Host Fish Species for Glochidia of *Anemina arcaeformis*
Revealed by Artificial Infection Experiment

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Abstract: In this study, host suitability for glochidia of the bivalve *Anemina arcaeformis* was tested in fish of 12 taxa in 4 families (*Lethenteron* sp. in Petromyzontidae; *Carassius* sp., *Rhodeus ocellatus ocellatus*, *Candidia sieboldii*, *Pseudorasbora parva*, *Gnathopogon elongatus elongatus*, *Biwia zezer*, and *Pseudogobio esocinus esocinus* in Cyprinidae; *Misgurnus anguillicaudatus* and *Cobitis* spp. (*C. biwae* and *C. minamorii tokaiensis*) in Cobitidae, and *Rhinogobius flumineus* in Gobiidae) by an artificial infection experiment. Metamorphosed juveniles were obtained from 9 taxa. Juveniles could not be obtained from *R. o. ocellatus*, *Carassius* sp., and *M. anguillicaudatus*. The highest metamorphosis rates from parasitized larvae to juveniles was 30.3% in *C. sieboldii*, 23.5% in *R. flumineus*, 16.8% in *Cobitis* spp., 13.6% in *P. parva*, 11.9% in *O. platypus*, 4.4% in *Lethenteron* sp., 2.8% in *B. zezer*, 2.1% in *G. e. elongatus* and 0.7% in *P. e. esocinus*. Only *Rhinogobius* spp. were previously known to be hosts of *A. arcaeformis*, so these represent new host fish.

Keywords: *Anemina arcaeformis*, glochidia, host fish, juvenile, metamorphosis

Introduction

Freshwater bivalves of the order Unionoida have a life cycle within which they parasitize fish in a larval stage called the glochidium and then metamorphose into juveniles. Fish species suitable as hosts vary depending on the species of Unionoida, and glochidia that parasitize non-hosts cannot metamorphose into juveniles. Host fish species are thus not only indispensable for reproduction, but also contribute to the dispersal of the Unionoida, which are otherwise known to have low dispersal ability. Unionoid populations have been decreasing in recent years. In Japan, 13 out of 17 species of Unionoida are designated as threatened or near threatened species in the Red List prepared by the Ministry of the Environment (Kondo, 2008; Ministry of the Environment, 2012). For the conservation of Unionoida, it is important to maintain or create suitable environments, and for this, the existence of host fish species is required. For the clarification of host fish species that contribute to the reproduction and dispersal of Unionoida in the field, it is important to examine the physiological host suitability of each fish species (such as metamorphosis rates of parasitizing larvae), the population dynamics of the fish and Unionoida in their habitats (such as density of inhabitation and configuration ratio of species), parasitization characteristics (such as incidence and intensity), and dispersal ability of the fish in a holistic manner (Itoh et al., 2003; Klunzinger et al., 2012; Levine et al., 2012; Kondo et al., 2012). Of these parameters, the physiological host suitability of each fish species is an important basis for clarifying which fish species contribute to

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the reproduction and dispersal of Unionoida.

*Anemia arcaeformis* belongs to the family Unionidae and is distributed in China, the Primoriye region of Russia, Korea and Japan (Kondo, 2008). In Japan, distribution (Kondo, 2008; Kondo *et al*., 2013a; 2013c), genetic variation among related species (Tabe & Fukuhara, 1995), and breeding seasons (Fukuhara *et al*., 2013) have been reported. But in regard to host fish species, there has been merely a record that juveniles were obtained from *Rhinogobius* spp. (Kondo, 2008). Therefore, the host suitability of *A.arcaeformis* was tested in this study with specimens from Gifu Prefecture by conducting experiments in parasitism on fish of 12 taxa in 4 families inhabiting the same water.

**Materials and Methods**

**Collection of glochidia and fish**

Gravid specimens of *A. arcaeformis* were collected from a pond in a biotope established in Ibigawa city, Ibi-gun, Gifu Prefecture, central Japan on February 25, 2012. This is where the presence of *A. arcaeformis* was confirmed for the first time in the Tokai region (Kondo *et al*., 2013c). With *A. arcaeformis*, the outer two hemibranchs become marsupia (Kondo, 2008) and turn brown when incubating. The condition of incubation of each mussel collected in the field was thus confirmed by opening the mussel shell with a spatula (total length: 200 mm, length of the spatula: 7 mm). The collected mussels were brought back to the laboratory and kept in an aeration tank. The water temperature was kept at about 15°C, the same temperature as that in the source pond. The mussels released glochidia two days after the start of the experiment. In accordance with the method of Zale & Neves (1982), some of the released larvae were collected and put in a saline solution to confirm activity by checking that the shells closed. Furthermore, some of the *A. arcaeformis* larvae were made to parasitize *Rhinogobius flumineus*, a related species of *Rhinogobius* spp. that is already known to be a host fish (Kondo, 2008). The glochidia were thus used in the experiment after checking their ability to parasitize fish.

In January and February 2012, 6 to 20 fish of 12 taxa in 4 families were collected with a long fishing net (opening dimension: 95 cm, mesh size: 2 mm) and a skimming net (opening dimension: 40 cm, mesh size: 2 mm) in canal connected to the source pond. The collected fish were *Lethenteron* sp. in Petromyzontidae; *Carassius* sp., *Rhodeus ocellatus ocellatus*. *Opsariichthys platypus*. *Candidia sieboldii*. *Pseudorasbora parva*. *Gnathopogon elongatus elongatus*. *Biwia zezea* and *Pseudogobio esocinus esocinus* in Cyprinidae; *Misgurnus anguillicaudatus* and *Cobitis biwae* and *C. minamorii tokaiensis* in Cobitidae, and *R. flumineus* in Gobiidae. Because it was difficult to distinguish two species of the genus *Cobitis*, they were treated together in the experiments as *Cobitis* spp. The fish may have already been parasitized by larvae because they were collected during the breeding season of *A. arcaeformis* (Fukuhara *et al*., 2013). In order to avoid mixing with the larvae participating in this experiment, the collected fish were kept in the tanks (water content: approximately 20 L, each) for about a month (Fig. 1) and used for the experiment after the larvae that had parasitized them in the field had dropped off completely. In the experimental device, 13 tanks, a filter system, an ultraviolet germicidal lamp, and a chiller were linked. Water was made to overflow from a hose, bridging the tops of the tanks, into a filter system. Gravel and sand were put in the filter system to gravitationally filter the water. The filtered water flowed into each tank via an ultraviolet germicidal lamp and a chiller. The water temperature was adjusted by the chiller and a heater installed in the filter system and each tank was kept at the same water quality and at the same water temperature. An artificial diet was given to the fish every other day or every three days during the experimental period. The water temperature in the tanks was kept at 17 ± 0.4 (SD) °C during the experiment. The light environment was not controlled.

The taxonomy and scientific names of the fish were determined from Nakabo (2013).
**Infection and collection of juveniles and glochidia**

The larvae of *A. arcaeformis* and all the fish were put in one tank (30×60×30 cm, water content: approximately 36 L) to allow the larvae to parasitize the fish for about 10 minutes. The water in the tank was stirred by aeration during parasitizing so that the larvae would be in a suspended state in the tank. Of the fish parasitized by larvae, 8 individuals of *G. e. elongatus*, 6 of *B. zeze*ra and 10 each of the other species were put in tanks separately by species and were designated test fish. The remaining fish were in other tanks separately from the test fish. Whenever test fish died during the experiment, fish of the same species similar in standard length were added to keep the number of test fish the same as that at the start of the experiment. A net with a mesh size of 5 mm was installed inside the tanks so that the fish would not prey upon the dropped-off larvae and juveniles (Fig. 1).

After the larvae parasitized the fish, the water in the tanks was filtered with a plankton net (mesh size: 100 μm) to collect the larvae and juveniles that dropped off from each of the fish species every day (every 24 hours) until the third day, and every third day (every 72 hours) thereafter. Periodically the tanks were emptied and cleaned by siphoning off the water, cleaning the inner sides and base, and returning the original water after filtering. The fish were kept in holding tanks in the original water during this operation. The collected larvae and juveniles were observed and counted with a stereoscopic microscope (at a magnification of 20 to 28x) and a biological microscope (200x). After completion of metamorphosis, new shells form inside the prodissoconch of the juveniles, and foot-stretching and shell opening / closing movements can be observed at this stage (Fukuhara *et al.*, 1990; Rogers & Dimock, 2003; Itoh *et al.*, 2010; Akiyama, 2011). Separation into juveniles with such characteristics and larval that had not metamorphosed was performed and individuals of each stage were counted. Based on the above, changes over time were assessed, both in the numbers of larvae and juveniles detaching and the rate at which this took place, and the metamorphosis rate of the parasitic larvae was determined. The metamorphosis rate (M%) was calculated using the number of detached larvae and juveniles as: $M = 100J/(J + L)$, where $J$ and $L$ were the number of detached juveniles and larvae. In the cases of *Lethenteron* sp. and *P. e. esocinus*, the number of test fish was reduced during the experimental period and larvae were parasitic on dead fish. Therefore, the metamorphosis rate of both species were corrected using the number of larvae which parasitize dead fish: $M_l = 100J/J_l + L_l$, $M = 100(J_l + J)/L_l + J_l + J_d + L_d)$, where $M_l$ was the metamorphosis rate of attached glochidia with live fish, $J_l$ was the number of detached juveniles from live fish, $L_d$ was the number of detached larvae from live fish. $J_d$ was the number of metamorphosed juveniles from dead fish and $L_{d}$ was the number of detached larvae from dead fish.
The experimental period was set at 40 days because the period required for the larvae of *A. arcaeformis* to metamorphose is said to be 12 to 24 days in a water temperature of 17°C (Kondo, 2008). On the 33rd day, 2 individuals of each fish species were taken out from among the test fish, fixed in 90% ethanol, and dissected to check for the presence of remaining larvae. The sites checked were the body surface, dorsal fins, pelvic fins, pectoral fins, anal fins, inside of the operculi and gills. Furthermore, the remaining other test fish continued to be kept and the water in the tanks was filtered on the 36th day and 40th day to check for the presence of dropped-off larvae and juveniles.

**Infection sites on fish body**

The fish that died during the experiment were fixed in 90% ethanol and dissected to check the parasitization sites and the number of parasitizing larvae. The sites checked were the body surface, dorsal fin, pelvic fins, pectoral fins, anal fin, inner sides of operculi and gills.

**Results**

**Glochidia and juveniles**

Metamorphosed juveniles were obtained from fish of 9 of the 12 taxa. Metamorphosed juveniles were not obtained from *R. o. ocellatus*, *Carassius* sp. or *M. anguillicaudatus*. The metamorphosis rates from parasitizing larvae to juveniles were: 30.3% in *C. sieboldii*, 23.5% in *R. flumineus*, 16.8% in *Cobitis* spp., 13.6% in *P. parva*, 11.9% in *O. platypus*, 4.4% in *Lethenteron* sp., 2.8% in *B. zezera*, 2.1% in *G. e. elongatus* and 0.7% in *P. e. esocinus* (Table 1).

Fig. 2 shows the change over time in the constituent ratio of the number of larvae that dropped off without metamorphosing, the number of juveniles that dropped off after completing metamorphosis, and the number of larvae that were still attached on each day of the experimental period. The number of larvae that were attached on each day was obtained by back calculation based on the number of dropped-off larvae and juveniles obtained at the end of the experiment. The unmetamorphosed larvae were obtained from all fish species. In regard to non-host fish species, all larvae dropped off *R. o. ocellatus* by the third day after the start of the experiment. With *Carassius* sp. and *M. anguillicaudatus*, most of the larvae that dropped off without metamorphosing (91.2%
and 84.4% respectively) had dropped off within 3 days, but some of them remained parasitic for a long time (18 to 23 days) before dropping off. This indicates that the fish species on which parasitizing larvae were confirmed in the field are not necessarily hosts. On the other hand, most of the larvae that dropped off the host fish species without metamorphosing (56.5 to 95.7%) dropped off within 3 days after the start of the experiment. The juveniles that had completed metamorphosis dropped off from the 12th to 24th day after the start of the experiment. In regard to the larvae that did not metamorphose, some dropped off with the shell opened, others with the shell closed, and both types had brownish transparent shells (Fig. 3A). With the juveniles, new shells formed inside the prodissococonch in the larval stage and the activities of opening and closing
the shell and to stretching the foot were observed (Fig. 3B). Also it was observed that the juveniles had cilia on the surface of the foot and in the margin of shells and moved them vigorously. Neither larvae nor juveniles were newly observed after the 25th day and no larvae were found in the fish body on the 33rd day, even when dissection was performed. It is safe to say that all of the parasitized larvae had dropped off by the 24th day after the start of the experiment. The average period of parasitization by the larvae that metamorphosed into juveniles was 14.2 days. The number of days required for metamorphosis might have been overestimated because the dropped-off individuals were observed every 72 hours from the 3rd day onwards after parasitization. However, this result seems to be reasonable because the average number of days required for the larvae of *A. arcaeformis* to metamorphose is 15.3 days at a water temperature of 17°C (Kondo, 2008).

### Infection sites on fish body

During the experiment, 2 individuals of *B. zezea*, 7 of *P. e. esocinus*, 4 of *R. flumineus*, 4 of *Lethenteron* sp., and 5 of *O. platypus* died. Dissection of these revealed parasitization by larvae in 1 individual of *B. zezea*, 4 of *P. e. esocinus*, 2 of *R. flumineus*, 2 of *Lethenteron* sp., and 1 of *O. platypus*. Approximately 83.3% of the larvae parasitized the fins, especially the pectoral fins (Table 2). With *O. platypus* however, all 7 larvae parasitized the gills. On the body surface, parasitization

### Table 2. Infection sites and number of glochidia on dead fish body.

<table>
<thead>
<tr>
<th>Date died</th>
<th>Fish species</th>
<th>Dorsal fin</th>
<th>Pelvic fin</th>
<th>Anal fin</th>
<th>Pectoral fin</th>
<th>Caudal fin</th>
<th>Body surface</th>
<th>Inside of operculum</th>
<th>Gill</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>29 Feb.</td>
<td><em>Biwia zezea</em></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1 March</td>
<td><em>Pseudogobio esocinus esocinus</em></td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td></td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>2 March</td>
<td><em>Pseudogobio esocinus esocinus</em></td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td></td>
<td>4</td>
<td></td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>6 March</td>
<td><em>Pseudogobio esocinus esocinus</em></td>
<td>1</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>7 March</td>
<td><em>Rhinogobius flumineus</em></td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>8</td>
<td>6</td>
<td>1</td>
<td></td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>7 March</td>
<td><em>Lethenteron</em> sp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>7 March</td>
<td><em>Lethenteron</em> sp.</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>7 March</td>
<td><em>Pseudogobio esocinus esocinus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>10 March</td>
<td><em>Opsariichthys platypus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>11 March</td>
<td><em>Rhinogobius flumineus</em></td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>3</td>
<td>12</td>
<td>6</td>
<td>22</td>
<td>8</td>
<td>9</td>
<td>0</td>
<td>12</td>
<td>72</td>
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<tr>
<td>Proportion</td>
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<td>4.2</td>
<td>16.7</td>
<td>8.3</td>
<td>30.6</td>
<td>11.1</td>
<td>12.5</td>
<td>0.0</td>
<td>16.7</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Fig. 3. Detached glochidium (A) and juvenile (B) of *Anemina arcaeformis*. The dashed lines indicate the areas with new shell formation, and the solid arrow indicates the foot. Scale = 100 μm.
Host Fish Species of Anemia arcaeformis

was observed on the labia and nares (in *P. e. esocinus*) and on the genitals (in *Lethenteron* sp.). With *P. e. esocinus*, a site (pelvic fins) where a cyst is formed and a site (labia) where a cyst is not formed were observed even on the same body (Fig. 4).

**Discussion**

In this study, it was demonstrated that 9 taxa (*C. sieboldii*, *R. flumineus*, *Cobitis* spp., *P. parva*, *O. platypus*, *Lethenteron* sp., *B. zezer*, *G. e. elongatus* and *P. e. esocinus*) are suitable hosts for *A. arcaeformis*. Only *Rhinogobius* spp. were previously known to be hosts of *A. arcaeformis*, so these are new discoveries. Furthermore, *Cobitis* spp. and *B. zezer* were revealed to be host fish of Japanese unionids for the first time. The metamorphosis rates of the larvae of *A. arcaeformis* in the host fish species were 0.7 to 30.3% depending on the fish species. Bivalve species in which the metamorphosis rate of parasitized larvae has been examined for more than one fish species as in the present study, include *Pronodularia japonensis* (Itoh et al., 2003), *Inversiunio jokohamensis* (Itoh et al., 2010) and *Unio douglasiae nipponensis* (Kondo et al., 2013b). All of these species were reported to differ in metamorphosis rate according to host fish species as in the case of *A. arcaeformis*. With some fish species, the parasitized larvae metamorphosed into juveniles at higher rates (80 to 90%) than those seen in the present study. Especially in the study of host suitability of *U. d. nipponensis* conducted by Kondo et al. (2013b) using the same fish species and methods as those in this study, higher metamorphosis rates of 95.3% and 88.3% were seen with *O. platypus* and *R. flumineus*. With *A. arcaeformis*, the metamorphosis rates were as low as 11.9% and 23.5% respectively, even with these two fish species. It may be possible that there are fish species with a higher metamorphosis rates for *A. arcaeformis* larvae that were simply not included among those used in this study. However, it has been said that defense mechanisms innate to fish and acquired resistance obtained through experience of parasitization by larvae of congeneric or heterogeneric species of Unionoida can affect the metamorphosis rates of juveniles after parasitization of fish. The metamorphosis rates may fall to a low level in fish with acquired resistance (Rogers & Dimock, 2003; Dodd et al., 2005). It is not possible to examine the influence of acquired resistance here because the parasitization history of the test fish used in this study is not known. If the fish had already experienced parasitization by larvae of Unionida species, this might have affected the metamorphosis rates and caused it to fall. However, the metamorphosis rate never becomes 0% even in fish that have acquired resistance (Strayer, 2008). The three species of *R. o. ocellatus*, *M. anguillicaudatus*, and *Carassius* sp., on which juveniles could not be found in this study, lack host suitability innately and physiologically. Furthermore, Itoh et al. (2003) suggested the possibility that the gills are better sites for parasitization than the fins because the metamorphosis ratio of the larvae of *P. japonensis* is higher with *Rhinogobius* sp. OR (orange type: see Suzuki et al., 2010), in which the larvae parasitize the gills rather than the fins as in other fish species. The dissection of the
dead fish performed in this study revealed that most of the larvae of *A. arcaeformis* parasitized the fins. It has been said that larvae of Unionoida parasitize the gills and fins of fish; some parasitize mainly the gills and others parasitize both the gills and fins (Kondo, 2008). Considering that many larvae of *A. arcaeformis* parasitized the fins, this may have been the cause of the low metamorphosis rates of the larvae of *A. arcaeformis* compared to those of Unionoida. However, these results were obtained by experimental verification in a tank. In the future, it is necessary to examine how the parasitization sites and situations of parasitization in the field affect the metamorphosis rates.

The tribe Anodontini, to which *A. arcaeformis* belongs, have lower host specificity (Strayer, 2008). In the present experiment, 9 out of 12 taxa were found to be hosts of *A. arcaeformis*. As a result of using the same fish species and experimental method to examine the metamorphosis rates of parasitizing larvae of *U. d. nipponensis*, which belongs to the Unionidae, 6 out of 12 taxa were found to be hosts (Kondo *et al*., 2013b). This suggests that *A. arcaeformis* is also a species with comparatively low host specificity. As a biological factor that affects the level of host specificity of Unionoida, Neves *et al*. (1985) suggested that the fins are an organ isolated from the immune system, unlike the gills, so larvae that parasitize the fins are less susceptible to immune responses from the fish and can thus use more fish species as hosts. Bauer (1994) reported that larvae of a bigger size have a shorter period of parasitization and are thus less susceptible to immunological influences from the fish. In regard to Japanese Unionoida, Kondo (1989) mentioned the relevance of breeding seasons and the number of incubated eggs. He reported that species breeding during the summer have fewer incubated eggs and higher host specificity, while those breeding during the winter have more incubated eggs and lower host specificity. The larvae of *A. arcaeformis* are bigger than those of other Japanese Unionoida and they parasitize the fins and gills over a comparatively longer period because they breed during the winter (Kondo, 2008). Therefore, *A. arcaeformis* does not fall into the category described by Bauer (1994) in which bigger larvae have a shorter period of parasitization, and it seems that the low host specificity is in accordance with the observations by Neves *et al*. (1985) and Kondo (1989) as mentioned above. However, Levine *et al*. (2012) reported that juveniles were obtained from 24 species as a result of making the larvae of *Popenaias popeii* of Unionidae parasitize the gills of 31 fish species. Itoh *et al*. (2003) also reported that juveniles were obtained from all 11 fish species used in an experiment on *P. japanensis* of the summer breeding type. Therefore, further examination is required to determine whether or not ecological factors of Unionoida, such as parasitization sites, parasitization period, and breeding season, affect the host specificity.

To identify which fish species actually contribute to the reproduction and dispersal in the wild based on these results, it is necessary to clarify the habitat of the fish and the state of parasitism in the field. It has been thought that *A. arcaeformis* is distributed over a wide range in Japan, but the current situation is probably still not fully understood because new distributions have been reported in some regions in recent years (Kondo *et al*., 2013a; 2013b). However, many Japanese Unionoida are on the verge of extinction, and environments suitable for *A. arcaeformis* have been lost and the populations have decreased unmistakably. This being so, it is essential to preserve the environments where *A. arcaeformis* can maintain populations in the long run, including host fish species. We hope that this study will be of some help for the subject.

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Host Fish Species of *Anemina arcaeformis*

References


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寄生実験により明らかとなったフネドブガイ幼生の宿主魚種

近藤美麻・伊藤健吾・千家正照

要約

フネドブガイ幼生の宿主適合性を調べるため，4科12分類群の魚類（スナヤツメ，ギンブナ，タイリクバラタナゴ，オイカワ，ヌマムツ，モツゴ，タモロコ，ゼゼラ，カマツカ，ドジョウ，シマドジョウ類，カワヨシノボリ）を対象として寄生実験を行なった。その結果，変態した稚貝が得られたのは12種中9種であり，タイリクバラタナゴ，ギンブナおよびドジョウからは稚貝は得られなかった。寄生幼生のうち稚貝へ変態した個体の割合が最も高かったのはヌマムツで30.3％，次いでカワヨシノボリが23.5％，シマドジョウ類が16.8％，モツゴが13.6％，オイカワが11.9％，スナヤツメが4.4％，ゼゼラが2.8％，タモロコが2.1％，カマツカが0.7％であった。既往知見で知られているフネドブガイの宿主魚種はヨシノボリ類のみであることから，本実験により新たに9種がフネドブガイの宿主としての適性をもつことが明らかになった。