposure period to determine UIA concentrations. After 24-h UIA exposure, the water flow was restored to all tanks and the fish were challenged with 750 colony-forming units (CFU) of *S. agalactiae*

per fish by intraperitoneal injection (Evans et al. 2003). The intraperitoneal challenge route is a reliable and reproducible method that ensures challenge of all individual fish with a uniform bacterial dose (Nordmo 1997; Nordmo and Ramstad 1997). Each group consisted of seven fish in three replicate tanks, and fish were observed for 21 d postchallenge. Dead fish were removed twice daily, and bacterial samples were obtained aseptically from the brain and anterior kidney. Samples were cultured onto sheep blood agar (Remel, Lenexa, Kansas), and *S. agalactiae* was identified as beta-hemolytic, Gram-positive, oxidase-negative cocci.

During the 24-h UIA challenge experiments, blood glucose levels were measured. Blood sampling time periods were as follows: immediately prior to exposure to sublethal UIA or no UIA; 24 h after exposure and immediately before challenge; 24 h after *S. agalactiae* challenge; and 48 h after *S. agalactiae* challenge if no increase in glucose levels after challenge. Blood glucose levels—a recognized, reliable indicator of stress responses in fish (Andersen et al. 1991; Chen et al. 1995; Cech et al. 1996; Reubush and Heath 1996; Ortuño et al. 2001)—were determined using the methods of Evans et al. (2003, 2004). Briefly, blood glucose levels were measured using a glucose meter and test strip (One-Touch Ultra Brand Meter, Lifescan, Milpitas, California). The blood sample was taken from the caudal vein using a tuberculin syringe and 27-gauge needle, and no anesthetic was used for the procedure. A 5–10-μL blood drop was placed onto a clean glass slide, the test strip was then dipped in the blood drop, and the confirmation window was allowed to completely fill with the blood drop (Diouf et al. 2000). The glucose concentration was displayed (mg/dL) in about 5 s. The accuracy of the glucose meter to determine Nile tilapia blood glucose levels was previously established by Evans et al. (2003, 2004). The authors used ten replicates of blood samples from healthy Nile tilapia to determine the sensitivity of the blood glucose protocol at 20 mg/dL and observed a 3.25% intra-assay variance (mean = 20.4 ± 0.66 mg/dL). The reaction of the glucose monitor was also correlated with a colorimetric commercial laboratory method, and the resulting correlation coefficient (r) was 0.928 at a P less than 0.001.

During the challenge experiments, the mean water quality conditions were as follows: temperature, 30.1 ± 0.5°C; DO, 3.9 ± 0.16 mg/L; pH, 7.53 ± 0.11. During the UIA exposure periods, the UIA concentrations were 0.37 ± 0.08 mg/L for the exposed fish and 0.01 ± 0.01 mg/L for the control fish in the first 24-h challenge experiment, and 0.32 ± 0.08 mg/L for the exposed fish and 0.03 ± 0.01 mg/L for the control fish in the second 24-h UIA challenge experiment.

Glucose time course study after short-term exposure to sublethal un-ionized ammonia.—Because no apparent stress responses were observed after 24-h UIA exposure in the challenge studies, a time course study was performed to determine glucose levels through several time points during a 24-h exposure. Larger fish were used in the time course study because fish were repetitively bled after exposure, and smaller fish presumably would not withstand such a rigorous bleeding schedule. Nonetheless, larger fish would give an indication as to the stress responses of fish exposed to elevated UIA. Nile tilapia (mean weight = 63.0 ± 9.9 g; $N = 5$ in quadruplicate tanks) were exposed to 0.32 mg/L UIA or no UIA for 24 h. After this 24-h exposure period, a 50% fresh water replacement was performed on each tank. Fish from each group were bled at 0, 1, 2, 4, 8, and 24 h postexposure and 24 h after the 50% freshwater replacement to determine blood glucose levels as described above. Water samples were also obtained at 0, 1, 2, 4, 8, and 24 h postexposure, and 6 and 24 h after the 50% fresh water replacement to determine UIA concentrations. The mean water quality conditions during the 24-h exposure were as follows: temperature, 30.08 ± 0.06°C; DO, 5.22 ± 0.14 mg/L; pH, 7.64 ± 0.05. The mean UIA in the control tanks was 0.09 ± 0.02 mg/L. After the 50% fresh water replacement, the water quality conditions were: temperature, 29.98 ± 0.28°C; DO, 4.97 ± 0.46 mg/L; pH, 7.73 ± 0.09. During this period, the mean UIA in the exposed tanks was 0.25 ± 0.09 mg/L, while the mean UIA in the control tanks was 0.17 ± 0.10 mg/L.

Statistical analysis.—Median lethal concentrations (LC50) of Nile tilapia to UIA were derived using the trimmed Spearman–Karber (TSK) method, version 1.5. All other data analysis was performed using SAS Institute software (Cary, North Carolina). Water quality, behavior, and blood glucose data were analyzed by analysis of variance (ANOVA) followed by means comparisons using Duncan's multiple-range test. Mortality patterns in control and UIA-exposed groups were compared by Lifetest procedure (Kaplan–Meier method). For all data analysis, differences were considered significant at P less than 0.05.

Results

Median Lethal Concentration (LC50) of Nile Tilapia to Un-Ionized Ammonia

Nile tilapia were exposed to different concentrations of UIA to determine the LC50, which remained constant at 1.46 mg/L for 24 and 48 h postexposure and decreased to 1.33 mg/L at 72 h postexposure, 0.98

mg/L at 96 h postexposure, and 0.85 mg/L at 120 h postexposure (Table 1). Within 24 h postexposure, all of the fish exposed to 2.0 and 4.0 mg/L UIA died, and 93% of the fish exposed to 3.0 mg/L died. Among the fish exposed to 1.0 mg/L UIA, 13% of the fish died within 72 h postexposure, and 67% of the fish died within 120 h postexposure. No deaths occurred among the controls or fish exposed to 0.5 mg/L UIA.

The behavioral scores of the exposed fish escalated with increased duration of exposure and UIA concentration (Table 3). At 3 and 4 h postexposure, the behavioral scores of all of the UIA-exposed fish were significantly greater than the control group. The increased behavioral scores in the 0.5 and 1.0 mg/L UIA groups were linked to changes in coloration, lethargy, and decreased swimming activity. The behavioral score for the 4.0 mg/L UIA group increased until 21-h postexposure, when all of the fish had died and the behavioral score was 3.0. Similar behaviors were observed in the 2.0 and 3.0 mg/L UIA groups, which also had increases in behavioral scores until all of the fish had died. The majority of fish failed to exhibit clinical signs before the onset of mortality, though a few fish showed dark coloration, hyperactivity, and head-up swimming.

The 0.5- and 1.0-mg/L UIA groups had significantly higher behavioral scores than the controls between 3 and 23 h postexposure, but the scores did not increase as drastically as in the higher concentration groups over 120 h. Behavioral scores of the 0.5 and 1.0 mg/L UIA groups dropped around 0 again between 23 and 96 h postexposure before they significantly increased again between 96 and 120 h postexposure. The highest behavioral scores for these groups were observed at 120 h postexposure. During the periods of increasing behavioral scores, the elevations again corresponded to decreased swimming activity, lethargy, darkened coloration, and mortalities, though the 0.5 mg/L UIA group exhibited hyperactivity and rapid operculation at 120 h postexposure, and the 1.0 mg/L UIA group experienced mortalities starting after 48 h postexposure.

Responses to Sublethal Un-Ionized Ammonia and Experimental S. agalactiae Challenge

In the first 24-h UIA challenge experiment, Nile tilapia were exposed to 0.37 mg/L UIA (as exposed groups) or 0.01 mg/L UIA (as control groups) for 24 h and then challenged with *S. agalactiae*. Blood samples taken before exposure to UIA showed that glucose levels were not significantly different between the two groups (Table 3). Twenty-four hours after sublethal UIA exposure, glucose levels were slightly higher than the preexposure levels. However, no significant differences were noted between the groups or between time periods. Twenty-four hours after bacterial challenge, blood glucose levels were significantly higher for the exposed fish (107.4 $\pm$ 18.6 mg/dL) than at earlier time points, and the exposed group had a significantly higher blood glucose level than the control group (43.5 $\pm$ 7.6 mg/dL). However, both the exposed and control groups experienced low-level, sustained mortalities, resulting in cumulative mortalities of 86% over 21 d (Table 4). Within the 21-d challenge period, the mean days to death was 9.1 $\pm$ 1.4 d for exposed fish and 10.9 $\pm$ 1.6 d for controls. Mortality data were not significantly different between the groups ($P < 0.6227$).

In the second 24-h UIA challenge experiment, Nile tilapia were exposed to 0.32 mg/L UIA (as exposed groups) or 0.03 mg/L UIA (as control groups) for 24 h and then challenged with *S. agalactiae*. Blood samples taken before exposure to UIA and 24 h after UIA exposure showed that glucose levels were not significantly different between the two groups (Table 3). No significant differences were noted between the groups or between time periods. Because no significant differences were noted at 24 h postchallenge as in the first 24-h UIA challenge experiment, blood was

TABLE 1.—Fraction dying and percent mortality of Nile tilapia after exposure to various un-ionized ammonia (UIA) concentrations (mg/L). The median lethal concentrations were as follows: 24 h, 1.46 mg/L; 48 h, 1.46 mg/L; 72 h, 1.33 mg/L; 96 h, 0.98 mg/L; and 120 h, 0.85 mg/L. The data are from triplicate aquaria for each UIA concentration, and each aquarium contained five fish.

	Hours postexposure									
	24		48		72		96		120	
UIA concentration	Fraction	%	Fraction	%	Fraction	%	Fraction	%	Fraction	%
0.0	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0
0.5	0/15	0	0/15	0	0/15	0	0/15	0	0/15	0
1.0	0/15	0	0/15	0	2/15	13	8/15	53	10/15	67
2.0	15/15	100	15/15	100	15/15	100	15/15	100	15/15	100
3.0	14/15	93	14/15	93	14/15	93	15/15	100	15/15	100
4.0	15/15	100	15/15	100	15/15	100	15/15	100	15/15	100

TABLE 2.—Mean behavioral scores of Nile tilapia exposed to various concentrations of un-ionized ammonia (UIA; mg/L). Low scores reflect normal behavior. The highest score attainable is 3, which would indicate death in all fish within a concentration. Different letters represent a significant differences ($P < 0.05$) within a time period.

UIA concentration	Hours postexposure										
	0	3	4	7	21	23	24	48	72	96	120
0.0	0 z	0 z	0 z	0 z	0 z	0 z	0 z	0 z	0 z	0.1 z	0.1 z
0.5	0 z	0.7 y	0.4 z	1.1 y	0.6 y	0.9 y	0 z	0 z	0 z	0 z	1.3 y
1.0	0 z	1 y	1 y	0.3 y	0.5 y	0 z	0 z	0 z	0.6 y	1.7 y	2.5 y
2.0	0 z	1 y	1 y	0.8 y	2.9 x	3 x	3 x	3 x	3 x	3 x	3 x
3.0	0 z	1 y	1.1 y	0.8 y	2.9 x	2.9 x	2.9 x	2.9 x	2.9 x	3 x	3 x
4.0	0 z	1.9 x	2.1 x	2.4 x	3 x	3 x	3 x	3 x	3 x	3 x	3 x

sampled again at 48 h postchallenge. Forty-eight hours after bacterial challenge, blood glucose levels were significantly higher for both the exposed fish (127.1 ± 10.8 mg/dL) and control fish (118.8 ± 21.7 mg/dL) compared with levels at prior sampling time points. No significant difference was noted between these two 48-h glucose levels. Both the exposed and control groups experienced low-level, sustained mortalities, resulting in cumulative mortalities of 86% for exposed fish and cumulative mortalities of 62% for control fish over 21 d (Table 4). Within the 21-d challenge period, the mean days of survival was 10.7 ± 1.4 d for exposed fish and 13.0 ± 1.8 d for controls. Again, mortality data were not significantly different between the groups ($P < 0.2859$).

Glucose Time Course Study after Short-Term Exposure to Sublethal Un-Ionized Ammonia

Nile tilapia were exposed to 0.32 mg/L UIA and blood was drawn to determine blood glucose levels following short-term exposure. Over the course of 2 h, the mean blood glucose levels in the exposed fish increased significantly from approximately 33.0 to 97.5 mg/dL (Table 5). Meanwhile, the mean blood glucose levels among the controls increased slightly, but the mean levels were not elevated to those of the exposed fish until 8 h had passed; the exposed fish mean blood glucose values were significantly higher than those of the controls at 1, 2, and 4 h postexposure. The exposed and control fish blood glucose levels peaked at 8 h at 120.5 and 103.3 mg/dL, respectively, but no significant differences were observed between the two groups. No mortalities were noted in controls or exposed fish in the 24-h period. After the 50% water replacement at 24 h postexposure, glucose levels among the exposed group fish decreased from 83.3 mg/dL at 24 h postexposure to 70.5 mg/dL at 24 h after the water replacement. This decrease was not significant. Glucose levels among the controls decreased significantly during the same time period from 88.0 mg/dL at 24 h postexposure to 65.3 mg/dL at 24 h after the water replacement. No significant differences were noted between the exposed and control fish at 24 h after the water replacement.

Discussion

Un-Ionized Ammonia Toxicity

Nile tilapia were less resistant to the toxic effects of UIA as compared with other tilapia species. The LC50

TABLE 3.—Mean blood glucose values (mg/dL) of control and un-ionized ammonia (UIA)–exposed Nile tilapia at time points before exposure to UIA, after exposure to UIA, and after challenge with 750 colony-forming units of *Streptococcus agalactiae* per fish. Tilapia were divided into triplicate aquaria and exposed to UIA or not exposed (control). Exposed fish were held in water containing 0.37 (first 24-h challenge experiment) or 0.32 mg/L (second challenge experiment) UIA. Data from control and exposed fish are represented as mean glucose levels ± SE, and different letters indicate significant ($P < 0.05$) differences between time intervals for each treatment. Asterisks denote significant differences between control and exposed fish within a challenge study at any time period; NS = not sampled.

Hour	First 24-h UIA challenge experiment		Second 24-h UIA challenge experiment	
	Control	Exposed	Control	Exposed
Pre-Exposure				
0	32.8 ± 2.2y	30.2 ± 1.1z	40.9 ± 1.3z	44.5 ± 1.8z
Post-Exposure				
24	36.7 ± 1.8y	40.5 ± 2.1z	42.1 ± 1.3z	42.6 ± 1.7z
Post-Challenge				
24	43.5 ± 7.6y*	107.4 ± 18.6y*	52.3 ± 13.1z	40.3 ± 2.8z
48	NS	NS	118.8 ± 21.7y	127.1 ± 10.8y

TABLE 4.—Mortality patterns among control and un-ionized ammonia (UIA)–exposed Nile tilapia challenged with *Streptococcus agalactiae*. Tilapia were injected intraperitoneally with 750 colony-forming units of *S. agalactiae* per fish after the exposed groups were held under increased UIA conditions for 24 h. Following challenge, fish were monitored for 21 d. No significant differences in mortality patterns were noted between groups during any of the two experiments.

Challenge study	Group	Duration of exposure (h)	Mean UIA concentration (mg/L)	Percent mortality	Days to death (mean ± SE)	*P*-value
1	Control	24	0.01	86	10.9 ± 1.6	
1	UIA exposed	24	0.37	86	9.1 ± 1.4	0.6227
2	Control	24	0.03	62	13.0 ± 1.8	
2	UIA exposed	24	0.32	86	10.7 ± 1.4	0.2859

for these fish was 1.46 mg/L UIA at 24 and 48 h, 1.33 mg/L at 72 h, and 0.98 mg/L UIA at 96 h. In contrast, the LC50 at 48, 72, and 96 h were 6.60, 4.07, and 2.88 mg/L UIA, respectively, for hybrid tilapia (Mozambique tilapia *Oreochromis mossambicus* × Nile tilapia *O. niloticus* [Daud et al. 1988]), and the LC50 at 24, 48, and 72 h were 2.46, 2.40, and 2.35 mg/L UIA, respectively, for blue tilapia *O. aureus* (Redner and Stickney 1979). However, the LC50 values from this study are comparable to those derived for other types of cultured fish, including reciprocal cross hybrid striped bass (white bass *Morone chrysops* × striped bass *M. saxatilis* [0.58 mg/L UIA at 24 h and 0.40 mg/L at 96 h; Harcke and Daniels 1999]), rainbow trout *Oncorhynchus mykiss* (Thurston and Russo 1983), and channel catfish *Ictalurus punctatus* (0.50 mg/L UIA at 96 h) (Tomasso et al. 1980). In addition, the UIA toxicity to Nile tilapia appears to be similar to that of common carp *Cyprinus carpio* fry, which had an LC50 of approximately 1.76 mg/L at 24, 48, and 96 h.

Relative tolerance to UIA may be attributed to water quality differences. For example, when DO is low, UIA is more toxic to fish (Merkens and Downing 1957; Daud et al. 1988). In our study, the mean DO concentration in the tanks was 5.6 mg/L compared with 8.35 mg/L (Daud et al. 1988) and a range of 5.7–7.3 mg/L (Redner and Stickney 1979) in the other tilapia studies. The apparent increased UIA tolerance of other tilapia species may have been influenced by different DO concentrations or by fish age and size. In our LC50 study, the Nile tilapia had a mean standard length of 89.3 mm, while the other tilapia studies used fish with mean standard lengths of 21 mm and 70–90 mm (Redner and Stickney 1979; Daud et al. 1988). The larger tilapia in our study seemed to have decreased UIA tolerance, suggesting that tilapia lose resistance as they grow. However, this conclusion does not fit with Thurston and Russo (1983), who found that rainbow trout UIA tolerance increased as the fish aged through the yearling stage. If the findings of Thurston and Russo are also applicable to tilapia, then our results suggest that tilapia UIA tolerance is determined mostly by tilapia species and DO.

The UIA no-effect levels (NOEL) calculated based on the methods of Tomasso (1994) corresponded to 0.131 mg/L UIA at 24 and 48 h, 0.120 mg/L UIA at 72 h, and 0.088 mg/L UIA at 96 h. These values show the maximum UIA concentrations at which the UIA would have no long-term effects on growth or survival. At 30°C and pH of 7.6, these NOEL correspond to TAN of 4.2 mg/L at 24 and 48 h, 3.9 mg/L at 72 h, and 2.8 mg/L at 96 h, respectively. Though UIA concentrations in aquaculture systems may not reach the LC50 concentrations found in this study, they may rise above the NOEL values and affect the long-term growth and survival of the Nile tilapia.

Behavioral changes were also noted with UIA exposure of the Nile tilapia. The Nile tilapia exposed to higher UIA concentrations first experienced mortalities within 4 h and exhibited decreased swimming activity, lethargy, and darkened coloration within 21 h. A few fish exposed to lower UIA concentrations displayed the same clinical signs throughout the study, but other fish in these groups exhibited hyperactivity and rapid operculation. Israeli-Weinstein and Kimmel (1998) observed that common carp exposed to sublethal concentrations of UIA exhibited altered behavior. Exposed fish initially settled on the bottom of the tank and later moved higher in the water column with increasing UIA concentrations and reached the surface with the highest UIA concentrations. These authors believed that the carp developed respiratory difficulties because of gill mucus accumulation, causing the fish to gather at the surface. With increased concentrations and duration of UIA exposure, fish became more lethargic and anorexic, and blood glucose levels increased significantly. Israeli-Weinstein and Kimmel (1998) proposed that the apparent lethargy was a factor of exhaustion or an attempt to retain energy needed to overcome stress. Randall and Tsui (2002) also point out that active fish have increased ammonia production and are increasingly affected by environmental ammonia. These authors indicate that resting would

TABLE 5.—Mean ± SE blood glucose values (mg/dL) of control and un-ionized ammonia (UIA)–exposed Nile tilapia from the glucose time course study. Tilapia were placed in triplicate aquaria and exposed to 0.32 mg/L or no UIA (controls), and glucose levels were monitored through 24 h postexposure and 24 h after 50% fresh water replacement. Different letters indicate significant ($P < 0.05$) differences between time intervals for each treatment. Asterisks denote significant differences between control and exposed fish at any time period.

Hour	Controls	Exposed
Preexposure		
0	31.3 ± 1.5 z	33.0 ± 1.9 z
Postexposure		
1	34.8 ± 4.7 z*	62.0 ± 3.1 y*
2	59.0 ± 5.6 xy*	97.5 ± 9.9 x*
4	42.3 ± 2.9 yz*	93.0 ± 8.9 x*
8	103.3 ± 14.9 w	120.5 ± 5.0 w
24	88.0 ± 11.0 w	83.3 ± 11.8 xy
After 50% fresh water replacement		
24	65.3 ± 10.6 x	70.5 ± 11.2 y

decrease ammonia production and susceptibility and also demonstrate that the LC50 for resting fish is higher than for active fish. In addition, Shingles et al. (2001) and Wicks et al. (2002) indicate that high ammonia concentrations probably decrease muscle membrane potential and alter muscle metabolism, subsequently causing decreased swimming activity. The behavioral changes may also be linked to branchial lesions caused by UIA exposure, which potentially causes respiratory difficulties: Daud et al. (1988) observed gill hemorrhage and erratic swimming among morbid tilapia fry, and Redner and Stickney (1979) found congestion, telangiectasia, and hemorrhage in the gills of tilapia subjected to sublethal and acute doses of UIA.

Because of the potential negative effects of UIA exposure and subsequent stress, fish have several physiological mechanisms of managing increased UIA concentrations. Adaptation to increased ammonia levels involves altered cell membrane permeability, improved ammonia detoxification or excretion, or a combination of the two (Lloyd and Orr 1969; Olson and Fromm 1971; Randall and Tsui 2002). Other researchers have suggested that one or both of these adaptive mechanisms occur in *Oreochromis* spp. In the study by Redner and Stickney (1979), *O. aureus* had an LC50 of 2.40 mg/L UIA at 48 h. However, all fish chronically exposed to sublethal UIA later survived 48-h exposure to 3.40 mg/L UIA, possibly because of enhanced glutamine synthetase and glutamate dehydrogenase activity after the sublethal exposure (Redner and Stickney 1979; Tomasso 1994). Lake Magadi tilapia *O. alcalicus grahami* have an especially high UIA tolerance (Walsh et al. 1993; Wilkie and Wood 1996), resulting in an LC50 of approximately 9.25 mg/L UIA at 24 h. This relative adaptation to high UIA concentrations also originates from elevated hepatic glutamine synthetase activity, which mediates the formation of glutamine from glutamate and ionized ammonia. This reaction detoxifies UIA and allows for the production of urea, which is excreted by the tilapia instead of ammonia (Mommsen et al. 1992; Walsh et al. 1993; Wilkie and Wood 1996). Though the mechanisms of UIA adaptation in Nile tilapia were not assessed here, the tilapia appear to have recovered from constant sublethal UIA concentrations within 24 h in the UIA challenge experiments.

Stress Responses

Before un-ionized ammonia exposure.—The baseline Nile tilapia glucose levels at 30°C were between 30 and 45 mg/dL, and these levels were similar to the baseline levels previously found in 39-g Nile tilapia held at 31°C as reported by Evans et al. (2004; 41.0–53.0 mg/dL). Slightly lower glucose levels have been reported in 30-g and 21-g Nile tilapia held at 25–26°C by Evans et al. (2003; 26.0–32.0 mg/dL) and by Benli and Yildiz (2004; 21.8 mg/dL), respectively. These marginal differences in Nile tilapia baseline glucose levels may reflect differences in physiological state at higher temperatures.

After un-ionized ammonia exposure.—In the UIA challenge experiments, Nile tilapia were exposed to a sublethal concentration of UIA (0.32 or 0.37 mg/L UIA) for 24 h and injected with 750 CFU of *S. agalactiae* per fish. These UIA values were meant to correspond with a concentration potentially found in aquaculture facilities and environmental settings (Plumb et al. 1974; Eldar et al. 1995; Glibert et al. 2002). While the UIA concentration was lower than the LC50 (1.46 mg/L UIA at 24 h), it was still considerably higher than the NOEL (0.131 mg/L UIA at 24 h) calculated for tilapia and would thus potentially promote stress responses, morbidity, and mortality. In the 24-h UIA challenge experiments, the exposed fish failed to exhibit significantly different glucose stress

responses compared with controls 24 h postexposure, and the glucose values were almost identical.

Because no apparent stress responses were observed after 24-h UIA exposure in the challenge studies, a time course study was performed to determine glucose levels through several time points during a 24-h exposure. Larger fish were used in the time course study because these fish presumably tolerated the repetitive bleeding schedule better than smaller fish and still gave an indication as to the stress responses of fish exposed to elevated UIA. Exposed fish exhibited significantly increased glucose stress responses between 1 h (62.0 mg/dL UIA) and 8 h (120.5 mg/dL UIA) postexposure. Therefore, the glucose stress responses may have been missed in the 24-h UIA challenge experiments that only assessed the glucose levels at 0 and then 24 h postexposure. The rapid, transitory glucose stress response shown in the time course experiment has not been demonstrated in previous studies of sublethal UIA exposures, since blood glucose is often not measured during the 24-h UIA exposure. Though some researchers have observed significant blood glucose level increases within 2 h and up to 24 h following exposure to a stressor (Vijayan et al. 1997), others have observed acute glucose increases that resolve within 24 h (Strange 1980; Evans et al. 2003; Small 2004). Strange (1980) determined basal glucose concentrations (approximately 30 mg/dL) among channel catfish held at 30°C and studied glucose concentrations in fish exposed to confinement stress. Glucose concentrations in stressed fish increased significantly within 0.5 h and increased steadily over 12 h (to 250 mg/dL), though fish subjected to confinement stress showed no significant increase in glucose at a 24-h time point. This indicates that glucose levels can increase within hours of stressor initiation but still may decrease to normal levels within 24 h. Likewise, Small (2004) did not observe significantly increased glucose levels between channel catfish exposed to 1.0 mg/L UIA for 24 h (60 mg/dL) and controls (approximately 45 mg/dL). Israeli-Weinstein and Kimmel (1998) indicated increases in blood glucose values in common carp exposed to one of three separate ranges (0.04–0.08; 0.12–0.27; or 0.4–0.8 mg/L) of fluctuating UIA concentrations for 24 h and also 24 and 48 h following removal of UIA. Glucose levels among controls ranged from 21.0 to 32.6 mg/dL, and values among exposed fish ranged from 39.4 to 41.4 mg/dL within 24 h of exposure initiation and from 40.4 to 52.5 mg/dL following UIA removal. Israeli-Weinstein and Kimmel (1998) reported that these postexposure glucose levels were significantly increased, but these glucose levels were surprisingly low and approximated baseline levels for Nile tilapia in our studies.

Because Nile tilapia adapt to increased UIA concentrations, this may limit detrimental stress responses and prevent histologic changes, increased susceptibility to infection, and mortality. Glucose values for exposed fish, although elevated, were not significantly different from control values at 8 h. This suggests that handling, confinement, and repetitive bleeding effects may manifest at 8 h, an effect documented in other stressor studies (Strange 1980; Vijayan et al. 1997; Gomes et al. 2003). Likewise, glucose values for exposed and controls were not significantly different at 24-h post-UIA exposure or 24 h after the 50% water replacement in the time course trial, but these values were twice the values of exposed and control fish in both 24-h UIA challenge experiments, suggesting continuing recovery from handling. In the absence of continued stressors, glucose levels recovery responses have been seen after removal of different types of stressors. Evans et al. (2003) demonstrated acute glucose stress responses in Nile tilapia after the introduction of low DO. In that time course study, glucose levels increased significantly in fish exposed to 1 mg/mL DO at multiple intervals during 24 h, and then fell to the levels of the control fish within 4 h after the restoration of optimal DO. Gomes et al. (2003) observed significant blood glucose increases in tambaqui *Colossoma macropomum* immediately after transportation. However, glucose levels decreased to those of control fish 24 h after transport completion.

Postchallenge infection stress.—An increase in glucose was not observed until 24 or 48 h postchallenge. Since these increases occurred in both the exposed and control groups after bacterial challenge, the observed physiologic responses must be due to handling, infection stress, or both rather than only the UIA exposure. Evans et al. (2003) observed that 24-h exposure to sublethal DO resulted in elevated glucose levels, but glucose values returned to baseline control levels 24 h after challenge with 750 CFU of *S. agalactiae* per fish. Nonetheless, this 24-h stress response appeared sufficient to suppress the protective mechanisms of innate resistance to *S. agalactiae*. In another study, Evans et al. (2004) indicated that *S. agalactiae* infection alone resulted in significant glucose stress responses at 24, 48, and 72 h postchallenge with a return to baseline glucose levels after 13 d postchallenge. In that study, blood glucose levels and mortality of infected controls were significantly correlated, suggesting that blood glucose levels were good indicators of infection stress. Benli and Yildiz (2004) presumptively attributed a glucose

stress response (234.0 mg/dL) in Nile tilapia spontaneously infected with *Edwardsiella tarda* to poor water quality conditions rather than infection, possibly because the authors did not equate stress responses to disease processes. Blood glucose values were obtained from infected fish from tanks where UIA was 1.78 mg/L and from control tilapia maintained at 0.47 mg/L UIA for unspecified lengths of time (Benli and Yildiz 2004). In our 24-h UIA challenge experiments, elevated glucose in both control and exposed fish 24 or 48 h postchallenge was, in part, the result of handling–infection stress that manifested at variable times. Future studies may involve sampling glucose from fish at the onset of mortality and at a sufficient duration of time postchallenge such that correlations between glucose levels, mortality, and disease susceptibility can be performed.

Disease Susceptibility

Unexpectedly, exposure to increased UIA concentrations and *S. agalactiae* challenge failed to produce significant differences in mortality between exposed and control fish. Overall, the mortality rates for UIA-exposed fish were similar for both of the 24-h UIA challenge experiments, and UIA exposure at 0.32–0.37 mg/L failed to significantly affect mortalities through 21 d postexposure. The experimental challenges indicated that 24-h exposure to sublethal UIA between 0.32 and 0.37 mg/L has a low probability of increasing susceptibility to *S. agalactiae*. These results were similar to those of Bowser et al. (1998), who found that Nile tilapia (20–30 g) were not increasingly susceptible to *S. iniae* when cohabitated with infected fish. In that study, Nile tilapia were held at 26°C and subjected to 8 h of low DO (<1.0 mg/L) and presumably high UIA (between 0.06 and 0.57 mg/L, depending on a pH ranging from 7.0 to 8.0). The authors concluded that fish may require longer term stressor exposure, increased temperatures, or both to increase susceptibility. Indeed, longer duration of exposure to sublethal low DO (Evans et al. 2003) and bacterial challenge at elevated temperatures (Evans et al. 2004) have been shown to increase disease susceptibility to *S. agalactiae*.

Our results were also surprising because other researchers have suggested a link between the environmental stressor, UIA, and mortalities among wild populations of fish exposed to group B *S. agalactiae*. Plumb et al. (1974) examined fish kills in estuarine bays of the Florida and Alabama Gulf Coast and group B streptococci from the eight affected fish species. The authors proposed that an underlying environmental condition influenced the bacterial pathogenicity and found that ammonium concentrations were at 0.14 mg/L among the most affected areas. Eldar et al. (1995) also investigated fish kills among four cultured fish species in Israel and linked mortality to *S. difficile*, presumably promoted by environmental changes such as increased UIA concentrations at 0.98 mg/L UIA. Glibert et al. (2002) described the roles of environmental stressors in a massive fish kill among wild Klunzingeri mullet *Liza klunzingeri* caused by *S. agalactiae* infection; these mortalities occurred in Kuwait Bay, where elevated ammonium concentrations ranged from 0.11 to 0.33 mg/L. The findings of these epizootic investigations indicate that elevated UIA concentrations may increase susceptibility to bacterial infection, something which contradicts the findings in this study. However, these fish kills involved a combination of environmental factors, such as changes in water temperature, low DO, and harmful algal blooms, which presumably contributed to disease susceptibility. Nonetheless, UIA alone may still increase susceptibility to *S. agalactiae* if fish are exposed to higher UIA concentrations, exposed to UIA for longer than 24 h, or challenged during an active stress response (1–4 h postexposure).

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