Water pH effects on survival, reproductive performances, and ultrastructure of gonads, gills, and skins of the Javanese medaka (*Oryzias javanicus*)

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Abstract: The Javanese medaka (*Oryzias javanicus*) has the potential to be developed as a test organism. It also exhibited many characteristics that are similar to those of well-known laboratory fishes. This study determined the effects of water pH on the survival, reproductive performances, and ultrastructure of the gonads, gills, and skins of the Javanese medaka. This study confirmed that Javanese medakas treated with pH 6–7 displayed the lowest mortality, but increased in egg production and hatchability. This supposition is supported by strong evidence from the oocytes and testes stage analyses, including histological examinations. The ultrastructure of gills and skins of fish treated in that range was also less histologically affected, indicating its suitability range. The sensitivity of the Javanese medaka towards environmental changes, in terms of the selected physiological performances and cellular level, indicate its potential as a test organism in tropical regions.

Key words: pH, Javanese medaka, survival, reproductive performances, ultrastructure

1. Introduction

The Javanese medaka (*Oryzias javanicus* Bleeker 1854) has the potential to be developed as a test organism, and exhibited many characteristics that are similar to well-known laboratory fishes (1). This small tropical fish has been widely used as a test organism in toxicology and ecotoxicology studies (2–5). This species has also been used in several studies to represent marine and freshwater fish due to its ability to occupy fresh, brackish, and saltwater (6). The Javanese medaka is widely distributed in the coastal areas of Peninsular Malaysia (7,8), Singapore, Indonesia, Thailand, and Western Borneo, and available all year round (9,10).

The water pH is very important in the survival and reproduction of medakas in captivity. Mass culture production of medakas also would be a great success if the optimal range of water quality could be determined, including the water pH (11). The pH preferences for wild and cultured medakas in captivity were found to be different from previous studies. In the natural environment, a study found that the mean pH of *O. javanicus* habitats (6.60 ± 0.40) tends to be higher compared to that of the Indian medaka *O. dancena* (5.9 ± 0.22), along the west of Peninsular Malaysia, Malaysia (7). However, for culture and breeding conditions in the laboratory, a pH range of 5.5–5.6 was used in an experimental study for *O. javanicus* (5), while for the Japanese medaka (*O. latipes*), a pH range between 6.8 and 7.5 was recommended for large-scale breeding (11).

Fish gonads have long been studied by biologists to identify annual reproductive cycles, length of breeding seasons, onset of reproductive maturity, spawning rhythms, fecundity, and various other aspects of reproductive biology (12). Besides gonads, another important organ of fish is the gills, which is a multipurpose organ that in addition to providing aquatic gas exchanges plays dominant roles in osmotic and ionic regulation, acid–base regulation, and excretion of nitrogenous wastes (13). The skin, which is the largest organ in an animal, provides an immunological and physical defense mechanism against harmful threats. However, fish skins differ from other exposed vertebrate skins, most notably at the surface, where living epidermal cells are in direct contact with the environment (14). The gonads, gills, and skins of fish have been widely used to study various changes and effects of chemicals, toxicants, and even physico-chemical water quality (15–17).
Information on the direct effect of pH on mortality, reproduction, and histological changes in organs in the Javanese medaka is limited compared to zebrafish (18–20) and other species of medaka (21). Understanding on these factors would be of great significance for future rearing programs of medakas in captivity and expand our current knowledge on the effect of pH on fish physiology, and even at the cellular level. This study investigates the effects of different pH on the survival, egg production, egg hatchability, and ultrastructure of the gonads, gills, and skins of Javanese medakas in captivity.

2. Materials and methods

2.1. Fish collection, acclimatization, and sex sorting
Javanese medakas were collected from the estuary area in Bagan Lalang, Selangor, Malaysia (2°37.15.2′N 101°42.38.4′E). The fish were placed in plastic aquarium containers provided with oxygen stones (Japan Pet Drugs Co. Ltd) and immediately transported back to the laboratory. In the laboratory, the fish were then acclimatized from the original salinity level where the fish were captured to the final 0 ppt at 1–2 ppt/day. The fish were placed in a 50-L holding tank with dechlorinated tap water and continuous aeration.

The fish were sorted according to their sex (10). After sorting, the fish were separated accordingly to different tanks. The fish were fed twice a day with commercial brine shrimps and dry foods.

2.2. Experimental design
The mean ± standard deviation (SD) of the total length and body weight of the fish prior to the experiment were 1.41 ± 0.23 cm and 0.109 ± 0.091 g, respectively. The aquariums used for the fish treatment were 18 cm height × 16 cm width × 26 cm length, with approximately 6-L volume of water. Each treatment was conducted in duplicate. Every replicate contained fish at a sex ratio of 3 females to 1 male during the experiment. The fish were placed in a combination of three males and nine females in an aquarium, making a total of 12 fish in each. A light source was provided on each aquarium, set up at a photoperiod of 14 h light:10 h dark. The fish were fed twice a day with commercial brine shrimps and dry foods. The fish ratio and photoperiod were set up as stated in order to induce breeding (11,22).

2.3. Fish treatment with different water pH
Five different water pH treatments were used in this study. The pH value was maintained at desirable pH 5, 6, 7, 8, and 9 using Neon pH-Up (Product No. PHU 2520) and Neon pH-Down (Product No. PHD 2521) (Jesco Trading, Malaysia). These buffers consisted of two types (pH down and pH up), which neutralize some of the carbonate hardness and alter the pH. The pH may rebound up to the original pH if the carbonate hardness changes. Based on the buffer contents, the pH up consisted of CaCO₃ and water, while the pH down consisted of H₂SO₄ and water. The water pH reading was checked every 12 h using a pH meter (Jenway Model 3450, UK) and maintained at the desired level throughout the study period.

The fish were exposed to different pH for 14 consecutive days. The water lost due to natural evaporation was replaced by the prepared water with desirable pH at 24-h intervals. The water temperature and dissolved oxygen were not disturbed during the study and were measured daily using a hand-held YSI meter (YSI, Yellow Springs, OH, USA), while ammonia was measured using multiparameter handheld colorimeter (DR900, Hach Company, Loveland, CO, US). The experiment was conducted in fully fresh water (0 ppt). The fish were handled and sacrificed according to the method approved by the Institutional Animal Care and Use Committee, Universiti Putra Malaysia (AUP No.: R006/2016).

2.4. Mortality, egg production, and egg hatchability
Fish mortality was recorded daily during the study period, and in order to maintain the accuracy of the results, the number of eggs produced from each pH treatment group was recorded only up to 3 h after the light was turned on daily.

The hatchability of fish eggs in each pH treatment group was determined based on the methods described by previous authors (4,5). Briefly, the newly spawned fish egg clusters were collected gently from the female's body by hand or from the bottom of the aquarium using plastic pipettes. The clusters of eggs were separated using forceps and then surface sterilized using a mixture of culture medium (NaCl, KCl, CaCl₂H₂O, MgSO₄·7H₂O) and methylene blue for 5 min (11). The eggs were then placed in a petri dish with new culture medium and incubated at 28 °C. The eggs were only considered successfully hatched when they were hatched within 14 days of incubation.

2.5. Histopathological procedures
On day 14, the surviving fish in each aquarium were fixed in 10% buffered formalin for 24 h before they were processed in a tissue processor (Leica TP 1020, Germany), embedded in paraffin, sectioned at 5-µm thickness (Leica Jung Multicut 2045, Germany), and stained routinely with Harris hematoxylin and eosin (HE) for histological study, while the freshly dead fish were also used for similar purpose.

The gills and skins of 10 fish from each treatment were observed for histopathological assessment. Complete sections of ovaries of four females and testes of three males from each group were randomly examined to determine their stages and assess any pathological changes. The photos were recorded and analyzed through a microscope (Nikon Eclipse 50i Japan) and the Nikon NIS-Element D 3.2 Image Analyzer (Nikon Instruments Inc., USA).
2.6. Histopathological assessment of the ovaries

The reproductive stages of the ovaries were determined according to previous studies with several modifications (23, 24). The oocytes stages were classified into stage II: previtellogenic, stage III: early vitellogenic, stage IV: mid-vitellogenic, stage V: mature oocytes, and stage VI: spawned oocytes. The stages of oocytes in each selected fish were counted, and an average value was presented. Any pathological changes, such as necrotic gonads, were recorded. The oocyte staging of each group was done using the formula below:

\[ \text{Number of oocytes of each stage} \times 100 \]
\[ \text{Total number of oocytes} \]

Fish testis stages were classified in the manner described in Blazer (25), with some modifications. The reproductive stages of the testes were classified into stage II: pre-spermatogenic, stage III: early spermatogenic, stage IV: mid-spermatogenic, stage V: late spermatogenic, and stage VI: spent testis. Any pathological changes, such as necrotic tubules, were also recorded.

2.7. Histopathological assessment of the gills and skins

The histological changes in gills were assessed based on specific descriptions, as described by Cengiz (15), which were i) desquamation and necrosis, ii) epithelium lifting and edema, iii) aneurysm, and iv) epithelium hyperplasia and lamella fusion.

For skins, the histopathological changes were evaluated based on the specific description by Noga (26), which were i) epithelium necrosis, ii) epithelium sloughing, and iii) epithelium hyperplasia. The lesions of gills and skins were scored as follows: none: 0% affected (−), mild: not more than 30% affected (+), moderate: 30%–60% affected (++), and severe: more than 60% affected (+++).

2.8. Statistical analysis

The mean of the water quality parameters, fish mortality, number of eggs produced, and the percentage of egg hatchability between the different treatments for 14 days of the study period were calculated and compared between each pH treatment using two-way ANOVA with Tukey’s LSD all-pairwise comparison test (IBM SPSS Statistics Version 22). The P value at < 0.05, which indicated statistical significance, was used. The percentages of gonad stages and histological changes in the gills and skins of fish exposed to the different pH levels were also determined.

3. Results

3.1. Water quality measurement

The range of water temperature, ammonia, and dissolved oxygen during the experimental trial were 28.05–28.40 °C, 0.00–0.19 mg/L, and 5.27–5.59 mg/L, respectively. There was no significant difference in the readings for temperature or dissolved oxygen for all of the treatments. However, water ammonia was significantly higher for the aquarium treated with pH 9, compared to the other treatments (data not shown). pH was maintained at the desired reading (pH 5, 6, 7, 8, and 9) throughout the study period.

3.2. Mortality, egg production, and egg hatchability

The highest mortality rate was observed for pH 9 (100.00 ± 0.00%), followed by pH 5 (25.00 ± 0.00%) and pH 8 (4.17 ± 4.17%) (Table 1). However, no mortality was recorded for fish treated with pH 6 and 7.

The number of eggs produced was highest for fish treated with pH 7 (39.00 ± 3.00), but showed no significant difference to the production of eggs from fish treated with pH 6 (35.50 ± 4.50) and pH 5 (30.50 ± 2.50). A total of 11.50 ± 3.50 eggs were produced in pH 8, while no egg was produced from fish treated with pH 9.

Percentage hatchability of eggs was significantly highest for fish treated with pH 7 (40.87 ± 1.98%), followed by pH 6 (33.47 ± 8.47%).

Table 1. Javanese medaka mortality, egg production, and egg hatchability after exposure to different pH. Data are presented as mean ± SE. Each treatment was conducted in duplicate, with a total of 12 fish in an aquarium. Every replicate contained fish at sex ratio of 3 female to 1 male during the experiment.

<table>
<thead>
<tr>
<th>Water pH</th>
<th>Parameters</th>
<th>Percentage of mortality</th>
<th>Number of eggs produced</th>
<th>Percentage of egg hatchability</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>25.00 ± 0.00b</td>
<td>30.50 ± 2.50c</td>
<td>6.60 ± 0.54a</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.00 ± 0.00a</td>
<td>35.50 ± 4.50c</td>
<td>33.47 ± 8.47b</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.00 ± 0.00a</td>
<td>39.00 ± 3.00c</td>
<td>40.87 ± 1.98b</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>4.17 ± 4.17a</td>
<td>11.50 ± 3.50b</td>
<td>6.25 ± 6.25a</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>100.00 ± 0.00c</td>
<td>0.00 ± 0.00a</td>
<td>0.00 ± 0.00a</td>
<td></td>
</tr>
</tbody>
</table>

a, b, c Different letters represent a significant difference (P < 0.05) between the same column.
6 (33.47 ± 8.47%). However, no significant difference was recorded for percentage of eggs hatchability for fish treated with pH 5 (6.60 ± 0.54%), 8 (6.25 ± 6.25%), and 9 (0.00 ± 0.00%).

3.3. Ovarian staging and pathology
Stage II was the most common reproductive stage counted in the treatment groups of pH 5, 6, 7, and 8, with values of 42.86 ± 1.83%, 63.64 ± 14.31%, 35.29 ± 2.58%, and 54.84 ± 6.58%, respectively (Table 2). For stage II oocytes, the group treated with pH 6 showed the highest value among all treatment groups. Stage III oocytes were highest in pH 8 (19.35 ± 0.82%), while the most counted stage IV oocytes were from the group treated with pH 7, with a value of 17.65 ± 1.29%. Stage V was highest at pH 5 treatment (28.57 ± 2.44%), while stage VI oocyte was highest in the group treated with pH 7 (23.53 ± 4.76%).

The histological appearance of Javanese medaka ovaries at different stages of gonadal maturation is presented in Figure 1. Only oocytes in treatment pH 9 demonstrated pathological changes. Severe necrotic oocytes, with no evidence of viable gonads, were observed in all fish in this group.

3.4. Testis staging and pathology
Most observed testes were in stage V, which were seen in pH 5, 6, and 7 (Table 3). Meanwhile, the least frequent testis stage was stage IV, which was only observed in pH 6 treatment. Stages II and III were mostly observed in treatments of pH 5 and 8, while stage IV was mostly seen in pH 6 and 7 groups.

The histopathological appearances of Javanese medaka testes showing various stages of spermatogenesis treated with different pH are presented in Figure 2. Testes of fish treated with pH 9 showed generalized severe necrosis of seminiferous tubules, but this was not observed for the other treatments.

3.5. Histopathological findings of the gills
Histopathological results indicated that the gills were one of the primary organs affected by direct exposure to different pH. Epithelium lifting and edema were the most frequent findings seen in all treatment groups, except for pH 7, which demonstrated no histopathological changes (Table 4). Treatment groups of pH 5 and 8 showed moderate lesions, while they were mild in groups of pH 6 and 9.

The most severe lesion found in the study was desquamation and necrosis of epithelium cells in the treatment group of pH 9 (Figure 3). The same lesion, but with a mild score, was established in treatment groups of pH 5 and 8, but was absent in treatment groups of pH 6 and 7.

Aneurysm was found to be mild in treatment groups of pH 8 and 9, but was absent in the other treatment groups. The least lesion found in this study was epithelium hyperplasia and lamella fusion, which was only present in the pH 8 group with a mild score.

3.6. Histopathological findings of the skin
In fish exposed to pH 6 and 7, no histopathological changes were observed (Table 5). However, severe epithelium necrosis and sloughing were observed in fish of group pH 9 (Figure 4). The fish of group pH 8 demonstrated moderate epithelium sloughing, mild epithelium necrosis, and hyperplasia of skin epithelium. Mild epithelium sloughing and hyperplasia and moderate score of skin epithelium necrosis were observed in fish treated with pH 5.

4. Discussion
This study indicates that water pH of 6–7 reduced mortality, but increased egg production and hatchability of the Javanese medaka. The influences of pH on the histology of fish gonads have not been studied at depth and are currently poorly understood. However, the pH effects were mostly

Table 2. Percentage of oocyte staging in Javanese medaka after exposure to different pH. Data are presented as mean ± SE. Number of female fish used for oocyte staging for each different pH is four. Egg staging was determined on histological sections.

<table>
<thead>
<tr>
<th>Stage (Oocyte)</th>
<th>pH 5 (mean ± SE)</th>
<th>pH 6 (mean ± SE)</th>
<th>pH 7 (mean ± SE)</th>
<th>pH 8 (mean ± SE)</th>
<th>pH 9 (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>42.86 ± 1.83</td>
<td>63.64 ± 14.31</td>
<td>35.29 ± 2.58</td>
<td>54.84 ± 6.58</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>14.29 ± 0.82</td>
<td>9.92 ± 1.63</td>
<td>5.88 ± 0.58</td>
<td>19.35 ± 0.82</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>9.52 ± 0.82</td>
<td>8.26 ± 1.29</td>
<td>17.65 ± 1.29</td>
<td>4.84 ± 1.29</td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>28.57 ± 2.44</td>
<td>11.57 ± 2.08</td>
<td>17.65 ± 1.29</td>
<td>17.74 ± 1.29</td>
<td>-</td>
</tr>
<tr>
<td>VI</td>
<td>4.76 ± 0.82</td>
<td>6.61 ± 0.82</td>
<td>23.53 ± 4.76</td>
<td>3.00 ± 0.82</td>
<td>-</td>
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</table>
noted on the endocrinial responses and relationships, which usually interrupted normal fish physiology, leading to cellular abnormality (27,28). Ikuta and Kitamura (29) demonstrated that when mature salmonid fish were exposed to sulfuric acid water of pH 4.5–5.0 inhibition of development and increases in malformation were observed in the embryos of their offspring. Moreover, as plasma levels of sex steroids and gonadotropin showed abnormally high levels in both male and female fish exposed to acid, the acid stress disrupted the endocrine system of reproduction of the fish. Kurita et al. (30) stated that the important stages to assess in oocyte development were the vitellogenic and atretic oocytes, since both stages mark the oogenesis capability of the fish. The present study revealed that the highest values of matured oocytes and spawned oocytes were observed in fish treated with pH 7. However, atretic oocytes tremendously decreased when the condition was acidic (pH 5) and alkaline (pH 9), which was thought to be due to hormonal effects (20). None of the fish in pH 9 survived, and histologically, the fish demonstrated generalized severe necrosis of oocytes, which indicate pH stress of the fish (25).

Figure 1. Histological appearance of Javanese medaka ovaries, in different stages of gonadal maturation. (A) Mixture of oocyte stages, which consist of stage II (O2): previtellogenic with basophilic cytoplasm; stage III (O3): early vitellogenic with central germinal vesicle (+) and cortical alveoli (arrow). Group pH 8. HE, ×200. (B) Oocyte stages, which predominantly showed stage V (O5): mature oocyte with the absence of germinal vesicle; an oocyte with stage IV (O4): mid vitellogenic with the main characteristic of acidophilic yolk sacs (arrow) with germinal vesicles at the center. Group pH 5. HE, ×100. (C) Partially spawned ovary with the evidence of stage VI (O6): atretic oocyte with thick wall and shrunken. Group pH 7. HE, ×100. (D) Severe oocytes necrosis (arrow) observed in all fish in the group treated with pH 9. Group pH 9. HE, ×200.
levels involved in alkaline environments (38), however, the increased ion losses during acid exposure are thought to be largely associated with the disruption of paracellular tight junctions (TJs) (19) and supposed to be caused by Ca\(^{2+}\) displacement from the TJs (20). Ca\(^{2+}\) is important in the assembly and sealing of TJ proteins between cell contacts (42), and thus leaching of Ca\(^{2+}\) by acid exposure may disrupt stable cell–cell contacts and increase paracellular permeability to ions (43). Moreover, elevation of water Ca\(^{2+}\) level reduces diffusive ion losses in fish exposed to acidic water (44). Histologically, the damage begins with epithelium necrosis at the upper layer, and becomes more severe in subsequent layers in a few days. In chronic conditions, if the fish survive, the epithelium becomes hyperplastic (26). In the present study, fish treated with pH 5 demonstrated mild to moderate skin epithelial lesions, with epithelium necrosis more frequently observed. The findings were similar to the cellular responses of the skin of rainbow trout when exposed to pH 5 for 7 days (45). The epithelium hyperplasia lesion was observed to be very mild. This lesion usually appears in chronic form (≥14 days of exposure) (45), which could not be observed in this study. All samples from fish treated with pH 9 exhibited generalized severe epithelium sloughing and necrosis. Furthermore, the high mortality rate confirmed that the environment is lethal to the fish.

The present study demonstrated that Javanese medakas cultured at pH 6–7 had better survival rates and increased egg production and hatchability, which is supported by strong evidence from the oocyte and testis stage analyses. Moreover, the ultrastructure of the gills and skins of fish treated in that range were also less histologically affected, indicating its suitability for rearing purpose. Moreover, the sensitivity of the Javanese medaka towards environmental changes in terms of physiological performance and cellular level indicates its potential as a test organism in tropical regions.
Table 4. Histopathological findings in the gill of Javanese medaka after exposure to different pH. Number of fish used in each pH treatment is 10. The lesions were scored as follows: none: 0% affected (–), mild: not more than 30% affected (+), moderate: 30%–60% affected (++) and severe: more than 60% affected (+++).

<table>
<thead>
<tr>
<th>pH treatment</th>
<th>Histopathological findings</th>
<th>Desquamation and necrosis</th>
<th>Epithelium lifting and edema</th>
<th>Aneurysm</th>
<th>Epithelium hyperplasia and lamella fusion</th>
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<tr>
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<td></td>
<td>+</td>
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<td>pH 6</td>
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<td>–</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>pH 8</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pH 9</td>
<td>+++</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
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</table>

Figure 2. Histopathological appearance of Javanese medaka testes showed various stages of spermatogenesis treated with different pH. (A) Stage III: consists of equal proportion of spermatocytes (a) and spermatid (b) and few spermatozoa (c). Group pH 5. HE, ×400. (B) Stage V: consists of all stages, predominantly spermatozoa (c). Group pH 6. HE, ×400. (C) Stage VI: spent testes that have empty tubule (arrow) with fewer spermatozoa remaining within tubules. Group pH 7. HE, ×400. (D) Severe necrosis of seminiferous tubule. Group pH 9. HE, ×400.
Table 5. Histopathological findings in the skins of Javanese medaka after exposure to different pH. Number of fish used in each pH treatment is 10. The lesions were scored as follows: none: 0% affected (–), mild: not more than 30% affected (+), moderate: 30%–60% affected (++) and severe: more than 60% affected (+++).

<table>
<thead>
<tr>
<th>pH treatment</th>
<th>Histopathological findings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Epithelium necrosis</td>
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<tr>
<td>pH 5</td>
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<tr>
<td>pH 6</td>
<td>–</td>
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<td>pH 7</td>
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<td>pH 8</td>
<td>+</td>
</tr>
<tr>
<td>pH 9</td>
<td>+++</td>
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</tbody>
</table>

Figure 3. Histopathological changes of Javanese medaka gills treated with different pH. (A) Normal structure. Group pH 7. HE, ×200. (B) Aneurysms were occasionally found (encircled). Group pH 8. HE, ×200. (C) Mild epithelium lifting and edema (black arrow), and lamella fusion (red arrow). Group pH 8. HE, ×400. (D) Severe necrosis (arrow). Group pH 9. HE, ×100.
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