Thiamethoxam affects the developmental stages of banded gourami (Trichogaster fasciata)

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A B S T R A C T
Thiamethoxam (THM), a type III systemic neonicotinoid insecticide, is widely used in agriculture in many countries, including Bangladesh. THM can enter the aquatic systems through the runoff, spray-drift and groundwater leaching and can affect the aquatic organisms, including fish. Current environmental levels of THM in Bangladesh waters are variable. However, the presence of this chemical in the aquatic environment and its possible effects on the fish inhabiting those water bodies is concerning. To understand the effects of environmental THM on the development of embryo and larvae, the present study used banded gourami (Trichogaster fasciata), a freshwater fish species distributed in different Asian countries, including Bangladesh. In laboratory setting, fertilized eggs (n = 100) and one-day-old banded gourami larvae (n = 100) were exposed to six concentrations of THM (0, 0.02, 0.2, 2, 20, 200 mg/L) in three replicates, in which three concentrations were within the environmentally relevant levels. Hatching rate, incubation period, mortality, and malformations of embryo and larvae were observed. The hatching success and survival of embryo and larve significantly decreased with increasing THM concentrations. The 24-h LC50 of THM for the embryo was 4.24 mg/L. The 24-h, 48-h, 72-h, and 96-h LC50 values of THM for one-day-old larvae were 12.20, 3.80, 0.78, and 0.27 mg/L, respectively. Overall developmental malformations included lordosis, notochord abnormality, yolk-sac edema, dark brown yolk sac, hemorrhage, and irregular caudal fin. These abnormalities in embryos were common across all the concentrations of THM applied. The results of the present study suggest that environmentally relevant concentrations of THM can induce developmental defects in the embryo and larvae of banded gourami.

1. Introduction
Neonicotinoid insecticides are widely used in agriculture since the 1990s due to their efficacy in improving crop protection and management [1]. These compounds are considered a potential replacement for other groups of pesticides. In recent years, neonicotinoids have been detected in aquatic systems all over the world due to surface runoff, spry-drift, and groundwater leaching [2-5]. Literature suggests that neonicotinoids show deleterious effects on non-target aquatic organisms [6-11]. The European Commission has restricted the use of neonicotinoid insecticide in the European Union to protect bees and other pollinator insects [3].

Thiamethoxam (THM) is one of the most extensively used insecticides in Bangladesh among many other different groups of pesticides [12]. THM [3-(2-chloro-1,3-thiazol-5-methyl)–5-methyl-4-nitroimino-perhydro-1,3,5-oxadiazine] is considered a second-generation neonicotinoid insecticide, having a half-life of 385–408 days in water and up to 3001 days in soil [1]. This insecticide is commonly used for the control of whiteflies, aphids, and Lepidoptera [13]. THM can be toxic to many aquatic organisms, including fish and invertebrates [5,14,15] due to low soil retention, high leaning capability, high solubility in water, and resistance to biological treatment [16]. Particularly in fish, THM is reported to cause hepatorenal damage, oxidative stress, immunotoxicity, hemato-biochemical alterations and metabolic disorders [10,11].

Banded gourami also called Striped Gourami (Trichogaster fasciata; family Osphronemidae; order Perciformis) is abundant in Bangladesh,
India, Myanmar, Nepal and Pakistan. Banded gourami usually inhabits freshwater pools, ditches, wetlands, marshes and waterways, and vegetative lakes [17]. This species is considered as an ornamental fish. Often it has also drawn consumer attention due to its unique taste and nutritional composition [18]. Banded gourami is used as an animal model for eco-toxicological studies in Bangladesh and some Asian countries [18,19]. It is convenient to maintain in the laboratory condition due to its small size, breeding in captivity, and their availability [20, 21].

Fish acute toxicity tests play important roles in environmental risk assessment across various aquatic toxicity assays [22–25]. Toxicity of a chemical varies with the species tested. Toxicity of THM in various fish species have been determined and reported (Table 1). It is not clearly understood whether current environmental levels of THM affect the developmental trajectories of banded gourami inhabiting the contaminated site. Hence, the present study determined the survival, hatching success, and the malformations of the early stages of banded gourami after acute THM exposures.

2. Materials and methods

2.1. Pesticide, brood fish selection, and pairing

Commercial grade THM (CAS number: 153719–23–4; trade name: spike 25WG; manufacturer: Syngenta) was purchased from an authorized dealer at Shambhugonj Market, Mymensingh, Bangladesh. Juvenile fish were obtained from Bangladesh Fisheries Research Institute (BFRI), Mymensingh, Bangladesh. The average body weight of fish before the experiment was 11.02 ± 1.05 g and body length 7.69 ± 1.04 cm. About 300 fish were reared in a cemented cistern (250 × 195 × 70 cm²; water height 30 cm). They were fed with commercial feed (Quality Fish Feed Ltd. with 30% protein) at 5%/kg body weight twice a day. Fish were reared for four months until they developed secondary sexual characteristics (i.e. size, color, swollen abdomen, and genital opening). Fish were released into the breeding aquarium for two days for appropriate pairing and conditioning before hormone treatments. Fish maintenance and the experimental procedures were approved by the Animal Care Committee of Bangladesh Agricultural University Research System (BAURES), Mymensingh, Bangladesh (registration number: 2018/696/MOE).

2.2. Aquarium set-up, hormone treatment, and induced-breeding

The aquarium used for induced-breeding was set up in the Wet Laboratory of the Faculty of Fisheries, Bangladesh Agricultural University. Water lettuce (Pistia stratiotes) was placed in the aquarium to help the male brood form a bubble nest where eggs are attached after fertilization. Fish were continuously aerated throughout the experiment.

Ten male (weight: 14 ± 1.85 g, length: 8.5 ± 0.8 cm) and ten females (weight: 11.08 ± 1.15 g, length: 7.5 ± 1.2 cm) broodfish were artificially induced by intramuscular injection of carp pituitary (PG) extract collected from Sharnalata Agro Fisheries Ltd. Hatchery, Mymensingh, Bangladesh. PG extract was prepared by dissolving PG powder in saline and administered intramuscularly at a dose of 2 mg/kg body weight for both male and female fish [18]. After two days of hormone administration, spawning took place and the fertilized eggs were deposited in the nest. The fertilized eggs were transparent, whereas the unfertilized eggs were opaque. Approximately 4000 fertilized eggs were collected from the breeding aquarium and used for embryo and larval bioassays.

2.3. Experimental design

For embryonic and larval bioassays, six concentrations (0, 0.02, 0.2, 2, 20, 200 mg/L) of THM were used in triplicates and the two independent experiments were conducted. The dosing regime for both experiments was selected based on a previous study by Yang et al. [27]. They reported the 96-h value of THM for rare minnow larvae as 201.6 mg/L, which is corresponding to the highest concentration of the present study. The stock solution was prepared by serial dilution of the stock. In the present bioassays, 18 plastic bowls containing 4 L dechlorinated tap water were used. For the evaluation of embryonic toxicity, 100 fertilized eggs were exposed to six different THM concentrations. Aeration was installed in each plastic bowl. The incubation period, hatching rate, and mortality of embryos were examined after 24 h of THM exposure. To evaluate the larval toxicity, a similar dosing regime and experimental design were used as used for embryo bioassay. The mortality of larvae was determined every 24 h of THM exposure during the experimental period of 96 h. Malformations were observed in embryos at 6 h interval and in larvae at 24 h interval using a digital microscope (Classic LED, Hamascope) connected with a camera (Magnus analytics, Model-MIPS). The cumulative mortality rate was calculated in the end of the experiment for embryonic and larval bioassays. Water quality parameters particularly DO, temperature, pH, and total alkalinity were measured during the experimental period, and the values of these parameters in

| Species                        | Life stage | Endpoint                                      | Threshold effects i.e.LC50 (mg/L) | NOEC (mg/L) | Reference                             |
|-------------------------------|------------|-----------------------------------------------|----------------------------------|-------------|---------------------------------------|
| Rainbow trout (Oncorhyncus mykiss) | Adult      | Mortality (96 h)                              | > 125                            | 125         | Finnegan et al. [26]                  |
| Bluegill sunfish (Lepoma macrochirus) | Adult      | Mortality (96 h)                              | > 114                            | 114         | Finnegan et al. [26]                  |
| Common carp (Cyprinus carpio)   | Adult      | Mortality (96 h)                              | > 120                            | 120         | Finnegan et al. [26]                  |
| Rainbow trout (Oncorhyncus mykiss) | Early life stage | Hatching success, larvae and fry survival (88 d) | –                                | 100         | Finnegan et al. [26]                  |
| Rare minnow (Gobiocypris rarus) | Larvae     | Mortality (48 h)                              | 386                              | –           | Finnegan et al. [26]                  |
|                               | Juvenile   | Mortality (96 h)                              | 202                              | –           | Yang et al. [27]                      |
|                               | Adult      | Mortality (96 h)                              | 55                               | –           | Yang et al. [27]                      |
| Rare minnow (Gobiocypris rarus) | Eggs       | Mortality (48 h)                              | 84                               | –           | Yang et al. [27]                      |
|                               |            | Mortality (96 h)                              | 463.7                            | –           | Wang et al. [28]                      |
| Banded gourami (Trichogaster fasciata) | Eggs      | Mortality (24 h)                              | 4.24                             | –           | The present study                     |
|                               | One-day-old larvae | Mortality (24 h)                              | 12.20                            | –           |                                       |
|                               |            | Mortality (48 h)                              | 3.80                             | –           |                                       |
|                               |            | Mortality (72 h)                              | 0.78                             | –           |                                       |
|                               |            | Mortality (96 h)                              | 0.27                             | –           |                                       |

Table 1

An overview of the toxicity of thiamethoxam on various stages of different fish species.
the experimental units were as follows: DO (7.6–8.0 mg/L), temperature (28.6–28.9 °C), pH (7.4–8.3), and total alkalinity (123–149 mg/L).

2.4. Statistical analysis

Statistical analysis was performed using RStudio (version 4.0.5). Data visualization packages ‘ggplot2’ and ‘agricole’ were used for creating graphics. The LC10 and LC50 were determined after probit analysis using SPSS (version 20; SPSS Inc., Chicago, IL, USA). To determine the toxic effects of different THM concentrations in embryo and larvae, and their hatching rate and malformation rate, a one-way analysis of variance (one-way ANOVA) was conducted using Duncan’s multiple comparisons test at a 5% level of significance using SPSS (version 20; SPSS Inc., Chicago, IL, USA). The one-way ANOVA assumptions of normality were determined by the Shapiro-Wilk’s test.

3. Results

The number of dead embryos significantly increased (one-way ANOVA; F5,17 = 283; P = 0.000) in 24 h with increasing THM concentration. The 24-h LC10 and LC50 values (with 95% confidence level of THM were 0.09 and 4.24 mg/L, respectively (Table 2). THM exposure resulted in a significant decrease in hatching success (one-way ANOVA; F5,17 = 283; P = 0.000). Approximately 37% hatching success of embryos was observed when exposed to 20 mg/L of THM, while 93% were observed in the control group. Furthermore, the incubation period slightly decreased in THM-exposed eggs compared to control (Table 2).

The number of dead larvae at 24 h (one-way ANOVA; F5,17 = 106; P = 0.000), 48 h (one-way ANOVA; F5,17 = 32; P = 0.000), 72 h (one-way ANOVA; F5,17 = 29; P = 0.000), and 96 h (one-way ANOVA; F5,17 = 35; P = 0.000) significantly increased with increasing THM concentrations (Table 2). The 24-, 48-, 72- and 96-h LC10 and LC50 values (with 95% confidence level) for THM are presented in Table 2.

In embryos, several malformations were observed, including eggshell broken (ESB), yolk sac edema (YSE), dark brown yolk-sac (DBYS), and un-hatched egg (UE) after exposure to different THM concentrations (Fig. 1A-F). Yolk sac edema (30%) was the most common form of deformity, while the dark-brown yolk sac (12%) was the least (Fig. 2). The cumulative malformation rate of banded gourami embryos significantly increased (one-way ANOVA; F5,17 = 17.23; P = 0.000) with the progression of THM concentrations (Table 3).

In one-day-old larvae, malformations included yolk sac edema (YSE), notochord abnormality (NA), hemorrhage (H), deformed posterior body part (DB), lordosis (L) and irregular caudal fin (ICF) (Fig. 3A-I). Yolk sac edema (57%) was the most common form of deformity, while hemorrhage (9%) was the least occurring effect (Fig. 4). The cumulative malformation rate of one-day-old larvae of banded gourami significantly increased (one-way ANOVA; F5,17 = 14.26; P = 0.000) with the increasing concentrations of THM (Table 3).

4. Discussion

The present study demonstrates that acute THM exposure can affect the hatching success, incubation period, and survival of embryo and larvae of Banded Gourami. Hatching success was null in the highest concentration (200 mg/L THM) exposed group. Previously, exposure to pesticides caused a significant decrease in hatching success of fish embryos, particularly banded gourami exposed to chlorpyrifos [18], common carp exposed to imidacloprid [6,9], and Ganges Mystus exposed to cypermethrin [29]. In common carp, imidacloprid exposure (31.2 mg/L) reduced hatching success to 55% [6]. Chlorpyrifos exposure in banded gourami had 3.7% hatching success in the 0.1 mg/L [18]. Similar findings were reported in previous studies when different fish species were exposed to various pesticides, such as deltamethrin [30], alphamethrin [31], diazinon [32], cyhalothrin [33], endosulfan [34] and thiacloprid [35]. The reduced hatching success of banded gourami may occur due to disturbance of the hatching process, which consists of several steps mainly choroiysis, hatching enzyme secretion, hatching enzyme biosynthesis, differentiation and maturation of hatching gland cells, genetic control of hatching enzyme synthesis, etc. [36]. A proteolytic enzyme secreted from the hatching gland cells of the embryo facilitates the digestion of chorion (egg envelope) during the normal hatching process. Although we did not examine hatching enzyme levels in the present embryos, it looks like the function of this hatching enzyme might be inhibited by THM causing a blockade of the pore channel of chorions, resulting in oxygen depletion in the embryo as suggested by Fan et al. [37].

In the present study, a decreased incubation period embryos were observed caused by different concentrations of THM. Tyr and Harkrishan [6] showed similar results of decreasing incubation period in common carp with the increasing imidacloprid concentrations. In contrast, a prolonged incubation period of banded gourami embryos was noticed when exposed to chlorpyrifos [18], and of Gangetic Mystus embryos exposed to cypermethrin [29].

The 24-h LC50 of THM for banded gourami embryo was 4.24 mg/L. Yang et al. [27] calculated the 48- and 96-h LC50 of THM for rare minnow embryos as 463.7 and 351.9 mg L−1, respectively, which is about 83–109 times higher than present results. Another study reported the 96-h LC50 of acetamiprid for zebrafish embryos as 13.33 mg/L [28], which is 3 times higher than those calculated for banded gourami embryos. Almost similar LC50 value of neonicotinoid pesticide imidacloprid was reported for zebrafish and common carp embryos [9,38]. However, other groups of pesticides than neonicotinoids had lower LC50 values for different fish species. For instance, Sumon et al. [18] found the 24-h LC50 value of chlorpyrifos for banded gourami embryo to be 0.0118 mg/L, which is several hundred folds lower than present LC50 values.

The 24-, 48-, 72- and 96-h LC50 of THM for the 1-day-old larvae were 12.20, 3.80, 0.78, and 0.27 mg/L, respectively. Yang et al. [27] estimated the 96-h LC50 of THM for rare minnow larvae to be 201.6 mg/L,

Table 2

Toxicity of thiamethoxam on the embryo and the larvae of banded gourami (n = 100).

| Concentration (mg/L) | Incubation period | Number of dead embryos at 24 h | Number of dead one-day-old larvae at 24 h | Number of dead one-day-old larvae at 48 h | Number of dead one-day-old larvae at 72 h | Number of dead one-day-old larvae at 96 h |
|----------------------|-------------------|-------------------------------|----------------------------------------|-----------------------------------------|----------------------------------------|----------------------------------------|
| 0                    | 24 h              | 7.33 ± 1.15                   | 0.00                                   | 0.00                                    | 0.00                                    | 2.66 ± 2.30                            |
| 0.02                 | 24 h              | 11.33 ± 1.15                  | 12 ± 4                                 | 16 ± 4                                  | 21.33 ± 2.31                           | 22.66 ± 2.30                           |
| 0.2                  | 24 h              | 13.33 ± 3.05                  | 22.66 ± 8.32                           | 42.66 ± 22.03                           | 55.33 ± 14.19                          | 66.66 ± 16.65                          |
| 2                    | 23 h              | 27.33 ± 7.02                  | 28 ± 8                                 | 32 ± 10.58                              | 45.33 ± 22.03                          | 52 ± 17.43                             |
| 20                   | 23 h              | 62.66 ± 5.03                  | 20 ± 8                                 | 36 ± 6.93                               | 58 ± 5.29                              | 78.66 ± 8.32                           |
| 200                  | ND                | 100                           | 100                                    | 100                                     | 100                                     | 100                                     |
| P value              |                   | 0.000                         | 0.000                                  | 0.000                                   | 0.000                                   | 0.000                                   |
| LC50 value with 95% confidence limit | 0.09 (0.02-0.24) | 0.05 (0.00-0.37)              | 0.000 (0.00-0.09)                      | 0.001 (0.00-0.02)                       | 0.001 (0.00-0.01)                       | 0.001 (0.00-0.01)                       |
| LC50 value with 95% confidence limit | 4.24 (1.96-9.92) | 12.20 (2.38-200.40)           | 3.80 (0.53-51.40)                      | 0.78 (0.11-3.59)                        | 0.27 (0.05-0.93)                        | 0.27 (0.05-0.93)                        |

ND, no data due to 100% mortality.
which are approximately 747 folds higher than the 96-h LC50 of banded gourami. Other studies calculated the 96-h LC50 of acetamiprid and imidacloprid for zebrafish larvae as 15.5 and 128.9 mg/L, respectively; which are again 57–477 folds higher than our findings [28,38]. However, Islam et al. [9] reported an almost similar LC50 value (1.2 mg/L) of imidacloprid for common carp larvae after 96 h of exposure. Based on above threshold values of neonicotinoid pesticides for different fish species, it can be reported that THM is more toxic than other neonicotinoid pesticides; and banded gourami is more sensitive to THM than other species tested in previous studies.

Several malformations were evident in the embryos and larvae exposed to different concentrations of THM. The most prevalent malformation in embryo and larvae of banded gourami was yolk-sac edema after THM exposures. The formation of edema in embryos and larvae can partially be explained by the fact that THM exposure disturbs the osmoregulatory functions that leads to the down regulation of pkt7 (a critical regulator of slc2a10/glut10) and wwox genes [39], however, the mechanisms of edema formation remains further investigation. The findings of our study are in accordance with previous investigations in

Table 3
Cumulative malformation rate (%) of banded gourami embryo and larvae exposed to different concentrations of THM in the end of the embryonic and larval bioassays.

| Concentrations (mg/L) | Embryo (24 h) | One-day-old larvae (96 h) |
|-----------------------|---------------|---------------------------|
| 0                     | 0.00          | 0.00                      |
| 0.02                  | 0.00 ± 0.00   | 10.99 ± 8.11              |
| 0.2                   | 3.03 ± 5.24   | 10.49 ± 3.91              |
| 2                     | 24.24 ± 2.48  | 20.49 ± 2.48              |
| 20                    | 38.78 ± 4.44  | 31.49 ± 5.42              |
| 200                   | ND            | ND                        |
| P value               | 0.000         | 0.000                     |

ND, no data due to 100% mortality

Fig. 1. Malformation observed in banded gourami embryos due to THM toxicity. (A) Normal fertilized embryo (zygote) after 6 h of exposure to 0mgL\(^{-1}\) of THM (B) Unhatched egg (UE) after 24 h of exposure to 20mgL\(^{-1}\) of THM (C) Dark-brown yolk sac (DBYS) after 18 h of exposure to 200mgL\(^{-1}\) of THM (D) Eggshell broken (ESB) after 6 h of exposure to 0.2mgL\(^{-1}\) of THM (E) Yolk sac edema (YSE) after 18 h of exposure to 200mgL\(^{-1}\) of THM (F) Yolk sac edema (YSE) after 18 h of exposure to 20mgL\(^{-1}\) of THM. Scale bar 40 µm, magnification 100X.

Fig. 2. The frequencies of each malformation observed in banded gourami embryo exposed to THM in the end of experimental period of 24 h. ESB: eggshell broken; UE: un-hatched egg; YSE: yolk-sac edema; DBYS: dark brown yolk-sac.
the sense that they also observed similar kinds of malformation for various fish species exposed to different pesticides. For instance, imidacloprid resulted yolk-sac edema and perichardial edema in common carp embryo and larvae after acute exposures as stated by Islam et al. [9]. Velisek and Stara [35] observed yolk-sac edema and pericardial edema in common carp larvae exposed to neonicotinoid thiacloprid. Similar malformation was reported for African catfish exposed to buprofezin [40], Stinging catfish exposed to fenitrothion [41], gangetic mystus exposed to cypermethrin [29], zebrafish exposed to sumithion [42], and banded gourami exposed to chlorpyrifos [22].

In conclusion, present results demonstrate that the neonicotinoid insecticide THM can affect hatching success, incubation period, and survival of embryo and larvae of Banded Gourami. The threshold values (LC10 and LC50 values) for embryos and larvae derived from this study will be useful for the environmental risk assessment of THM for fish in the future. However, we will design long-term mechanistic studies to understand the underlying mechanisms of the toxicity of THM in the

Fig. 3. Malformations observed in one-day-old larvae of banded gourami due to THM. (A) A representative larva after 24 h of exposure to 0 mg L\(^{-1}\) of THM (B) Notochord abnormality (NA) and deformed posterior body part (DB) after 24 h of exposure to 20 mg L\(^{-1}\) of THM. (C) Hemorrhage (H), yolk sac edema (YSE) and irregular caudal fin (ICF) after 24 h of exposure to 0.2 mg L\(^{-1}\) of THM. (D) Notochord abnormality (NA), yolk sac edema (YSE), and irregular caudal fin (ICF) after 48 h of exposure to 0.2 mg L\(^{-1}\) of THM. (E) Yolk sac edema (YSE) after 72 h of exposure to 0.2 mg L\(^{-1}\) of THM. (F) Lordosis (L) and yolk sac edema (YSE) after 24 h of exposure to 20 mg L\(^{-1}\) of THM. (G) Irregular caudal fin (ICF) after 24 h of exposure to 2 mg L\(^{-1}\) of THM. (H) Notochord abnormalities (NA) after 96 h of exposure to 2 mg L\(^{-1}\) of THM. (I) Yolk sac edema (YSE) and notochord abnormalities (NA) after 24 h of exposure to 0.02 mg L\(^{-1}\) of THM. Scale bar 50 μm, magnification 40X.

Fig. 4. The frequencies of each malformation observed in one-day-old larvae of banded gourami exposed to THM in the end of the experimental period of 96 h. YSE: yolk-sac edema; H: hemorrhage; ICF: irregular caudal fin; L: lordosis; NA: notochord abnormality.
developing Banded Gourami.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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