# Acute ammonia toxicity during early ontogeny of ide *Leuciscus idus* (Cyprinidae)

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**Abstract**   Acute ammonia toxicity was investigated in four developmental stages of the juvenile ide, *Leuciscus idus*: 1, 10, 20 and 30 days after the first feeding. Mean ($\pm$SD) total length of the larvae was $8.5 \pm 0.3$, $15.7 \pm 0.7$, $23.0 \pm 2.0$ and $29.7 \pm 2.0$ mm, and standard weight was $1.6 \pm 0.3$, $9.2 \pm 5.5$, $94.9 \pm 31.0$ and $196.0 \pm 31.7$ mg, respectively. The larvae used for toxicity tests were reared in the experimental, closed recirculating system. Groups of fishes ($n$ from 7 to 10; in respect of fish size) were exposed to the ammonium chloride solutions in 1-L glass units. Water temperature was $25 \pm 0.1$ °C for both the rearing and the toxicity tests. pH was not adjusted and ranged between 8.4 and 8.7. The ammonium chloride solutions were renewed every 12 h. At the same time, dead larvae were counted and removed, and the pH and temperature measurements were taken. Each acute toxicity test duration was 96 h, and lethal concentration $LC_1$, $LC_{50}$ and $LC_{99}$ values were calculated for 24, 48, 72 and 96 h. The susceptibility of the ide larvae to ammonia decreased linearly with age up to 20th day and surprisingly increased during the next 10 days. The $LC_{50}$ (48 h) values ranged from 0.27 mg $L^{-1}$ of unionized ammonia nitrogen for 1 day after the first feeding (AFF) larvae to 1.42 mg $L^{-1}$ at day 20 after first feeding. The $LC_{50}$ (48 h) for 30 days AFF was as high as 0.67 mg $L^{-1}$. The critical level of the unionized ammonia nitrogen for ide larvae was suggested as 0.21 mg $L^{-1}$.

**Keywords**   Toxicity test · Fish larvae · Unionized ammonia · $LC_{50}$

**Abbreviations**
AFF    After the first feeding
TAN    Total ammonia nitrogen

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UIAN    Unionized ammonia nitrogen
RAS     Recirculated aquaculture system


## Introduction

In contrast to mammals, most of the fish species do not excrete urea. The main waste product of the protein catabolism in fish is ammonia excreted through the gills (Wood 1993). Ammonia is a highly toxic chemical compound, and fish experience its toxic levels in both natural environment and intensive culture systems. Toxicity of ammonia depends on the water parameters including pH and temperature, mainly. These two parameters affect the concentration of more toxic form, which is unionized ammonia (UIAN) (Randall and Tsui 2002). UIAN diffuses through the gill membranes easily. Central nervous system is the main target organ for the ammonia intoxication (Randall and Ip 2006; Svobodova et al. 2007). Low ammonia concentration in the water can stimulate fish growth (Wood 2004); however, chronic exposure to ammonia reduces survival and growth in the fish at the early stages of development (Brinkman 2009).

Adhibition of RAS-based rearing at comparatively high temperatures and stocking densities, and application of the high-protein feeds allow for intensification of the fish larvae and juvenile production (van Rijn 1996; Remen et al. 2008). On the other hand, RAS generates high risk of the waste nitrogen compound accumulation. According to Biswas et al. (2006), ambient ammonia and nitrite concentration is a key factor for the optimization of the stocking density as even small concentration of these compounds decreases fish growth, health and survival rate.

Ide *Leuciscus idus* (L.) is an Eurasian native fish species. In the recent years, a serious decline of the ide populations has been observed in the Central Europe (Hamáčková et al. 2007, Kucharczyk et al. 2008, Aleksejevs and Birzaks 2011). This triggered the need for the production of the ide for the restocking purposes. Golden ide *Leuciscus idus melanotus* are often kept as ornamental fish and furthermore are used as test fish for toxicological monitoring. According to Krejszeff et al. (2009), ide contains 69–91 % of rheophilic cyprinid production in Poland.

It was recently reported that rearing of the cyprinids larvae may be conducted in very high stocking densities, up to 600 ind. $L^{-1}$, without the negative effect on their survival (Kupren et al. 2011; Żarski et al. 2011). However, application of such intensive procedures together with the intensive feeding may result in increased level of the ammonia excretion. There is still very little information regarding the effect of the increased ammonia level on freshwater fish larvae. Recent studies have shown that susceptibility of the chub, *Leuciscus cephalus* (L.), to ammonia decreased during the larval development and the larvae at the onset of the exogenous feeding are extremely sensitive to the ammonia (Gomułka et al. 2011). There are no data on the susceptibility of the ide larvae at different ontogenetic stages to ammonia.

The aim of this study was to determine the acute toxicity of ammonia to ide at four different stages of larval development.


## Materials and methods

Broodstock and hatched larvae management

Ide larvae were obtained in the course of the controlled reproduction of the wild spawners caught in the Mosąg Lake (northeast Poland) at the beginning of the reproductive season.

The reproduction was performed according to the method described by Krejszeff et al. (2009) with the application of the single hormonal treatment with Ovopel (40 µg of mammalian analogue of gonadoliberin and 20 mg of dopamine antagonist—metoclopramide). The eggs were incubated in Weiss jars at 14 °C. Hatched larvae were transferred to the rearing fiberglass tank (150 L) where larvae were kept till the inflation of the swim bladder at the temperature of 20 °C. Next, larvae were acclimated to the temperature of 25 °C (for 1 day) and transferred to the experimental rearing units.

Rearing procedure

Fifteen 1-L glass rearing units placed in a small reticulating system (described in detail by Krejszeff et al. 2008) were used at the experimental rearing starting from the onset of the exogenous feeding of larvae. That moment of the onset of the exogenous feeding was determined according to the procedure described by Gomulka et al. (2011).

The experimental rearing lasted 30 days. The stocking density for the first 20 days was 150 ind. $L^{-1}$. Larvae were then randomly restocked for the further rearing at 50 ind. $L^{-1}$ in the rearing unit. The temperature (25 °C $\pm$ 0.1) and photoperiod (12L:12D) were constant throughout the experiment. Fish were fed *ad libitum* three times daily with the hatched *Artemia* nauplii. Tanks were cleaned twice daily. Dead larvae were counted and removed. Every 3 days, oxygen saturation (HI 91410, Hanna Instruments, Italy) and the concentrations of ammonia (HI 83214, Hanna Instruments, Italy) and nitrites (using an LF205 photometer, Slandi, Poland) were monitored.

Before each toxicity test, 30 larvae were randomly chosen, euthanatized in a solution of 2-phenoxyethanol (0.4 mL $L^{-1}$) and photographed under a stereoscopic microscope (Leica MZ 12.5, Germany). These photographs were then used for larvae measurements ($\pm$0.01 mm, with ProgRes_ Capture Pro 2.5 software, Jenoptik, Germany). The wet body weight (WBW) of the larvae was determined ($\pm$0.1 mg).

Toxicity testing

Test procedure followed the OECD Guideline No. 203 "Fish acute toxicity test." The first experimental group (D1) was sampled the day when first feeding began (day 1). Food was supplied to all rearing tanks at the day. Before second feeding, 100 randomly chosen (from three tanks) specimens were removed to a separate tank and were starved for the next 24 h. These larvae were then used for the first toxicity test. The next three groups of larvae representing group D10, D20 and D30 were sampled on days 10, 20 and 30 after first feeding (AFF), respectively.

Next, fish were randomly divided into nine experimental groups subjected to a set of increasing ammonia concentrations. Larvae ($n = 10$ for the D1, D10 and D20 tests and $n = 7$ for the D30 test) were then randomly allocated in 1-L test glass units placed in a water bath. The test water temperature was 25 $\pm$ 0.1 °C. Each experiment was conducted in duplicate. Each replicate was provided with the control group. Each unit was gently aerated. Ammonia solutions were renewed every 24 h. The test duration was 96 h. The water temperature, pH and total ammonia nitrogen (TAN) concentration were measured twice a day. The TAN concentration was measured with a multiparametric analyzer (HI 83214, Hanna Instruments, Italy). The number of dead fish was recorded, and dead fish were removed twice a day.

Ammonia solutions

A new stock solution of 10 g L$^{-1}$ of ammonium chloride (NH$_4$Cl pure p.a.; Chempur, Poland) in the redistilled water was prepared every day. Defined volumes of the stock solution were added to the clean experimental units according to the established set of concentrations. Each vessel was then filled with laboratory tap water up to 1 L. The tap water was aerated and heated to 25 $\pm$ 0.1 °C for 24 h before use. The tap water parameters were as follows (mean $\pm$ SD): hardness 274 $\pm$ 15 ppm CaCO$_3$ and conductivity 548 $\pm$ 24 μS cm$^{-1}$. No pH adjustment was applied.

Calculations and data analysis

The mean temperature and mean pH were calculated for each test unit and for each exposure time point (12, 24, 36, 48, 60, 72, 84 and 96 h). Then, mean UIAN concentration was calculated according to the algorithm given in "Update of Ambient Water Quality Criteria for Ammonia" (USA Environmental Protection Agency publication number EPA-822-R-99-014; 1999). The concentrations of UIAN that kills 1, 5, 50, 95 and 99 % of exposed fish (LC$_1$, LC$_5$, LC$_{50}$, LC$_{95}$ and LC$_{99}$, respectively) were calculated with Probit software (EPA, version 1.5) for each exposure time point, if possible. The relationships between measured parameters were analyzed using the nonlinear regression method. The significance level of 0.05 was used.

## Results

Controlled rearing

Throughout the rearing period, a very low mortality rate was observed, which did not exceed 4 % in any of the rearing units at day 30. At the first, tenth, twentieth and thirtieth days of the experiment, the length of the larvae and the wet body weight were 8.5 $\pm$ 0.3, 15.7 $\pm$ 0.7, 23.0 $\pm$ 2.0 and 29.7 $\pm$ 2.0 mm and 1.6 $\pm$ 0.3, 9.2 $\pm$ 5.5, 94.9 $\pm$ 31.0 and 196.0 $\pm$ 31.7 mg, respectively. The correlation between age and size was almost full ($r^2 = 0.998$ for age and length, $p < 0.05$). Throughout the rearing period, the concentration of ammonia and nitrites did not exceed 0.05 and 0.05 mg L$^{-1}$, respectively. The oxygen level in rearing tanks did not drop below 80 % saturation during 30 days of rearing.

Acute ammonia toxicity

The susceptibility of the ide larvae to ammonia decreased linearly with age till 20th day after the first feeding and then surprisingly increased within next 10 days (Table 1). The high positive correlation was found between fish weight and LC$_{50}$ values ($R^2 = 0.9446$, $p < 0.05$) (Fig. 1a) for the first 20 days AFF. The correlation between fish length and LC$_{50}$ values was almost full ($R^2 = 0.9996$, $p < 0.05$) for the same period (Fig. 1b).

The LC$_{50}$ (48 h) values ranged from 0.27 mg L$^{-1}$ of UIAN for D1 larvae to 1.42 mg L$^{-1}$ for D20 larvae (Table 1). The LC$_{50}$ (48 h) for D30 was estimated at 0.67 mg L$^{-1}$. The last value was calculated from the equation of the linear regression: $y = -0.003x + 0.82$, where $y$ is the LC$_{50}$ value and $x$ is the time of the exposure (the Pearson's correlation coefficient $r = 0.93$, $p < 0.05$).

**Table 1** Results of acute ammonia toxicity tests for larval ide *Leuciscus idus* in the ages of 1, 10, 20 and 30 days after first feeding

| Days after first feeding | LC | 24 h UIAN (mg L⁻¹) | 95 % Confidence limits Lower | Upper | 48 h UIAN (mg L⁻¹) | 95 % Confidence limits Lower | Upper | 72 h UIAN (mg L⁻¹) | 95 % Confidence limits Lower | Upper | 96 h UIAN (mg L⁻¹) | 95 % Confidence limits Lower | Upper |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | $LC_1$ | 0.21 | 0.14 | 0.25 | 0.17 | 0.11 | 0.20 | 0.12 | 0.07 | 0.14 | 0.00 | 0.00 | 0.01 |
|   | $LC_5$ | 0.24 | 0.18 | 0.28 | 0.19 | 0.14 | 0.22 | 0.13 | 0.09 | 0.15 | 0.01 | 0.00 | 0.01 |
|   | $LC_{50}$ | 0.36 | 0.32 | 0.42 | 0.27 | 0.25 | 0.31 | 0.17 | 0.15 | 0.19 | 0.03 | 0.01 | 0.06 |
|   | $LC_{95}$ | 0.53 | 0.44 | 0.80 | 0.39 | 0.33 | 0.56 | 0.23 | 0.20 | 0.33 | 0.23 | 0.12 | 0.60 |
|   | $LC_{99}$ | 0.62 | 0.50 | 1.07 | 0.45 | 0.37 | 0.74 | 0.25 | 0.22 | 0.42 | 0.50 | 0.24 | 2.03 |
| 10 | $LC_1$ | 0.48 | 0.41 | 0.76 | 0.45 | 0.25 | 0.58 | 0.4 | 0.25 | 0.51 | 0.3 | 0.08 | 0.44 |
|   | $LC_5$ | 0.62 | 0.49 | 1.05 | 0.57 | 0.37 | 0.7 | 0.5 | 0.35 | 0.6 | 0.39 | 0.15 | 0.52 |
|   | $LC_{50}$ | 1.16 | 0.92 | 1.64 | 1.00 | 0.86 | 1.17 | 0.86 | 0.75 | 0.99 | 0.71 | 0.52 | 0.97 |
|   | $LC_{95}$ | 2.16 | 1.62 | 2.94 | 1.75 | 1.42 | 2.79 | 1.47 | 1.23 | 2.08 | 1.29 | 0.95 | 3.49 |
|   | $LC_{99}$ | 2.80 | 1.84 | 3.06 | 2.21 | 1.69 | 4.15 | 1.84 | 1.46 | 2.91 | 1.66 | 1.13 | 6.35 |
| 20 | $LC_1$ | <1.30 to >1.90[a] |  |  | 1.05 | 0.8 | 1.18 | 0.85 | 0.52 | 0.99 | 0.78 | 0.42 | 0.92 |
|   | $LC_5$ |  |  |  | 1.15 | 0.94 | 1.26 | 0.95 | 0.66 | 1.08 | 0.88 | 0.56 | 1 |
|   | $LC_{50}$ |  |  |  | 1.42 | 1.3 | 1.59 | 1.25 | 1.13 | 1.41 | 1.15 | 1.02 | 1.28 |
|   | $LC_{95}$ |  |  |  | 1.76 | 1.58 | 2.29 | 1.65 | 1.45 | 2.43 | 1.51 | 1.34 | 2.3 |
|   | $LC_{99}$ |  |  |  | 1.93 | 1.69 | 2.71 | 1.84 | 1.57 | 3.13 | 1.7 | 1.45 | 3.03 |

**Table 1** continued

| Days after first feeding | LC | 24 h UIAN (mg L$^{-1}$) | 95 % Confidence limits Lower | Upper | 48 h UIAN (mg L$^{-1}$) | 95 % Confidence limits Lower | Upper | 72 h UIAN (mg L$^{-1}$) | 95 % Confidence limits Lower | Upper | 96 h UIAN (mg L$^{-1}$) | 95 % Confidence limits Lower | Upper |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 30 | LC$_1$ | 0.51 | 0.31 | 0.58 | <0.48 to >0.73[b] | | | 0.43 | 0.31 | 0.48 | 0.44 | 0.35 | 0.48 |
|  | LC$_5$ | 0.56 | 0.4 | 0.62 | | | | 0.46 | 0.37 | 0.51 | 0.47 | 0.39 | 0.51 |
|  | LC$_{50}$ | 0.72 | 0.66 | 0.82 | | | | 0.57 | 0.52 | 0.62 | 0.55 | 0.5 | 0.59 |
|  | LC$_{95}$ | 0.92 | 0.82 | 1.43 | | | | 0.7 | 0.64 | 0.85 | 0.64 | 0.59 | 0.76 |
|  | LC$_{99}$ | 1.02 | 0.87 | 1.85 | | | | 0.76 | 0.69 | 1 | 0.68 | 0.62 | 0.85 |

[a] 0 % mortality at 1.30 UIAN mg L$^{-1}$ and lower levels; 100 % mortality at 1.90 UIAN mg L$^{-1}$ and higher levels; too few mortality points to calculate LC values

[b] 0 % mortality at 0.48 UIAN mg L$^{-1}$ and lower levels; 100 % mortality at 0.73 UIAN mg L$^{-1}$ and higher levels; too few mortality points to calculate LC values
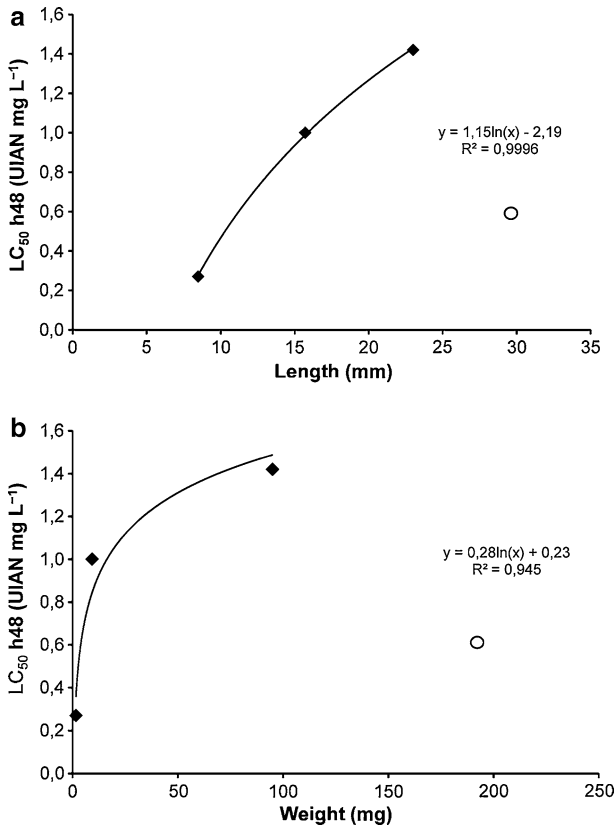
**Fig. 1** The correlation between 48 h $LC_{50}$ of ammonia and both length (**a**) and weight (**b**) of larval ide. Best-fit regression lines and equations are provided for the period from 1 to 20 days after first feeding. *Open circle* 48 h $LC_{50}$ value for 30 days after first feeding larvae

## Discussion

The relationship between fish age and susceptibility to ammonia toxicity has been studied in various fish species. It is not clear and different results are reported by numerous authors (see Gomułka et al. 2011).

Following Vedel et al. (1998) and Wicks and Randall (2002), Gomułka et al. (2011) hypothesized that increasing resistance to ammonia results from a general increment in both physiological efficiency and muscle capability for glutamine storage in larval chub. However, in case of the ide, the correlation between age and size was almost full during 30 days of the experiment and thus other factor was responsible for the drop of ide resistance to ammonia. Larval development of ide and chub is similar; however, the time for reaching particular developmental stage is species specific and dependent on the environmental conditions (Kupren et al. 2011). We can only speculate that some developmental changes (e.g., rapidly increasing gill surface) enabled a rise of ammonia inside the fish body and, at the same time, the ability to ammonia removal or temporal storage did not increase as quickly as changes enabling the rise of blood ammonia level. Further studies on these phenomena are needed.

Ide is relatively susceptible to UIAN during its larval development when compared to other fish species (for details, see the review of US Environmental Protection Agency publication "1999 Update of Ambient Water Quality Criteria for Ammonia" EPA-822-R-99-014).

The drop of the resistance to ammonia at the end of larval development may result in mortality during transport or other manipulations involving stress and high stocking densities. In Poland, 5-month-old fry are usually used for stocking purposes. Older fish seems to be more resistant to ammonia. Hendriks and Stouten (1993) reported ammonia LC$_{50}$ for 24 h for adult ide as high as 28.0 mg L$^{-1}$.

## Conclusions

We can assume that larval ide is particularly susceptible to ammonia toxicity at the beginning of larval development. Thus, during that period, special attention should be paid to ammonia concentration monitoring. We suggest using the LC$_5$ for 96 h (0.01 UIAN mg L$^{-1}$) for the 1st day AFF as a warning level and the LC$_1$ for 24 h (0.21 UIAN mg L$^{-1}$) (Table 1) as a critical unionized ammonia nitrogen concentration for ide larvae. Larval care procedures should provide efficient ammonia removal or freshwater supply enough to maintain ammonia level below 0.01 UIAN mg L$^{-1}$ during the first 10 days AFF at least.

Any procedures involving stress or high stocking densities should be avoided during larval development.

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