First evidence of bitterling larvae's minute tubercles as an adaptation to prevent premature ejection by host mussels

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Abstract

1. Bitterlings are small freshwater fish that use long ovipositors to place eggs in host mussels and have morphological adaptations to increase larval survival. The most well-known adaptations are the minute tubercles on the skin surface of larvae, which are developed in early-stage larvae with weak swimming ability and disappear in free-swimming larvae before they leave the host mussel. 2. In the present study, a comprehensive analysis of the developmental stages of Rhodeus pseudosericeus larvae, their morphological and physiological characteristics, their migration inside mussels, and the development of minute tubercle are presented as direct evidence of the morphological function of the minute tubercles. These tubercles began to develop 1 day after hatching (formation stage), grew for 2–5 days (growth stage), reached the peak height after 6–7 days (peak stage), abruptly reduced in height after 8–10 days (abrupt reduction stage), and went through a final gradual reduction (reduction stage) until completely disappearing 27 days after hatching (disappearance stage). 3. The larvae remained in the mussels' interlamellar space of the gill demibranchs until 10 days after hatching, and began to migrate to mussels' suprabranchial cavity 11 days after hatching. At this time, the larvae had clear components of heart rate and caudal fin began to develop. At 24 days after hatching, the minute tubercles had almost disappeared, and some individuals were observed swimming out of the mussels. 4. The experiment results herein presented prove that the minute tubercles are a first direct evidence that the bitterling larvae are morphologically adapted to prevent premature ejection from the mussel.

1. INTRODUCTION

Coevolution is a process that consists of reciprocal evolutionary changes resulting from the interrelationship between a group of organisms and associated populations and plays an important role in the adaptation and speciation of almost all living organisms (Thompson, 1994). Prey-predator, host-parasite, and symbiont relationships are typical examples of coevolution (Thompson, 2002; Liu et al., 2006). Theoretical evidence of coevolution and adaptive traits have been obtained mainly from studies on host-parasite interactions (e.g., avian brood parasitism) (Thompson & Burdon, 1992; Takasu, 1998; Rothstein & Robinson, 1998; Davies, 2000).

Oviparous fishes with parental care use different reproductive strategies to select and prepare spawning sites to increase the number and survival rate of larvae, for instance, by defending their eggs or oxygenating the water around them (Smith & Wootton, 1995). Besides the reproductive success of an individual, the choice of type and site of spawning in species with parental care are also important factors that influence larval survival (Smith et al., 2000; Mills & Reynolds, 2002; Kitamura, 2005; Refsnider & Janzen, 2010). In contrast, species without parental care are vulnerable to abiotic (e.g., low oxygen rates and extreme temperatures) and biological factors (e.g., predators, parasites, and competitors) during the larval stages (Smith & Wootton, 1995).

Bitterlings (Acheilognathinae) are small freshwater fish predominantly distributed in Europe and Northeast Asia, and they have a unique relationship with freshwater mussels (Bivalves: Unionidae) (Smith et al., 2004; Damme et al., 2007). During the spawning season, the female bitterlings elongate their ovipositors and spawn on the gills of mussels through the mussels' exhalant siphons. The male fish, who have nuptial coloration and form territories around the mussels, release sperm that enters the mussels' inhalant siphons through their feeding and breathing actions. The eggs are, therefore, fertilized in the gill cavity of mussels where, depending on the temperature, they remain for 3–4 weeks feeding on their own reserves until they become free-swimming larvae; at this stage, the larvae leave the mussels and begin external feeding (Aldridge, 1999; Smith et al., 2004).

Although bitterlings do not have parental care, they have very few eggs. The host-parasite relationship between these fish and mussels presents difficulties mainly during the moment of spawning and when a premature ejection of larvae by mussels occurs; however, the period in which the fish are most vulnerable (i.e., as eggs) is spent safely inside the mussels, from where the larvae only exit after acquiring swimming ability (Zale & Neves, 1982; Smith et al., 2004; Kitamura, 2008). Host-parasite interaction and choice of oviposition site are critical aspects of vertebrate ecology that have not been sufficiently studied (Refsnider & Janzen, 2010). The relationship between bitterlings and mussels is a notable example of coevolution between host and parasite (Reynolds et al., 1997; Mills & Reynolds, 2003; Reichard et al. , 2010; Rouchet et al. , 2017).

Recent studies have shown that the bitterling-mussel relationship is in fact a type of host-parasite interaction (Reichard et al., 2001; Mills & Reynolds, 2003; Spence & Smith, 2013). Bitterlings make sophisticated oviposition decisions to prevent ejection and have several unique physiological, behavioral, and morphological adaptations for spawning on host mussels (Aldridge, 1997; Smith et al., 2004; Kitamura, 2006a, 2006c; Spence & Smith, 2013; Methling et al., 2018). The fish larvae develop single-celled epidermal cells, called 'minute tubercles', on their skin surface, which are known to play an important role in preventing the larvae from being prematurely ejected from the gills (Suzuki & Hibiya, 1984a, 1985; Suzuki et al., 1985; Suzuki & Jeon, 1987). Minute tubercles are common in all developmental stages of bitterlings, even though the larval morphology is diverse. Previous studies have reported that the minute tubercles are mainly developed in the frontal part of the larvae and on the eyes of larvae, forming a wing-like projection (Suzuki & Jeon, 1988a, 1988b, 1988c, 1988d, 1989, 1990; Kim et al., 2008; Park et al., 2008).

Many studies using in vitro insemination have briefly described the development of the minute tubercles and the morphological characteristics of larvae, and based on their results the minute tubercles were assumed to prevent premature ejection of larvae from their host mussels. However, no comprehensive studies correlating the developmental stages of larvae with their morphological and physiological characteristics, their migration inside the mussels, and the development of the minute tubercles have been conducted. Therefore, in the present study, my goal was to find direct evidence indicating that the development of minute tubercles in bitterlings prevents premature ejection. For this, I focused on the relationships among the height of the minute tubercles, morphological and physiological characteristics of the larvae during development, and the position of the larvae in the mussels. Further I discussed the evolutional advantages of the development of the minute tubercles and migration of larvae inside mussel for better survival.

2. MATERIALS & METHODS

2.1. Study site

This study was performed at the Heukcheon stream of the Namhangang river in Yangpyoeng-gun, Kyeongki-do, Korea in March–April 2017. The experiment site was a small pond (30 m wide × 10 m long; maximum and mean depth of 1.5 m and 0.8 m, respectively) connected to the Heukcheon stream. The stream bed was comprised of silt and mud. The site had eight species of freshwater fishes, but only one bitterling species,*Rhodeus pseudosericeus* , and one mussel species, *Unio douglasiae sinuolatus* . *R. pseudosericeus* is considered an endangered species by the Ministry of the Environment of Korea, thus we received permission from the Ministry of Environment of Korea (Permission number, 2017-22) to perform this study.

2.2. Induction of *R. pseudosericeus* spawning

To study the development of the minute tubercles at each larval developmental stage and the position and migration of larvae in mussels, the induction of *R. pseudosericeus* spawning on mussels was performed simultaneously in large quantities. Mussels were collected on 5 March 2017, before the spawning period of *R. pseudosericeus* . A total of 150 mussels (shell length 35–70 mm) were collected using a kick net (mesh size 3 × 3 mm) and placed in the small pond where the spawning experiments would be conducted. The captured mussels were then placed on fine sand inside a plastic box (60 cm length × 60 cm width × 20 cm height) through which water could pass but not *R. pseudosericeus* individuals. The sealed box was placed in another pond next to the pond where the experiment would be performed. The spawning induction experiment was prepared on 27 March 2017, at night. The mussels were then divided into three boxes with 50 mussels each, and the boxes were places at 3-m intervals. On the morning of 29 March 2017, 36 hours later, the plastic box containing the mussels was removed to complete the spawning induction experiment. Water from the pond was collected in three plastic boxes (100 cm length × 100 cm width × 60 cm height), which were transferred to a laboratory with an oxygen generator.

2.3. Mussel rearing in the aquarium

In the laboratory, an experimental aquarium (60 cm width × 60 cm length × 60 m height) was prepared, and sand was evenly spread (10 cm height) on the bottom of the aquarium. For spawning induction, the three groups of 50 mussels were separately placed in three glass tanks. Oxygen was supplied so that dissolved oxygen (DO) was maintained above 7 mg/L, and aquarium heaters were used to keep water temperature around 20 ± 1°C. A natural 13:11 h light:dark photoperiod was used. The mussels were maintained in the experimental aquarium and fed daily with a live*Chlorella* sp. suspension derived from an indoor aquarium.

2.4. Observation of *R. pseudosericeus* larval development stage and position inside the mussels

After 1 day in the tanks, three mussels per day for 30 days were checked for *R. pseudosericeus* larvae. The presence of larvae on the four gills (left or right, outer or inner) of the *U. d.sinuolatus* mussels was checked by using a mussel-opening device that enabled mussels to be opened to approximately 1 cm. Mussels with spawn had their adductor muscle cut and were examined for the position, number, and developmental stage of larvae. Mussels without spawned were housed in different tanks.

To evaluate the changes in larval position in the mussels, the gills were divided into nine parts (Figures 1, 2); from the gill demibranch to its point of contact with the suprabranchial cavity, the gill was divided into lower part (L), middle part (M), and upper part (U); it was also divided into three parts in the other direction, 3 being the farthest from the outlet, followed by 2 and 1. Moreover, the larvae's position was accurately recorded and photographed (Canon, Mark II, Japan) by measuring the transverse length of the siphon of the mussel and the longitudinal length from the suprabranchial cavity to the gill demibranch. The developmental stages of the *R. pseudosericeus*larvae were determined under a stereoscopic microscope (Nikon, SMZ-10, Japan) using the AxioVision LE program (version 4.5, Carl Zeiss, Germany), following Kim et al. (2006).

2.5. Observation of the larvae's minute tubercles

The development trend of the minute tubercles at each larval developmental stage was determined using scanning electron microscopy (SEM), and the height of the minute tubercles was measured. For the SEM analysis, three specimens at each stage of larval development were fixed for 24 h under carcodylate-buffered 2.5% glutaraldehyde, dehydrated in an ethanol graded series, and dried to a critical point with liquid CO_2. The dried samples were sputter-coated with gold and then examined under SEM (Supra40VP, Carl Zeiss, Germany). For photographic documentation and assessment of the minute tubercles, a Carl Zeiss vision camera (LE REL. 4.4, Carl Zeiss, Germany) was used during SEM.

To facilitate the description of the distributional patterns, the surface of the larvae was divided into three regions (Figure 3) following Kim et al. (2008): (1) Anterior yolk sac projection covering eyes and head (hereafter referred to as EHR), (2) surface of wing-like projection composed of a pair of dorsal and one

ventral yolk sac (hereafter referred to as WLP), and (3) posterior regions of yolk sac and most parts of the body including caudal fin-fold region (hereafter referred to as PR). During the 30 days of experiment, no dead mussels were found, and the minute tubercle heights were measured at each larval developmental stage. Thirty minute tubercles per region were measured from three regions per larva removed from the tank.

2.6. *R. pseudosericeus* utilization of host mussel

Host use of *R. pseudosericeus* was determined by recording the position of larvae within the four gill demibranchs and the number and frequency of larvae. To compare the size of mussels with and without larvae, mussel shell lengths were measured to the nearest 0.01 mm.

2.7. Statistical analyses

Statistical analyses were conducted using SYSTAT softwater (Systat version 18.0, SPSS Inc.,, Chicago, IL, USA). A two sample t-test was performed to compare the size of mussels with and without larvae. The Kruskal-Wallis H test was used to test the difference in number and frequency of larvae among different gill parts related to mussel size. Statistical significance was considered when $P < 0.05$.

3. RESULTS

3.1. General features of the minute tubercles on *R. pseudosericeus* larvae

The larvae's minute tubercles were observed in three sites: EHR, WLP, and PR; the different heights of the tubercles at each region are shown in Table 1 and Figures 4, 5. The shape and degree of development of the minute tubercles clearly differed among regions. Two types of tubercles were found: hemispheric and vestigial-shaped. The minute tubercles on the surface of EHR and WLP were of a well-developed hemispheric shape, whereas those on the PR had a shrunken and flattened vestigial shape. Immediately after hatching, the larvae had many minute tubercles on their surface, and the tubercles' distribution and development changed with larval growth and development.

The hemispheric minute tubercles on the EHR and WLP were observed immediately after hatching. Their height gradually increased from day 1 after hatching, and the highest values were found on the WLP on day 7 [11.4 ± 2.0 μm (6.9–17.1)]. The height of the tubercles rapidly decreased approximately 60% from day 8 to day 10. The height of the tubercles continuously decreased from day 11 on, and on day 24, unidentifiable small protuberances were observed only around the eyes. No tubercles were observed on the epidermis of the larvae from day 27 on. The minute tubercles were mostly hemispheric but slightly inclined towards the posterior region; they were also denser and higher on the WLP than on the EHR. The minute tubercles on the posterior region were first observed on day 6, and they remained vestigial until day 11, when they became shrunken or flattened.

3.2. Larval migration inside the mussels

The changes in larval position in the mussels at each larval developmental stage are shown in Table 1 and Figure 4. The spawning ovipositor of *R. pseudosericeus* entered the gill demibranch (U and M parts) or suprabranchial cavity (L part) of the mussel through the exhalant siphon (Figures 1, 2), and the position of the larvae varied according to developmental stage. Changes in the position of the larvae per developmental stage were different before and after day 11 day, when larval migration from the gill demibranch to the suprabranchial cavity was first observed. Until day 10, the larvae were found in the interlamellar space of the demibranch (U and M parts, prevalence 100%, n = 110); from day 11 on, the larvae were more common in the suprabranchial cavity (L part; prevalence 92.4%, n = 134) than in the gill demibranch (U and M parts; 7.6%, n = 11). The larvae in the L part were faster than those found in the M and U parts at the same time. All the larvae found in the gill demibranchs and suprabranchial cavities had their heads facing the direction opposite to the exhalant and inhalant siphons.

3.3. Relationship between the height and position of the minute tubercles and the morphological and physiological characteristics of the larvae

The position of larvae inside the mussels and the larvae's external morphological and physiological characteristics were closely related as larval development progressed (Table 1; Figures 4, 5). The changes in height of the minute tubercles were divided into six stages: formation, growth, peak, abrupt reduction, reduction, and disappearance.

3.3.1. Formation stage (from hatching to day 1 after hatching)

During this stage, the EHR and WLP of the larvae were already covered with hemispheric minute tubercles. On day 1, the heights of the tubercles on the EHR and WLP were 2.6 ± 0.5 μm (1.8–4.1) and 4.8 ± 1.7 μm (2.3–8.9), respectively; i.e., tubercles on the WLP were larger than those on the EHR. Tubercles on the PR of the larvae were not observed at the stage.

No larval migrations were detected immediately after hatching, but a pair of WLP, which were small and started to develop on the dorsal and ventral regions, were identified. The fin-fold of the caudal region was very small at this stage. Moreover, all larvae were found in the M part of the mussels.

3.3.2. Growth stage (day 2 to day 5 after hatching)

At this stage, the minute tubercles developed very rapidly, reaching approximately twice the size compared to the previous stage, and was abundant on the EHR and the WLP. Their heights on the EHR and WLP were 3.3 ± 1.0 μm (1.9–5.6) and 5.9 ± 1.4 μm (3.2–8.8) on day 2 and 4.2 ± 0.9 μm (2.7–7.1) and 7.5 ± 1.5 μm (4.5–12.0) on day 5, respectively. Vestigial minute tubercles began to appear on the posterior region, but were still very small.

On day 4, the larvae's head developed slightly anterior to the egg yolk and the tubercles on the dorsal and ventral regions greatly developed. Between days 2 and 5, larvae were found only in the U and M parts of the mussels.

3.3.3. Peak stage (day 6 to day 7 after hatching)

The tubercles' height was the highest at this stage (reaching approximately thrice the size compared to the formation stage), and their density on the EHR and WLP was very high. The heights of the tubercles on the EHR and WLP were 6.0 ± 1.3 μm (4.0–9.5) and 9.7 ± 1.8 μm (6.0–13.6) on day 6 and 6.6 ± 1.5 μm (4.2–11.0) and 11.4 ± 2.0 μm (6.9–17.1) on day 7—when they reached their peak heights—, respectively. Vestigial minute tubercles on the PR were first observed during this stage, but they were still very small and flat.

The larvae began to form eyes, and their heartbeat could be observed under their heads. Red blood circulation could be seen in front of the yolk, and the epidermis on the dorsal side began to shrink slightly, with the yolk lengthening backwards. No larval migration was observed, and the larvae were only found in U and M parts of the mussels.

3.3.4. Abrupt reduction stage (day 8 to day 10 after hatching)

At this stage, the minute tubercles on the the EHR and WLP became drastically smaller and shorter than in the previous stage. On day 8, the height of the tubercles on the EHR and WLP decreased to 4.7 ± 0.9 μm (2.8–7.0) and 9.0 ± 1.5 μm (5.2–12.9), respectively; and on day 10, it rapidly decreased to 2.4 ± 0.5 μm (1.7–3.8) and 4.3 ± 0.6 μm (3.1–5.7), respectively, reaching a height similar to that of the formation stage.

At this stage, the development of the lens in the larvae's eyes was completed, their heart components were clearly differentiated, and the caudal fin began to develop. The tubercles on the dorsal region were significantly contracted and shortened. The larvae remained in the demibranchs, and no migration was observed in the suprabranchial cavity. The larvae were only found in the U and M parts of the mussels.

3.3.5. Reduction stage (day 11 to day 26 after hatching)

At this stage, the tubercles at all sites were smaller than in the previous stage. On day 11, the heights of tubercles on the EHR and WLP were 1.7 ± 0.3 μm (1.2–2.6) and 3.3 ± 0.4 μm (2.2–4.4), respectively.

On day 24, some larvae without minute tubercles were found. On day 26, almost all minute tubercles had disappeared, only traces of them were left.

The pectoral and caudal fins of the larvae developed at this stage, and their eyes became clear and silver brown. Their heads, with complete upper and lower jaws, markedly developed. Their color darkened as the melanin pigment expanded, and their air bladders became complete, with two parts and a slightly larger front. The tubercles on the anterior side completely reduced, followed by the reduction of those on the dorsal side; some parts of the yolk remained. Most of the larvae were found in the suprabranchial cavity. From day 24 on, free-swimming individuals were found in the experimental tanks; the larvae that had remained inside the mussels also swam freely when removed from the mussels. A total of 2.3%, 6.2%, and 91.5% of the larvae were found in the U, M, and L part of the mussels, respectively.

3.3.6. Disappearance stage (day 27 to free-swimming larvae)

At this stage, only parts of minute tubercle were observed and only in some larvae. The pectoral, ventral, and caudal fins of the larvae were completely developed, the mouth and anus were open, and the yolk sac was completely absorbed. Larvae were found only in the L part of the mussels.

3.4. Host mussel utilization by *R. pseudosericeus* larvae

No significant differences in shell length were found between the mussels that had larvae (53.66 ± 6.65 mm; range, 38.42–68.82; n = 85) and those that did not (54.05 ± 7.20 mm; 40.10–69.96; n = 65; two sample t-test; $t_{148} = 0.341$, $P = 0.734$). The number of larvae inside mussels was 3.01 ± 2.27 (range, 1–13; n = 127). The number of larvae in the left outer, left inner, right inner, and right outer gills of the mussels was 1.89 ± 1.17 (1–5; n = 56), 1.30 ± 0.67 (1–3; n = 10), 1.00 ± 0 (1; n = 5), and 2.36 ± 1.92 (1–10; n = 56), respectively (Figure 6A); no significant difference among the four demibranchs was found (Kruskal-Wallis H test, $P = 0.148$; Figure 6A).

A total of 49, 30, 6, and 0 larvae were found in one, two, three, and four parts of the mussels' gills. The frequency of appearance of larvae in the left outer, left inner, right inner, and right outer gills of the mussels was 44.09% (n = 56), 7.87% (n = 10), 3.94% (n = 5), and 44.09% (n = 56) among each of the four demibranchs (Figure 6B). The larvae were significantly more frequent in the two outer demibranchs than in the inner demibranchs (Kruskal-Wallis H test, $P< 0.001$; Figure 6B).

4. DISCUSSION

This study investigated the relationship among three factors (larval developmental stage, minute tubercle height, and position of the larvae inside the host mussel) (Table 1; Figures 4, 5), and three main results were found. Firstly, the minute tubercles were concentrated on EHR and WLP of the *Rhodeus* bitterling larvae, being more developed in the latter region than in the former. Second, during the formation, growth, peak, and abrupt reduction stages, larval development occurred in the interlamellar space of the demibranchs (U and M parts); larval migration to the suprabranchial cavity (L part) occurred only during the reduction stage, when the minute tubercles became shorter (Figures 5, 6). Thirdly, when the larvae migrated to the suprabranchial cavity, morphological and physiological changes regarding their locomotion ability were apparent; in fact, individuals that migrated to the suprabranchial cavity clearly developed faster than those that remained in the demibranches.

Two types of minute tubercles of *R. pseudosericeus* larvae were found: hemispheric and vestigial-shaped. Among the *Rhodeus* bitterlings, *R. atremius* , *R. suigensis* , *R. ocellatus* , and *R. o. smithi* have only hemispheric minute tubercles, whereas *R. uyekii*and *R. pseudosericeus* have both hemispheric and vestigial-shaped tubercles (Suzuki & Hibiya, 1984a, 1984b; Suzuki & Jeon, 1988b; Suzuki et al., 1985). The minute tubercles are unique features in bitterlings, and as wing-like projections exist in *Rhodeus* bitterlings but not in *Acheilognathus* and *Tanakia* , they were used as taxonomic characteristics to differ among acheilognathinae genera (Kim, 1982, 1997). Suzuki & Hibiya (1984a, b) proposed the existence of three types of yolk projections in bitterlings, and *Rhodeus* was considered to have type-C projections, which was confirmed for *R. pseudosericeus* in the present study. Suzuki & Jeon (1987) reported that the type and morphology of

these minute tubercles change over time and from species to species. In the present study, *R. pseudosericeus* larvae with two types of minute tubercle shapes similar to those of *R. uyekii* larvae were found; however, *R. pseudosericeus* larvae had a high concentration of hemispheric tubercles only on the EHR and WLP, whereas *R. uyekii* larvae developed these tubercles throughout most of their PR (Suzuki et al., 1985). The two species are very similar not only in the shape of the wing-like projection and the egg yolk during the development and disappearance stages, but also in the morphology of the adult fish; however, there were differences in the developmental area and height of the epidermis (Kin et al., 2006). Moreover, *R. pseudosericeus* eggs are not sticky and are laid in the interlamella space of the demibranches, whereas those of *R. uyekii* are sticky and laid in egg masses in the suprabranchial cavity (Kim et al., 2015). The reason for these similarities and differences cannot be determined based on the results of this study. Thus, in-depth studies on speciation based on ecological characteristics and specific factors are necessary (Mayr, 1969; Arai et al., 2001).

The hemispheric minute tubercles on the WLP were approximately twice as large as those in the EHR, and the direction of the minute tubercles was slightly inclined posteriorly, making it easy for them to fixate on the gills but difficult to be removed, like a harpoon. The WLP was the largest and most developed part of the entire larva, have also the largest surface area. The minute tubercles on the WLP began to develop shortly after hatching and began to shrink during the abrupt reduction stage. The hatched larvae that entered through the mussel's exhalant siphon settled on the demibranches, growing in their interlamellar space, during which time the larvae's widest surface area is the WLP (Song & Kwon, 1994). Mortality of bitterling larvae occurs by two main factors: premature ejection by the mussel and death in the mussel gill by asphyxiation or nutrient deficiency (Smith et al., 2000; Kitamura, 2005; Kawamura & Uehara, 2005). The minute tubercles are formed by large unicellular epidermal cells and are presumed to be polysaccharidal in nature; studies have shown that they perform an attachment function that enables them to attach to vegetation and submerged objects (Laale, 1980). The minute tubercles occur only in larvae with no swimming ability; when fins (and consequently, the larvae's swimming ability) start to develop, the minute tubercles are abruptly reduced (Table 1; Figures 4, 5). The minute tubercles in *Acheilognathus* and *Tanakia* bitterlings, which do not have a wing-like projection, develop most intensively in the foremost part of the head, and the form of the yolk projection is scaly or hilly, different from that of *Rhodeus* bitterlings (Fukuhara et al., 1982; Suzuki & Hibiya, 1985; Suzuki & Jeon, 1987, 1988a, 1988c, 1988d, 1989, 1990; Park et al., 2008). The development of larger and sharper minute tubercles in *Acheilognathus* and *Tanakia* larvae compared to those of *Rhodeus* larvae (20–40 μm vs. 3–15 μm) is an adaptation strategy that also prevents premature ejection and allows larvae to tightly fit in the interlamellar space of the hosts' demibranchs (Suzuki & Hibiya, 1985; Kitamura, 2006b). Further research will be required to compare with migration inside mussels in *Acheilognathus* and *Tanakia* larvae for investigating the role of minute tubercle as their types.

Mussels have one exhalant and one inhalant siphon. The bitterling's ovipositor enters a mussel's exhalant siphon, and eggs are placed in the suprabranchial cavity or interlamellar space of the demibranch (Wu, 1998). As the inhalant siphon is connected to the mantle cavity, when the mussel's shell opens, the larvae would become exposed to the environment; therefore, bitterling spawning must occur in the exhalant siphon to increase larval survival (Tankersley & Dimock, 1993a). The interlamellar space of the demibranchs expands as the larvae grow and becomes a limiting factor. The larvae that remained in the interlamellar space for more than 11 days after hatching were found to have a slower development than those that migrated to the suprabranchial cavity. By migrating to the suprabranchial cavity, which is larger than the interlamellar space, ventilation rates can be increased, thus increasing oxygen supply and space (Davenport & Woolmington, 1982; Mills & Reynolds, 2002). Song & Kwon (1994) reported that *A. yamtsutae* larvae return to the U part as they gain physical abilities over the developmental stages. *A. signifier* and *R. sericeus* larvae, in contrast, remain in the interlamellar space only during the initial developmental stages, and as their swimming ability increases, they migrate to the suprabranchial cavity, in the direction opposite to the exhalant siphon (Aldridge, 1997; Back & Song, 2005). *A. rhombeus* was reported to initially remain in the suprabranchial cavity and then migrate in the direction opposite to the exhalant siphon (Kim et al., 2018). The bitterlings' eggs inside the gills may compete with glochidia for oxygen and space (Smith et al., 2001; Kitamura, 2005). The migration of larvae from the interlamellar space of the demibranchs

to the suprabranchial cavity may reduce intraspecific competition and lower larval mortality rate in the suprabranchial cavity by providing space for growth and increased oxygen supply (Kitamura, 2006b; Spence & Smith, 2013; Methling et al., 2018).

Many previous studies have reported that mussel gill structure and conditions such as size, water flow speed, and dissolved oxygen content vary among gill positions, sexes, and density of larvae (Tankersley & Dimock, 1993a, b; Aldridge, 1999; Mills & Reynolds, 2002, 2003; Smith et al., 2004; Kitamura, 2005, 2006a, b). No glochidia were found during the present study, so the sex of the mussels was unknown. However,*R. pseudosericeus* larvae were mainly found in the two outer demibranchs of the four gills. *U. d. sinuolauts* is known to brood glochidia only in the outer demibranchs, but as the spawning season is after May, no glochidia care was observed during this study. Aldridge (1997) and Mill and Reynolds (2003) reported that the bitterlings mainly use the inner demibranchs, which had more larvae than the outer demibranchs, because of four reasons: active choice, space availability, ovipositor accessibility, and ejection ability. Studies have reported that *A. rhombeus, A. cyanostigma* , and *R. o. kurumeus* eggs were found at a higher rate in inner demibranchs than in outer demibranchs, suggesting that it is these species' choice to avoid competition for oxygen and space with glochidia of mussels that use the outer demibranchs as brood pouches (Kitamura, 2006b, 2006c; Kim et al., 2018). Tankersley (1992a) proposed that the total flow in gills during brooding would be approximately 16% and 4% of those in non-gravid and non-marsupial gills, respectively. Kitamura (2006c) reported that female bitterlings may have been more constrained in their spawning inside the inner demibranchs irrespective of mussel sex during group spawning. Moreover, Mills and Reynolds (2003) reported that when mussels brood larvae, bitterlings spawn in inner demibranchs, but that after the mussels release their larvae, the widened outer demibranchs can be used as spawning sites. Interestingly, when the spawning patterns of mussels in March and April (i.e., before mussels brood the larvae) and in May and June (after the brooding season) were analyzed, *R. pseudosericeus* was found to have higher spawning rates in outer demibranchs than in inner demibranchs (per. observation). For *A. signifier* , twice as many larvae were identified in inner demibranchs without brooding pouches compared to the outer demibranchs with brooding pouchs (Kim et al., 2014). Further studies are necessary to elucidate the selectivity of bitterlings regarding gill position and whether it is related to gill structure or active selectivity of bitterlings (Tankersley & Dimock, 1993b).

Bitterlings have a unique early life history. The bitterlings' eggs can be classified into four types: bulb-like, pear-shape, spindly, and ovoid; moreover some eggs are sticky (Kim et al., 2006; Kim et al., 2011). They lay a small number of eggs, develop unique tissue structures called minute tubercles during the early stages of larval development, have a very fast hatching time, and are unique in laying eggs in mussels. However, this species, of which 60 types are known worldwide, evolved due to various factors such as type of maturation, development, spawning type, spawning position and larval migration in mussels, and host selection (Smith et al., 2004; Nelson, 2006). In conclusion, the present study, by examining the development of minute tubercles, the migration of larvae inside mussels, and the physiological characteristics of the larvae, provided direct and comprehensive evidence that minute tubercles are developed to prevent the premature ejection of larvae by their mussel hosts. Thus, this finding may enhance our understanding of the evolutional advantages of the development of the minute tubercles and migration of larvae inside mussel for better survival. In this present study, however, the investigation was limited to the determination of the main factors causing growth or reduction of minute tubercles development and advantages of migration of larvae. Therefore further physiological research will be required to determine the role physiological factors.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

Kim H.S. conceived, designed, and executed this study and wrote the manuscript. No other person is entitled to authorship.

DATA AVAILABILITY STATEMENT

Data will be available at figshare (https://figshare.com/s/10cfe2fee4bc89aeb268).

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Table 1. Mean (± SD) height of minute tubercle on the surface of two regions and characteristics of *Rhodeus pseudosericeus* larvae

Days after hatching	Developmental stage	Height of minute tubercles (um)	Height of minute tubercles (um)	Characteristics of larave
		eyes and head	Wing-like projection	

Days after hatching	Developmental stage	Height of minute tubercles (um)	Height of minute tubercles (um)	Characteristics of larave
1	Formation	2.57±0.51	4.85±1.68	no movement of larvae yolksac begin to develop in dorsal and ventral regions
2	Growth	3.26±0.99	5.91±1.38	larvae start to movement
3		3.57±0.82	6.27±1.71	head slightly develop
4		3.83±1.15	6.80±1.83	
5		4.22±0.90	7.45±1.52	
6	Peak	6.05±1.26	9.68±1.76	eyes form, heartbeat observes
7		6.62±1.53	11.42±2.04	red bood circulation confrim dorsal and ventral yolksac shrink
8	Abrupt reduction	4.67±0.88	8.95±1.51	minute tubercle contract and reduce
9		3.51±0.75	6.24±1.02	caudal fin begin to develop
10		2.43±0.46	4.28±0.58	lens complete
11	Reduction	1.72±0.30	3.27±0.40	Larvae were observed in the suprabranchial cavity minute tubercle completely reduce
12		1.61±0.23	3.09±0.51	pectoral and caudal fin develop
13		1.42±0.24	2.51±0.43	air bladder divide into two rooms
14		1.28±0.20	2.08±0.51	
15		1.13±0.19	1.78±0.49	
16		1.07±0.17	1.70±0.54	
17		1.01±0.19	1.57±0.35	
18		0.98±0.16	1.45±0.28	
19		0.95±0.17	1.41±0.29	
20		0.87±0.20	1.41±0.26	
21		0.84±0.16	1.25±0.29	
22		0.72±0.16	1.19±0.29	
23		0.56±0.13	1.03±0.28	
24		0.38±0.29	0.65±0.50	free-floating individuals appear
25		0.26±0.20	0.47±0.38	
26		0.11±0.16	0.19±0.29	
27	Disappearance	-	-	all kind of fins complet

Days after hatching	Developmental stage	Height of minute tubercles (um)	Height of minute tubercles (um)	Characteristics of larave
28		-	-	anus open and
29		-	-	yolk sac completely absorb
30		-	-	larvae become free swimmer

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