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Original Research

Effects of Total Hardness and Calcium:Magnesium Ratio of Water during Early Stages of Rare Minnows (*Gobiocypris rarus*)

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The ionic composition of water is important for all fish. In the present study, the effects of total hardness and Ca^{2+}:Mg^{2+} ratio on early life stages of rare minnows (*Gobiocypris rarus*), a promising laboratory fish in China, were evaluated. Paired parent fish were transferred to spawning aquaria (16 L) containing water at different total hardness and Ca:Mg ratios, and their offspring were further cultured at 25 ± 1 °C and 12:12-h light:dark photoperiod. Fertilization rates were not affected by total hardness to 480 mg L^{-1} $CaCO_3$, but egg size decreased with increasing total hardness. Ca:Mg ratios less than 1:20 or greater than 8:1 had adverse influences on hatching, feeding, development, larval growth, and survival. Embryos and larvae incubated in Mg^{2+}- and Ca^{2+}-deficient waters exhibited high malformation rates and high mortality. Our results demonstrate that rare minnows can adapt to a wide range of total hardness and Ca:Mg ratios, although an imbalance between Ca^{2+} and Mg^{2+} in water is toxic to this species. To increase the comparability and usefulness of test results, we recommend the use of reconstituted or drinking water of defined total hardness and Ca:Mg ratio for the culture and toxicity testing of rare minnows.

Abbreviations:dph, days posthatching; TH, total hardness

The ionic composition of water is important for all fish. The concentrations of the divalent cations Ca^{2+} and Mg^{2+} play a vital role in the ionic regulation of freshwater fish because these ions modulate branchial permeability.[52] Water is also an important source of the Ca^{2+} and Mg^{2+} required for fish growth.[5,17] Ca^{2+} and Mg^{2+} are the main contributors to the total hardness (TH) of water, which is generally considered to be an important factor in fish culture. Numerous studies have demonstrated that TH has significant influences on fertilization, hatching, and larval culture. In particular, inappropriate TH has been associated with fertilization failure,[30] hatching failure,[11,18,28,36] larval abnormality,[28] retardation of growth and development,[4,28,40] and mortality.[23,28,40] In addition, the relative proportions of Ca^{2+} and Mg^{2+} in the water (expressed as the Ca:Mg ratio in the present study) are important in fish culture, and imbalances between Ca^{2+} and Mg^{2+} adversely affect embryonic development,[7,33,34,42,43] larval growth, and survival.[35] An appropriateTH and Ca:Mg ratio of the ambient water is essential for the successful culture of fish.

Rare minnows (*Gobiocypris rarus*) are an endemic cyprinid fish inhabiting the upper Yangtze River. Wild populations of this species are sparsely distributed in several counties of Sichuan Province, China.[16,49] Rare minnows usually live in small water systems, such as paddyfields, puddles, and ditches, especially weedy ditches with flowing water.[18,44] Tubificidae, *Chironomus* larvae, copepods, and cladocerans are their favorite foods.[45] In general, rare minnows achieve maturity in 4 mo and spawn every 3 or 4 d as night falls,[8,46] with a clutch size ranged from 96 to 655 eggs.[46] Rare minnows are easily cultured in the laboratory.[8] Newly spawned eggs are adhesive and attach to aquarium walls[10,44] and then gradually hatch out during the following 4 d at 25 °C.[10] These features make it possible to generate copious biologic material for scientific research. Moreover, rare minnows have proven to be a sensitive species to chemicals and pollutants.[55,57-59] To date, as a recommended test organism for the toxicity testing of chemicals in China,[14] rare minnows havewidely been applied to toxicologic studies on endocrine disruptors[60,62] and heavy metals.[27,63]

High-quality natural water, drinking water, or reconstituted water are usually recommended for the culture of and toxicity testing using *G. rarus*.[13,14] However, the ionic compositions of natural and drinking water vary among water systems because of differences in bedrock geology, land use, anthropogenic activities, and rainfall.[26,64] Both TH and Ca:Mg ratio vary markedly among these water sources in China,[39,56] and whether all are suitable for the culture of this endemic species is unknown. In particular, TH, especially Ca^{2+}, reduces the acute or chronic toxicity of many compounds for fish.[51] Using reconstituted water of consistent quality in toxicity tests would help improve the comparability of the results. Currently, the 'standard dilution water' (TH, 250 mg/L $CaCO_3$; Ca:Mg ratio, 4:1) recommended by the Organization for Economic Cooperation and Development has been used

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Table 1. Composition of water of varied total hardness and the stock solutions used to generate the experimental conditions

	Total hardness (mg/L $CaCO_3$)						Stock solutions		
Reagent(mg/L)	60	120	240	480	720	960	Mg^{2+}-free	Ca^{2+}-free	Ca^{2+}- and Mg^{2+}-free
$CaCl_2 \cdot 2H_2O$	44.10	88.20	176.40	325.80	502.20	705.60	325.80	0.00	0.00
$MgSO_4 \cdot 7H_2O$	73.80	147.60	295.20	590.40	885.6	1180.80	0.00	590.40	0.00
$NaHCO_3$	64.75	64.75	64.75	64.75	64.75	64.75	64.75	64.75	64.75
KCl	5.75	5.75	5.75	5.75	5.75	5.75	5.75	5.75	5.75

The stock solutions contained 240, 240, and 0 mg/L $CaCO_3$, respectively.

widely in acute toxicity tests.[29] Whether standard dilution water is also applicable for toxicity tests using rare minnows needs to be assessed.

In the present study, the effects of TH and Ca:Mg ratio on the fertilization, hatching, development, larval growth, and survival of rare minnows were evaluated. Our experimental aim was to test whether this experimental fish has specific requirements for TH and Ca:Mg ratio during its early life stages. The present study is the first evaluation of the effects of TH and Ca:Mg ratio on rare minnows and provides a scientific basis for experimental applications of this promising model fish.

Materials and Methods

Fish and rearing conditions. Rare minnow parent fish (age, 1 y; weight: female, 1.58 ± 0.38 g; male, 0.96 ± 0.38 g; n = 10 per sex) were obtained from a conventional closed colony (Ihb:IHB) that was free from acute and highly infectious disease including virulent *Aeromonas*, *Mycobacterium marinum*, *Vibrio cholera*, *Flavobacterium columnare*, *Balantidium coli*, *Ichthyophthirius multifiliis*, *Trichodina* spp., *Chilodonella* spp., *Dactylogyru* spp., and *Gyrodactylus* spp. and held at the Institute of Hydrobiology (Chinese Academy of Sciences, Wuhan, China). Random breeding pairs were generated and housed in a custom-built recirculating aquaculture system, which contained 3 rows of plastic aquaria (10 independent aquaria per row), one pump (Shinhoo, Hefei, China), and one filter tank (Shanghai Haisheng Biotech, Shanghai, China). Every aquarium (Suhang Instruments Equipment for Lab Animal, Suzhou, China) has an independent inlet valve to regulate water flow. Water-quality parameters were determined weekly and maintained as follows: temperature, 25 ± 1 °C; dissolved oxygen, 7.0 to 8.5 mg/L; pH, 7.80 to 8.55; ammonia, less than 0.5 mg/L;nitrite, less than 0.1 mg/L; nitrate, less than 1.0 mg/L; conductivity, 420 to 480 µS/cm; alkalinity, 140 to 174 mg/L $CaCO_3$; and TH, 167.33 ± 5.39 mg/L $CaCO_3$. The photoperiod was maintained asa 12:12-h light: dark cycle. Rare minnows were fed to satiety with ozone-disinfected frozen *Chironomus* larva (Yuerle, Tianjin, China) twice daily. Spawning data were recorded daily.

Experiments were conducted according to the Chinese Ministry of Science and Technology Guiding Directives for Humane Treatment of Laboratory Animals[15]. All animals were treated humanely and with the aim of alleviating any suffering.

Exposure solutions. Seven experimental media that varied in TH(nominal TH: 60, 120, 240, 480, 720, and 960 mg/L $CaCO_3$; all with a Ca:Mg molar ratio of 1:1) and 3 stock solutions (Mg^{2+}-free; Ca^{2+}-free; Ca^{2+}- and Mg^{2+}-free) were combined to prepare media with a range of Ca:Mg ratios after the addition of appropriate quantities of 4 analytical grade salts ($NaHCO_3$, KCl, $CaCl_2 \cdot 2H_2O$, and $MgSO_4 \cdot 7H_2O$; Sinopharm Chemical Reagent) according to a previous study[31] (Table 1). Aerated tap water (TH, 167.33 ± 5.39 mg/L $CaCO_3$; Ca:Mg ratio, 4:1) was used for the control group.

Effect of TH. On the basis of previous observations of their spawning interval (3 to 4 d) and prenatal behavior, parent fish were transferred to the spawning aquaria (16 L) containing different TH exposure solutions at 18:00 and removed after spawning at 23:00. On the following day, eggs were siphoned into glass cylinders (600 mL; diameter, 12 cm; Huaou Industry, Yancheng, China) containing the same exposure solution as they were spawned in, and the diameters of random eggs were measured under a dissecting microscope (model SMZ 168, Motic, Hong Kong). Unfertilized and fertilized eggs were counted to calculate the fertilization rate. Thereafter, fertilized eggs were allocated to cylindrical glass containers at a density of 30 eggs per container for further incubation in same exposure solution.

When they hatched, larval morphologic characteristics were measured immediately under the dissecting microscope. The body length of newly hatched larvae was measured as shown in Figure 1. Hatchability was calculated when hatching had finished. Rare minnow larvae were fed with newly hatched brine shrimp (*Artemia* spp.) nauplii (Heading, Tianjin Red Sun Aquaculture, Tianjin, China) to satiety twice daily beginning at 2 d posthatching (dph). At 8 dph, larvae were transferred to square glass aquaria (20 cm × 20 cm × 20 cm) containing 5 L of the same exposure solution. Body lengths and survival rates were determined at 15 and 22 dph in larvae anesthetized in tricaine methanesulfonate solution (MS222, Sigma-Aldrich, St Louis, MO) with a buffered pH of 7.5. In total, 10 pairs of parent fish were used in this experiment. Ten batches of eggs (one batch per pair) were collected for the measurement of body length of newly hatched larvae and fertilization rate, and 3 replicates were further cultured for the measurement of body length and survival rate at later life stages.

Effect of Ca:Mg ratio. On the basis of previous observations of their spawning interval and prenatal behavior, parent fish were transferred to spawning aquaria (16 L) containing Ca:Mg exposure solutions at 18:00 and were removed, after spawning, at 23:00. Eggs were collected, and the larvae were cultured as described in the previous experiment on TH, but the Ca:Mg ratio experiment was terminated at 7 dph. Egg diameters, fertilization rate, hatchability, body length (0 and 7 dph), and survival rate were measured as before. In addition, the number of larvae with functional swim bladders at 1 dph and those with *nauplii* in the gut at 3 dph were counted to calculate the rate of swim bladder inflation and feeding incidence according to the following equations:

$$\text{The rate of swim bladder inflation \%} = \frac{\text{no. of larvae with functional swim bladder}}{\text{no. of larvae surviving at 1 dph}} \times 100\%$$

$$\text{Feeding incidence \%} = \frac{\text{no. of larvae with } \textit{nauplii} \text{ in gut}}{\text{no. of larvae survived at 3 dph}} \times 100\%$$

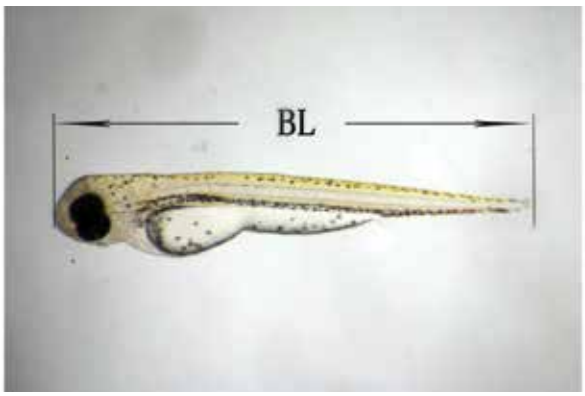

Figure 1. A newly hatched rare minnow larva. BL, body length.

In total, 10 pairs of parent fish were used in this experiment. Ten batches of eggs (one batch per pair) were collected for the measurement of the body length of newly hatched larvae and fertilization rate, and 3 additional replicates were further cultured to measure the rate of swim bladder inflation, feeding incidence, and body length over the following 7 d.

Water-quality monitoring. Throughout the experiments, exposure solutions were renewed twice daily (at 2 h after feeding) by removing and replacing more than 90% of the total volume. The pH, dissolved oxygen, and temperature of the exposure solutions were monitored and recorded daily by using a water-quality analyzer (model HQ30d, Hach, Loveland, CO). TH, calcium hardness, ammonia, and nitrite were measured before and after water replacement and determined titrimetrically according to standard methods.[13]

Statistical analysis. Egg diameter, fertilization rate, body length, hatchability, rate of swim bladder inflation, feeding incidence, malformation rate, and survival rate were checked for assumptions of homogeneity of variance by using the Levene test. Wherever the assumption was met, data were analyzed by ANOVA, followed by Waller–Duncan multiple-comparison tests to compare with the controls. If the assumption was not met, data were analyzed by using the nonparametric Kruskal–Wallis test followed by the Mann–Whitney *U* test. The significance level was set at a *P* level of less than 0.05. Except when stated otherwise, the software SPSS 19.0 (IBM, Armonk, NY) was used. All figures were created by using Excel 2007 (Microsoft, Redmond, WA) and Photoshop CS 7.0 (Adobe, San Jose, CA).

Results

Water quality. Dissolved oxygen concentrations were between 7.0 and 8.5 mg/L in all experiments. Mean values of ammonia and nitrite were controlled at less than 0.5 mg/L NH_4^+-N and less than 0.1 mg/L NO_2^--N, respectively. The pH values of the exposure solutions were stable at 7.75 ± 0.12 except for the control, which was 8.25 ± 0.05. Measured TH values of exposure solutions did not differ by more than 5% from their nominal values. Measured values of Ca^{2+} hardness and Mg^{2+} hardness of the

Table 2. Measured Ca^{2+} and Mg^{2+} hardness of Ca:Mg solutions

Ca:Mg ratio	Ca^{2+} hardness	Mg^{2+} hardness
Ca^{2+}- and Mg^{2+}-free	2.20 ± 0.53	1.40 ± 0.53
Ca^{2+}-free	2.50 ± 0.48	243.08 ± 3.51
1:80	6.20 ± 0.66	237.50 ± 2.69
1:20	13.95 ± 1.95	231.65 ± 2.23
1:8	27.60 ± 1.04	217.18 ± 2.21
1:4	49.15 ± 1.62	193.85 ± 3.05
4:1	194.73 ± 3.17	47.60 ± 1.71
8:1	216.93 ± 3.26	26.75 ± 1.11
20:1	225.68 ± 4.34	15.01 ± 0.62
80:1	237.80 ± 2.25	4.53 ± 1.13
Mg^{2+}-free	245.48 ± 4.64	1.10 ± 0.39

Data are expressed as mean ± SD mg/L $CaCO_3$.

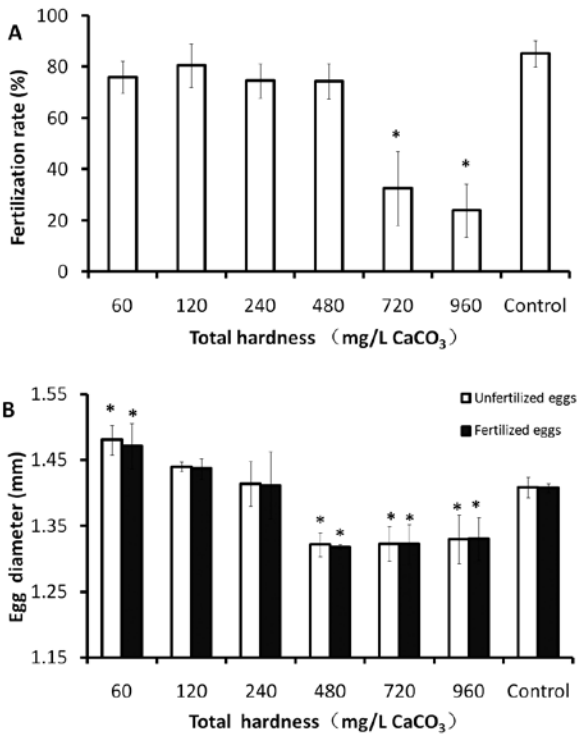

Figure 2. (A) Fertilization rate and (B) egg diameter of rare minnows spawned in solutions of different total hardness. Data are presented as mean ± SEM (error bars). *, Value is significantly ($P < 0.05$) different from that of the corresponding control group (aerated tap water).

Table 3. Fertilization rate, hatchability, malformation rate, rate of swim bladder inflation, feeding incidence, and survival rates (%) of rare minnows incubated in solutions with different Ca:Mg ratios

Ca:Mg ratio	Fertilization rate	Hatchability	Malformation rate	Rate of swim bladder inflation	Feeding incidence	Survival rate
Ca^{2+}- and Mg^{2+}-free	82.33 ± 6.82	98.89 ± 1.92	0.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	96.59 ± 3.45
Ca^{2+}-free	79.39 ± 6.96	21.11 ± 13.88[a]	96.97 ± 5.25[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
1:80	85.45 ± 4.67	97.78 ± 1.92	5.71 ± 4.02[a]	85.17 ± 8.06	30.61 ± 6.27[a]	34.18 ± 7.36[a]
1:20	81.14 ± 2.22	98.89 ± 1.92	0.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
1:8	85.73 ± 8.49	98.89 ± 1.92	0.00 ± 0.00	98.89 ± 1.92	98.89 ± 1.92	98.89 ± 1.92
1:4	83.29 ± 9.96	100.00 ± 0.00	0.00 ± 0.00	100.00 ± 0.00	97.78 ± 3.85	97.78 ± 1.92
4:1	81.18 ± 8.88	98.89 ± 1.92	0.00 ± 0.00	100.00 ± 0.00	98.85 ± 1.99	97.78 ± 1.92
8:1	83.21 ± 5.45	95.56 ± 1.92	0.00 ± 0.00	100.00 ± 0.00	98.85 ± 1.99	94.17 ± 2.07
20:1	84.14 ± 2.98	94.44 ± 5.09	0.00 ± 0.00	100.00 ± 0.00	83.02 ± 14.71[a]	96.46 ± 0.19
80:1	87.65 ± 7.53	100.00 ± 0.00	15.56 ± 10.72[a]	31.11 ± 40.32[a]	0.00 ± 0.00[a]	8.89 ± 12.62[a]
Mg^{2+}-free	81.14 ± 1.69	82.22 ± 11.71[a]	91.24 ± 7.67[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
Control	86.87 ± 9.66	100.00 ± 0.00	0.00 ± 0.00	98.89 ± 1.92	97.78 ± 3.85	98.89 ± 1.92

Data are expressed as mean ± SD.
[a]Value significantly ($P < 0.05$) different from that of the corresponding control group (aerated tap water).

reconstituted water used in the Ca:Mg ratio experiment are shown in Table 2.

Effect of TH on early life stages. The fertilization rate of rare minnows was not affected by TH concentrations between 60 and 480 mg/L$CaCO_3$. The fertilization rates in the 720 and 960 mg/L $CaCO_3$ groups were significantly ($P < 0.05$) lower than in the control group (Figure 2 A). Egg size decreased significantly with increasing TH ($P<0.01$)(Figure 2 B). Egg size did not differ between unfertilized and fertilized eggs (Figure 2 B). Hatchability and body length of newly hatched larvae were not significantly different among groups. At 22 dph, larval survival rates were between 70% and 84% and were not significantly different among treatments. There were no significant differences in body length at 22 dph among treatment groups.

Effect of Ca:Mg ratio on early life stages. Fertilization rates were not affected by the Ca:Mg ratio, even under Ca^{2+}-free, Mg^{2+}-free, and Ca^{2+}- and Mg^{2+}-free conditions (Table 3). Egg size was not affected by Ca:Mg ratios, except in the treatment lacking both Ca^{2+} and Mg^{2+}. However, Ca:Mg ratios less than 1:20 or greater than 8:1 adversely effected hatching, feeding, development, and larval growth and survival (Table 3, Figure 3). High malformation rates and mortality occurred in the Mg^{2+}- and Ca^{2+}-deficient groups (Table 3). Larvae incubated in Mg^{2+}-deficient water displayed arch-shaped bodies, cardiocoelomic edema, and yolk sac necrosis, whereas those in Ca^{2+}-deficient water presented corkscrew-shaped and S-shaped bodies (Figure 4). Surprisingly, the feeding, development, and larval growth and survival of the group that was deficient in both Ca^{2+} and Mg^{2+}were comparable to those of larvae in aerated tap water (Table 3, Figure 3).

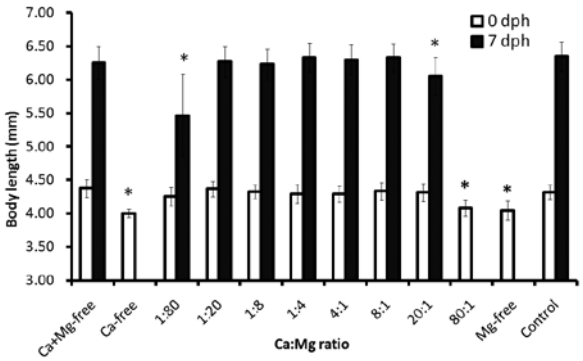

Figure 3. Body length (mean ± SEM [error bars]) at 0 and 7 d posthatching (dph) of rare minnows incubated in water with different Ca:Mg ratios. *, Value is significantly ($P < 0.05$) different from that of the corresponding control group (aerated tap water).

Discussion

In the present study, TH of greater than 480 mg/L $CaCO_3$ had an obvious adverse effect on fertilization in rare minnows (*G. rarus*). A similar effect was observed in vundu catfish (*Heterobranchus longifilis*).[30] The low fertilization rate in hard water is possibly related to its high osmolality, which is believed to inhibit sperm motility in freshwater fishes.[2] High levels of Ca^{2+} and Mg^{2+} might also cause the low fertilization rate. Some authors[37] suggest that Ca^{2+} was a major contributor to fertilization failure in salmonids when the concentration of total dissolved solids was higher than 250 mg/L. In another study,[24] Ca^{2+} and Mg^{2+} hardness higher than 150 mg/L $CaCO_3$ suppressed sperm motility in perch (*Perca fluviatilis*). Further studies should be conducted to explore the inhibition of sperm motility by osmolality and ionic concentration in rare minnows.

Although egg size in rare minnows was linearly dependent on TH, hatching success was not affected by the tested values of TH or Ca:Mg ratio. In a previous study,[18] silver carp (*Hypophthalmichthys moltrix*) eggs incubated at TH of 100 to 200 mg/L $CaCO_3$ absorbed excess water and burst prematurely. Similarly, striped bass (*Morone saxatilis*) eggs swelled excessively in soft water, becoming more buoyant and floating out of the jar and affecting hatchability.[36] Furthermore, African catfish (*Clarias gariepinus*) eggs that hatched in water of TH greater than 200 mg/L $CaCO_3$ showed a high incidence of larval abnormality.[28] These effects were not detected in the present study, which suggests that the chorion of rare minnows may have an increased ability to protect embryos from osmotic disturbance.

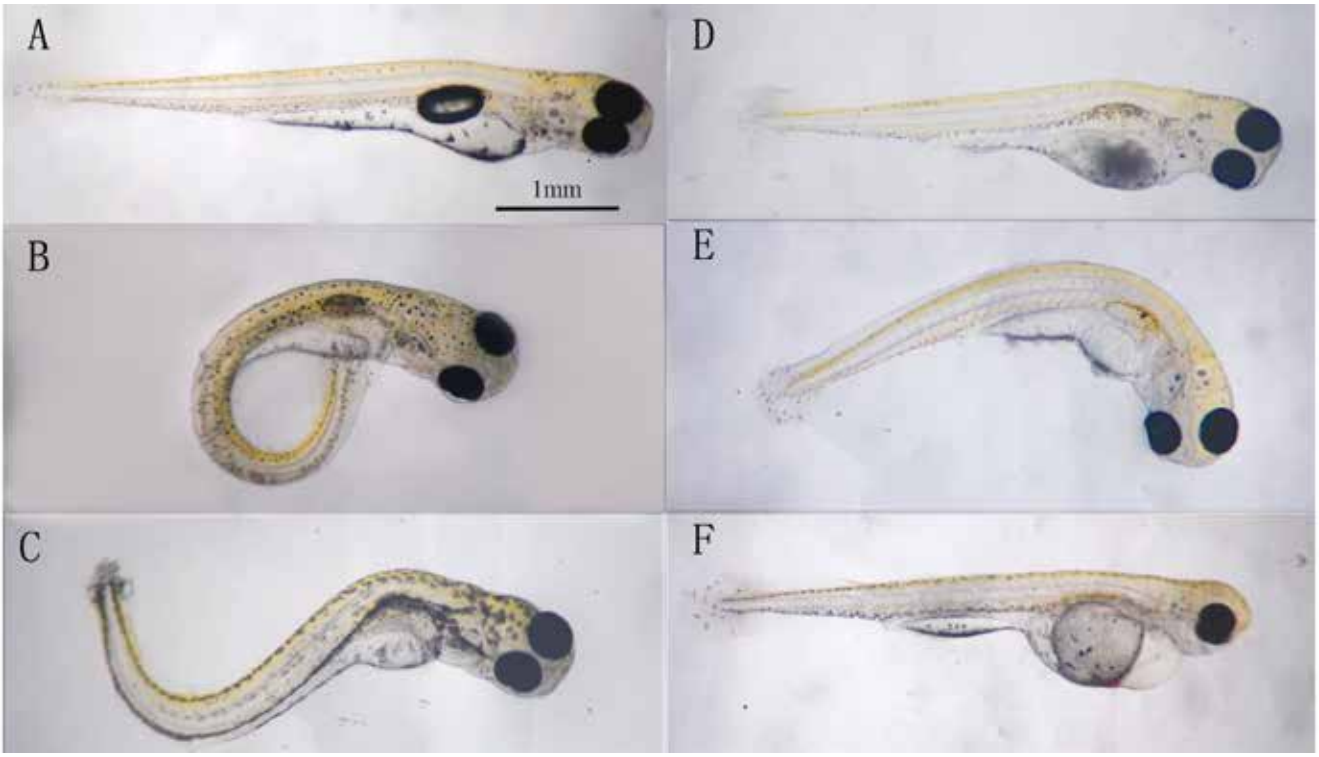

Figure 4. Morphologic abnormalities at 1 d posthatching of rare minnow larvae incubated in Ca^{2+}- and Mg^{2+}-deficient water. (A) Normal body structure. (B) Corkscrew-shaped body (Ca^{2+}-deficient water). (C) S-shaped body (Ca^{2+}-deficient water). (D) Yolk sac necrosis (Mg^{2+}-deficient water). (E) Arch-shaped body (Mg^{2+}-deficient water). (F) Cardiocoelomic edema (Mg^{2+}-deficient water).

Our results revealed that an imbalance in the ratio of Ca^{2+} to Mg^{2+} concentration in the water was toxic to rare minnow embryos and larvae. A similar phenomenon was found in black sea bream (*Sparus macrocephalus*),[33] Japanese eel (*Anguilla japonica*),[43] common carp (*Cyprinus carpio* L.),[42] and brown trout (*Salmo trutta* L.).[7] A Mg^{2+} hardness less than 10 mg/L $CaCO_3$ impeded the embryonic development of common carp when the Ca^{2+} hardness was 80 mg/L $CaCO_3$.[42] Another study found that magnesium sulfate is toxic to aquatic organisms and that its toxicity is dependent on the Ca^{2+} concentration.[41] In the present study, Ca^{2+}- and Mg^{2+}-deficient water had adverse effects on the development of rare minnows. It is plausible that there is a minimum requirement for waterborne Ca^{2+} and Mg^{2+}during the early life stages of rare minnows. However, these adverse effects might not be symptoms of Ca^{2+} or Mg^{2+} deficiency. In general, the nutrients in the yolk sac are sufficient to meet the requirementsfor larval growth to 3 dph.[50] In the present study, fertilized eggs incubated in Ca^{2+}- or Mg^{2+}-free water were abnormal, and most of the larvae that hatched died before the first feeding. This finding indicates that the adverse effects observed in the present study may be not caused by Ca^{2+} or Mg^{2+} deficiency.

In the present study, rare minnows grew normally in water free of both Ca^{2+} and Mg^{2+}. This absence of adverse effects could be related to an ability to modulate the efficiency and mechanisms of uptake of these ions in different environments. To acclimate to low-Ca^{2+} medium, fish modulate their Ca^{2+} uptake capacity through proliferation of the lamellar mitochondrial cells.[12,22] Moreover, fish are able to absorb Ca^{2+} and Mg^{2+} from the diet to meet the requirements for growth and development.[5,20] Brine shrimp nauplii (*Artemia* spp.) are rich in nutrients and are considered to provide an excellent diet for commercial larviculture of aquatic animals. Brine shrimp eggs reportedly contain 72.10 mg Ca^{2+} and 22.34 mg Mg^{2+} per 100 g.[38] The nutrients in brine shrimp nauplii might be sufficient to meet the Ca^{2+} and Mg^{2+} requirements of rare minnow larvae. Further studies should be conducted to confirm this finding. However, water deficient in either Ca^{2+} or Mg^{2+} was toxic to rare minnow larvae. High levels of waterborne Mg^{2+} or Ca^{2+} can lead to hypocalcemia or hypercalcemia, respectively, as found in tilapia (*Sarotherodon mossambicus*).[52] An excessively high or low Ca:Mg ratio in the incubation water might lead to the accumulation of cellular Ca^{2+} or Mg^{2+} because of the competitive relationship between these cations[42] and thus produce toxic effects.

Previous studies have demonstrated that rare minnows can adapt to a broad range of water temperatures and are highly tolerant to hypoxia and hypercapnia.[47,48] Another study[54] found that this species rapidly adapts to changes in culture conditions. In the present study, rare minnows showed broader adaptability to TH than did other freshwater fishes, including silver catfish (*Rhamdia quelen*),[40] silver carp (*H. molitrix*),[18] vundu catfish (*H. longifilis*),[30] African catfish (*C. gariepinus*),[28,30] and hybrid catfish (channel catfish *Ictalurus punctatus* × blue catfish *Ictalurus furcatus*).[11] Our results similarly demonstrated that rare minnows were able to adapt to a wide range of Ca:Mg ratios, although water deficient in either Ca^{2+} and Mg^{2+} typically is toxic to this laboratory fish. Given that the current recommended maximal TH of drinking water is 500 mg/L $CaCO_3$[53] and that the surface water is rich in Ca and Mg[56], , we conclude that TH and Ca:Mg ratio are not likely to be barriers to more widespread distribution of *G. rarus* and to its application as a model fish. However, other parameters, such as alkalinity and pH, need to be considered when determining

whether drinking water is suitable for maintaining rare minnows. These parameters can affect water hardness through effects on chemical equilibrium[25] and are significant in fish growth and survival.[1,9]

Water hardness, especially Ca^{2+} hardness, is a well-known modifying factor of toxicity for heavy metals and other chemicals.[6,32,51] A previous study has demonstrated that water hardness in both rearing water and test solutions had important effects on the results of toxicity tests.[3] As a promising model fish recommended by the China Registration, Evaluation, Authorization, and Restriction of Chemicals,[14] rare minnows have been used widely for toxicity testing and risk assessment of chemicals, including hormones,[55] herbicides,[65] fungicides,[62] pesticides,[60] heavy metals,[27,63] and others.[61] High-quality natural or drinking water and reconstituted water are usually used for culturing and toxicity testing involving rare minnows.[14] Although the different TH and Ca:Mg ratios of these types of water are safe and suitable for rare minnows, they might induce significant variability in test results.[21] To increase the comparability and effectiveness of test results, we recommend using drinking or reconstituted water of definedTH and Ca:Mg ratio for culturing and toxicity tests involving rare minnows. According to our current results, we consider that standard dilution water (TH, 250 mg/L $CaCO_3$; Ca:Mg ratio, 4:1) is appropriate and should be used consistently for rare minnows.

Acknowledgments

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