



## Who wins in the weaning process? Juvenile feeding morphology of two freshwater mussel species

Journal:	<i>Journal of Morphology</i>
Manuscript ID	JMOR-17-0104
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	17-May-2017
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Keywords:	Development, juvenile mortality, metamorphosis, biodiversity conservation

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Who wins in the weaning process? Juvenile feeding morphology of two freshwater mussel species

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Short title: Juvenile feeding morphology of freshwater mussel species

## Abstract

The global decline of freshwater mussels can be partially attributed to their complex life cycle. Their survival from glochidium to adulthood is like a long obstacle race, with juvenile mortality as a key critical point. Mass mortality shortly after entering into a juvenile state has been reported in both wild and captive populations, thus weakening the effective bivalve population. A similar phenomenon occurs during metamorphosis in natural and hatchery populations of juvenile marine bivalves. Based on a morphological analysis of newly formed juveniles of the freshwater species *Margaritifera margaritifera* (Margaritiferidae) and *Unio mancus* (Unionidae), we show that a second metamorphosis, consisting of drastic morphological changes, occurs that leads to suspension feeding in place of deposit feeding by the ciliated foot. We hypothesise that suspension feeding in these two species develops and improves gradually due to several morphological features including the contact between cilia of the inner gill posterior filaments, the inner gill reflection, the appearance of the ctenidial ventral groove and the formation of the pedal palps. Regardless of the presence of available food, a suspension feeding mode replaces deposit feeding, and juveniles unable to successfully transition morphologically or adapt to the feeding changes perish.

## Keywords

Development, juvenile mortality, metamorphosis, biodiversity conservation

## Introduction

Freshwater mussels of the Order Unionoida have a very complex life cycle. To ultimately reproduce, they have to survive a long obstacle race in which more than the 99% of offspring die (Bauer, 1991). These organisms must first come into contact with their host fish during the glochidium stage, survive metamorphosis in the fish's gills until juvenile stages and then detach in a suitable settlement habitat for further maturation. Despite reaching this stage, juveniles are still very fragile and experience high mortality, although the reasons for this are unclear (ASTM, 2006; Augspurger *et al.*, 2007; Strayer & Malcom, 2012; Archambault, Cope & Kwak, 2014). We hypothesise that this critical juvenile stage is related to a second metamorphosis event that occurs just after emergence as juveniles, resulting in drastic morphological changes that, regardless of the presence of available food, substitutes deposit feeding for suspension feeding (Lasee, 1991; Lima *et al.*, 2006; Kovitvadhi *et al.*, 2006, 2007;

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3 Trump, 2010; Schartum *et al.*, 2016), similar to the metamorphosis event observed  
4 during settlement in marine juvenile bivalves (ÓFoighil *et al.*, 1990; Reid *et al.*, 1992;  
5 Helm, Bourne & Lovatelli, 2004; Cannuel *et al.*, 2009).  
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8 The majority of bivalves are rhythmic filter and/or deposit feeders (Morton, 1973).  
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10 Adult freshwater bivalves are normally considered suspension feeders (phytoplankton,  
11 zooplankton and particulate detritus) or deposit feeders (particulate detritus in  
12 sediment); however, some authors have stated that deposit-feeding bivalves are never  
13 found in fresh water (Nicol, 1984). In terms of food sources, various authors have  
14 reported that some mussels maintain diets consisting of suspended bacteria, fungal  
15 spores, dissolved or sediment organic matter (Vaughn & Hakenkamp, 2001) or clearing  
16 coliform bacteria (Silverman *et al.*, 1997). River and lake unionids always ingest  
17 bacterial carbon instead of algal carbon, and are not always primary consumers or  
18 omnivores (Nichols & Garling, 2000). Nevertheless, the importance of algae in the diet  
19 is derived from the phytosterol, and cholesterol and vitamin B12 are also key dietary  
20 items (Nichols & Garling, 2000). Naiads feed directly on the living microbial and algal  
21 components of fine organic material on the sediment surface (Raikow & Hamilton,  
22 2001). Other authors have hypothesised that the food required by two species of  
23 *Margaritifera* comes from a healthy rhizosphere, and eutrophication of the immediate  
24 environment was responsible for the absence of food (Hruska, 1999; Howard, Cuffey &  
25 Solomon, 2005; Negishi *et al.*, 2014). Species and seasonal differences in feeding and  
26 metabolism have also been reported for different mussel species (Baker & Hornbach,  
27 2001; Vaughn, Spooner & Galbraith, 2007; Vaughn, Nichols & Spooner, 2008; Strayer,  
28 2008; Yasuno, 2014). Despite having much information, a good understanding of the  
29 food sources and feeding mechanisms of freshwater mussels (Vaughn & Hakenkamp,  
30 2001; Raikow & Hamilton, 2001; Strayer, 2008; Vaughn *et al.*, 2008; Welker & Walz,  
31 1998; Nichols & Garling, 2002) is still lacking, hindered by the abstract concept of  
32 detritus (Lefevre & Curtis, 1912; Coker *et al.*, 1921; Howard, 1922), a seemingly 'catch  
33 all' term for less clearly defined food sources.  
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38 Little is generally known about juvenile and adult feeding modes of most freshwater  
39 mussel groups. However, some data exist for adults of the Sphaeriidae family (Yeager,  
40 Cherry & Neves, 1994), which may share common feeding mechanisms with juvenile  
41 freshwater mussels of other families. Several sphaeriid species have been maintained  
42 with bacterial suspensions. However, whether they are deposit feeders, using the foot  
43 for particle collection, or suspension feeders, filtering the overlying water, is unclear.  
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3 Sphaeriids are highly mobile, and adopt an infaunal position with the dorsal surface  
4 facing downwards, from just below the sediment surface to a depth of several  
5 centimetres (Meier-Brook, 1969; Holopainen, 1985), indicating that the overlying water  
6 drawn into the mantle cavity by the mantle aperture is not inhaled (Lopez &  
7 Holopainen, 1987). Indeed, in these species, the short siphon does not reach the  
8 sediment surface and pumping of the overlying water is low, indicating they are  
9 infaunal suspension feeders siphoning interstitial water (Efford & Tsumura, 1973)  
10 and/or filtering microbes from the sediment (Holopainen & Hanski, 1979). Glucose  
11 uptake only accounts for a small proportion of the metabolic demands of *Pisidium*  
12 *casertanum*; therefore, utilization of dissolved organic matter from interstitial water is  
13 likely a less important feeding mode in sphaeriids (Efford & Tsumura, 1973).  
14 Nevertheless, some authors have claimed sphaeriids deposit feed, using the ciliated foot  
15 to create inhalant currents that collect food, including microbes (Mitropolskii, 1966;  
16 Mackie & Qadri, 1978; Hornbach, 1984; Burky, 1983). In contrast, other authors  
17 suggest this mechanism is not an important feeding mode for sphaeriids (Lopez &  
18 Holopainen, 1987), concluding that interstitial suspension feeding on bacteria is the  
19 primary mode and that the small size of these bivalves is likely the result of selection on  
20 this feeding mode. Finally, food choice may also differ between genera: plant detritus or  
21 herbivory appears to be more important for *Sphaerium*, and microbes for *Pisidium*  
22 (Holopainen & Lopez, 1989).

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36 Generally speaking, what little information is known of feeding modes in freshwater  
37 mussels stems from rearing captive cultures. Recently emerged juveniles appear to first  
38 pedal feed, then switch to deposit and suspension feeding (e.g. bacteria, bacteria-sized  
39 particles and algae in interstitial water) (Yeager *et al.*, 1994) or pedal deposit feeding  
40 (e.g. algae and detritus but not bacteria) before finally becoming algae filter feeders  
41 (Gatenby, Neves & Parker, 1996; Gatenby, Parker & Neves 1997; Beck & Neves,  
42 2003). However, captive breeding of freshwater mussels for conservation are still  
43 hindered by the lack of detailed knowledge of the diet and feeding mode of juveniles,  
44 with very few studies available describing changes in their feeding morphology during  
45 breeding (Lasee, 1991; Lima *et al.*, 2006; Kovitvadhi *et al.*, 2007; Trump, 2010;  
46 Schartum *et al.*, 2016).

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Based on the knowledge gained to date and information from marine bivalve  
aquaculture, some authors have developed controlled rearing systems, providing extra  
food in the form of algae (Kovitvadhi *et al.*, 2006, 2007, 2008; Gatenby *et al.*, 1996,

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3 1997; Beck & Neves, 2003; Hudson & Isom, 1984; O'Beirn, Neves & Steg, 1998;  
4 Henley *et al.*, 2001; Araujo, Quirós & Ramos, 2003; Gatenby *et al.*, 2003; Liberty,  
5 2004; Guyot, 2005; Jones, Mair & Neves, 2005; Barnhart, 2006; Liberty, Ostby &  
6 Neves, 2007; Eversole, Stuart & Brume, 2008; Schmidt & Vandr , 2010; Thielen, 2011;  
7 Gum, Lange & Geist, 2011; Malo, 2012; Eybe *et al.*, 2013).

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11 In this study, using information from the literature plus our personal experience in  
12 freshwater mussel captive care, we investigated the high level of early juvenile  
13 mortality observed in natural (and captive) habitats, in which both pedal and filter  
14 feeding are available (Araujo *et al.*, 2015). Using juveniles of two species of freshwater  
15 mussels belonging to two different families, Margaritiferidae and Unionidae, we aimed  
16 to test two hypotheses. Our first hypothesis is that young juveniles experience another  
17 round of metamorphosis that causes a change from deposit feeding by the ciliated foot  
18 to suspension feeding (Trump, 2010; Schartum *et al.*, 2016; personal observations) and  
19 that, regardless of the availability of food, many juveniles at this stage are unable to  
20 successfully transition feeding modes, thus resulting in high mortality. A similar  
21 metamorphosis occurs in natural and hatchery populations of marine juvenile bivalves,  
22 which results in mass mortality (due to inefficient particle processing during the  
23 transitional phase) ( Foighil *et al.*, 1990; Reid *et al.*, 1992; Helm *et al.*, 2004; Cannuel  
24 *et al.*, 2009). Our second hypothesis is that juvenile freshwater mussel suspension  
25 feeding development and efficiency can be attributed to the following features: 1) the  
26 contact between the cilia of the posterior filaments of the inner gill, creating a gill  
27 basket, 2) the appearance of the ventral groove in the inner gill and 3) the formation of  
28 the pedal palps.  
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#### 43 Material and Methods

44 The anatomical feeding organs of *M. margaritifera* and *U. mancus* juveniles were  
45 studied. Juveniles of *M. margaritifera* were collected from infections of 0+ *Salmo salar*  
46 Linnaeus, 1758 in a mussel rearing facility in Galicia (NW Spain) with glochidia  
47 specimens from the Arnego River (Ulla basin). After six months, the water of the  
48 infected fish was increased incrementally from 10 to 18  C (Eybe *et al.*, 2015), and  
49 juveniles emerged between 29 March and 8 April 2015. Four hundred juveniles were  
50 maintained in a box filled with 475 ml of river water and 25 ml of detritus without  
51 substrate. Juveniles were fed algae once weekly during a water exchange. The algae  
52 consisted of 120  l of Shellfish Diet 1800 (*Isochrysis* sp., *Pavlova* sp., *Thalassiosira*  
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3 *weissflogii* and *Tetraselmis* sp., with a diameter of 4-20  $\mu\text{m}$ ) and 200  $\mu\text{l}$  of  
4 *Nannochloropsis* sp. (1.5-2  $\mu\text{m}$ ) suspended in 10 L of river water (Eybe *et al.*, 2013;  
5 Scheder *et al.*, 2014). The boxes were kept in a conditioning cabinet at a constant  
6 temperature of 17 °C. The algal diet was doubled after the first month and tripled after  
7 6 months. After one year, juvenile mortality was about 90% in this species.  
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11 Juveniles of *U. mancus* were collected from infections of *Barbus meridionalis* Risso,  
12 1827 with glochidia specimens from Banyoles Lake (Spain); they emerged on 12 June  
13 2015. Juveniles were then maintained in a PVC cylinder measuring 16 cm in diameter  
14 and 17 cm in height (total volume capacity of 2.2 L) without sediment but with a 200  
15  $\mu\text{m}$  mesh at the bottom. This cylinder was suspended within another receptacle  
16 (33x46x44 cm) filled with 70 L of water from Banyoles Lake (Spain). This culture was  
17 fed 3 times weekly with 2 mg/L of frozen Easy Reefs (1/3 *Nannochloropsis gaditana* 2-  
18 4  $\mu\text{m}$  + 1/3 *Tetraselmis chuii* 12-14  $\mu\text{m}$  + 1/3 *Phaedactylum tricornutum* 2-6  $\mu\text{m}$ ). The  
19 outer receptacle was artificially aerated, and daily, the inner cylinder was moved up and  
20 down 5 times first to empty the water through the mesh and then to homogenize the  
21 inner cylinder water with the surrounding water. Water exchanges of 100% were  
22 performed every two days. Juvenile mortality after one year was about 40% in this  
23 species.  
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33 Between five and ten juveniles of each species were sacrificed every 10 days until day  
34 90 and then once a month until day 360. Prior to fixation in 2.5 % glutaraldehyde (2-24  
35 hours), specimens were first relaxed by slowly adding  $\text{MgCl}_2$  until the valves opened. If  
36 the valves did not open, one valve was broken before dehydration. Specimens were then  
37 cleaned with PBS buffer and dehydrated through a graded ethanol series (30-60 minutes  
38 each in 30, 50, 70, 90, 96, 100 and 100% ethanol). Specimens were stored in 100%  
39 ethanol until graded into solutions of 100% ethanol and hexamethyldisilazane (HMDS)  
40 (15-30 minutes each in 2:1; 1:1; 1:2 ethanol:HMDS).  
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46 Electron scanning microscopy analyses were made focusing on the following features:

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48 1. Gill buds and inner gill growth. 2. Inner gill reflection and ventral groove. 3. Mouth.  
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50 4. Labial palps. 5. Mantle cilia (border and inner side). 6. Cilia and ventral groove on  
51 the foot. 7. Gill buds and the appearance of the external gill.  
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55 Additional observations were made of ethanol preserved specimens under a dissecting  
56 scope.  
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## Results

### *M. margaritifera* (Tables 1, 2)

The mean length of newly emerged juveniles was 350  $\mu\text{m}$ . The most conspicuous characters were the very ciliated foot, with a marked ventral groove (Fig. 1), the ciliated border of the mantle (Figs. 2, 3) and the presence of three filaments and one posterior developing gill bud (Fig. 4). The ventral pedal groove is the byssus groove with the byssus pit (the posterior) and the byssus disc pit (the anterior) at either end (Fig. 1). Frontal, lateral and latero-frontal cirri were observed in the gill filaments, which connected adjacent and opposing filaments. The outer demibranchs of the internal gill were clearly developing (Fig. 5); however, they did not bend until 130 days, in specimens greater than 1 mm. There were also two diagonal rows of long cilia on the inner side of the posterior mantle wall (Fig. 6).

At 10 days (430  $\mu\text{m}$ ) post-emergence, these features are maintained, and a shell ring had grown around the original shell. At this time point, there were many more gill filaments, with greater connections between them, resulting in a basket-like structure having an oval-shaped orifice posteriorly surrounded with long cilia (Fig. 7). Diagonal rows of long cilia on the inner mantle wall were present.

By 30 days (580  $\mu\text{m}$ ), there were five gill filaments covered with cilia and cirri (Fig. 8) and one developing posterior gill bud. We observed the anterior adductor muscle and a very ciliated mouth (Fig. 9), but the labial palps were not yet present. The foot and the mantle border were heavily ciliated.

The cilia around the mantle margin began to diminish between 40 and 80 days (700-800  $\mu\text{m}$ ), although there were ciliary tufts on the margin (Fig. 10). The cilia on the foot and on the inner mantle also began to disappear. The labial palps and the ventral groove of the gills were still absent.

The labial palps were present at 150 days (1.4 mm) (Fig. 11). At 180 days, 13 filaments were present on each side of the gill, but the gill ventral groove was still not present (Fig. 12). Ciliary tufts were present on the posterior inner mantle roof above the ulterior exhalant aperture.

At day 210 (1.8 mm), the gill had 21 filaments, the ventral groove between the inner and outer lamellae was already marked (Fig. 13) and the labial palps were highly discernible (Figs. 14, 15). The palps were folded, and the inner sides ciliated (Fig. 15). The cilia and the ventral groove of the foot were shorter than at 100 days (Fig. 14). Two pseudodiaphragms were well developed at the posterior mantle margin, increasing the

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3 separation between the gill cameras (Fig. 16). More cilia were present on the mantle  
4 margin near the pseudodiaphragm that in other places of the mantle border. The first gill  
5 bud of the external gill was present at 210 days.  
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8 At day 240 (2 mm), the cilia of the mouth were shorter than at younger stages. Cilia  
9 were present on the pseudodiaphragm (Fig. 17) as were external gill filaments (Fig. 18).  
10 In adults, these filaments, along with the pseudodiaphragm, will form the gill cameras.  
11 At 300 days (2.1 mm), the byssal pit on the ventral foot and ciliary tufts on the posterior  
12 roof of the mantle were both present (Fig. 19), and the papillae of the inhalant aperture  
13 were discernible. The area around the inhalant aperture was darkly pigmented in live  
14 animals at 200 days. The developing external gill was apparent and possessed bent  
15 filaments (Fig. 20). The pseudodiaphragm and the borders of the inhalant and exhalant  
16 apertures were both ciliated. Ciliary tufts on the mantle border were evident, leaving  
17 circular marks (Fig. 21). At day 360 (2.2 mm), the folded labial palps were covered with  
18 cilia (Fig. 22), the external gill was more well developed and the papillae on the  
19 inhalant aperture were more visible.  
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28 At no time point were muscular connections between gill filaments observed.

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30 *U. mancus* (Tables 1, 2)

31 The mean length of newly emerged juveniles was 260  $\mu\text{m}$ . The ciliated mouth of *U.*  
32 *mancus* was much larger than that of *M. margaritifera*; however, the foot, mantle  
33 margin (Figs. 23, 24, 25) and gills were very similar in the two species at this initial  
34 time point.  
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38 At 60 days (1.1 mm), the outer demibranch was reflected, and the ventral groove of the  
39 inner gill had formed, and at 70 days, primordial labial palps with cilia were present  
40 (Fig. 26). The mantle border was also covered with cilia, though these were shorter than  
41 those observed at younger stages.  
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45 At 120 (1.6 mm) days, the inner gill had 18 filaments. The external gill became evident  
46 at 180 days (1.9 mm) and was associated with the posterior part of the inner gill, where  
47 a separation between the upper and lower gill chambers will eventually form on the  
48 inner wall of the mantle (Fig. 27), rather than a pseudodiaphragm as in margaritiferids.  
49 The developing external gill also already had bent filaments.  
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53 The papillae of the inhalant siphon were very clear at 345 days (2.8-3 mm) (Fig. 28),  
54 resembling spheres on the inner mantle border (Fig. 29). By this time, the overall  
55 appearance of the juvenile was very similar to that of the adult, although with unfused  
56 mantle borders (Fig. 30). The inner sides of the labial palps had ciliary folds (Fig. 31)  
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3 and a flat external surface with a ciliary flange (Fig. 32). Also at 345 days, 45 inner gill  
4 filaments with complete cilia and cirri were present and connected by horizontal  
5 muscular bridges (Figs. 33, 34). The foot was covered with short cilia, and at 395 days  
6 (3.8 mm), the ventral groove only remained in the posterior part (Fig. 35). The exhalant  
7 siphon was completely developed (Fig. 36), but the ventral borders of the inhalant  
8 siphon were not yet connected.  
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12 Based on these observed anatomical features, newly emerged juveniles of both *M.*  
13 *margaritifera* and *U. mancus* first feed by pedal feeding using the cilia of the foot and  
14 of the mantle margin. However, initiation of suspension feeding likely begins when the  
15 cilia of the posterior parts of the inner gill filaments join to form a virtual hole for water  
16 current. In effect, the gill filaments build a basket with a dorsal and a ventral chamber  
17 with a ciliated posterior opening. This opening allows water to enter, facilitated by the  
18 mantle and gill cilia, similar to the ulterior inhalant aperture (or siphon) in the adult. In  
19 this new mode of suspension feeding, a posterior-anterior current, opposite to the one  
20 used for pedal feeding (i.e. anterior-posterior), is generated. Furthermore, the function  
21 of this basket improves after the formation of the labial palps and the corresponding  
22 ventral groove between the two inner gill demibranchs, which direct food into the  
23 mouth, and as suspension feeding improves, the cilia on the foot become shorter and  
24 less numerous.  
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### 36 Discussion

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38 Freshwater mussels are one of the most imperilled animals in the world, and one of the  
39 most fragile stage in a mussel's life is as recently emerged juveniles (ASTM, 2006;  
40 Augspurger *et al.*, 2007; Strayer & Malcom, 2012; Archambault *et al.*, 2014). Based on  
41 these facts, and to improve artificial rearing of these molluscs, a better understanding of  
42 the natural diet and the development of the feeding organs of newly emerged juveniles  
43 is crucial (Tucker, 1927; Lasee, 1991; Uthaiwan *et al.*, 2001; Lima *et al.*, 2006;  
44 Kovitvadhi *et al.*, 2006, 2007; Trump, 2010; Schartum *et al.*, 2016).  
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47 Pedal feeding is likely the first method employed in young juvenile-staged benthic  
48 bivalves as an evolutionary constraint of a plesiomorphic character (Stasek, 1963; Reid  
49 *et al.*, 1992). This feeding method has been observed in some juvenile and small adult  
50 marine and freshwater bivalves (e.g. *Macoma balthica*, *Corbicula fluminea* and *Mysella*  
51 *bidentosa*) (Caddy, 1969; Reid *et al.*, 1992; Schartum *et al.*, 2016). The transition to  
52 suspension feeding, at least in phytoplankton fed juvenile freshwater mussels or in the  
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3 wild, seems to be a gradual process (Lasee, 1991; Trump, 2010; Yeager *et al.*, 1994;  
4 Beck & Neves, 2003) related to the development of new organs, particularly of two  
5 basic bivalve feeding organs, the gills and the labial palps (Kellogg, 1915; Stasek, 1963;  
6 Schartum *et al.*, 2016; Galbraith *et al.*, 2009). Once these two structures are well  
7 developed, definitive filter feeding is initiated. Our results are in agreement with other  
8 authors (Trump, 2010; Schartum *et al.*, 2016): the anatomical development of feeding  
9 structures is a result of an overall increase in an animal's size rather than strictly as a  
10 function of age (Tables 1, 2).

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16 Based on our observations, newly emerged *M. margaritifera* and *U. mancus* use pedal  
17 feeding to collect food, as evidenced by the densely ciliated mobile foot, similar to what  
18 has been observed in other post-metamorphosed juveniles (Lasee, 1991; Kovitvadhi *et*  
19 *al.*, 2007; Trump, 2010; Uthaiwan *et al.*, 2001). In *M. margaritifera* and *U. mancus*, as  
20 in *Lampsilis ventricosa* (Lasee, 1991), we did not find evidence of an elaboration of the  
21 byssal thread; nevertheless, in both these species, the two byssus pits on the ventral  
22 pedal groove are present in newly metamorphosed juveniles, whereas in *L. ventricosa*,  
23 the byssal complex forms between 4 and 8 weeks post-metamorphosis (Lasee, 1991).  
24 Although pedal feeding is facilitated by the ciliated foot and mouth, initial suspension  
25 feeding is initiated by currents made by the lateral filaments on the posterior mantle  
26 margin and by the cilia from the left and right gills forming the gill basket (Lasee, 1991;  
27 Gatenby *et al.*, 1996; Trump, 2010; Schartum *et al.*, 2016). Rejected food likely reaches  
28 the outside current by the cilia forming diagonal rows on the inner mantle wall and  
29 those on the posterior mantle roof and anterior mantle margin (Beninger, Veniot &  
30 Poussart, 1999). In *Villosa iris* juveniles 14 days post-metamorphosis, pedal and  
31 filtering feeding are both utilised but always using the pedal cilia and not the posterior  
32 apertures (Yeager *et al.*, 1994). These authors concluded that the cilia of the foot creates  
33 the filtering current, although in this case, the foot does not extended as it does during  
34 pedal (deposit) feeding (Yeager *et al.*, 1994). However, as observed in 500-800  $\mu\text{m}$   
35 larvae of the marine bivalve *Pecten maximus*, particle capture is likely inefficient  
36 (Beninger, Dwiono & Le Pennec, 1994). In 60-day-old *Hyriopsis myersiana* juveniles,  
37 both the ciliated foot and the cilia on the mantle and gills are used to transport  
38 phytoplankton (Kovitvadhi *et al.*, 2006, 2008). The development of inner gill frontal  
39 cilia and laterofrontal cirri is important to form the gill basket for initial filter feeding  
40 (Schartum *et al.*, 2016). In *L. ventricosa*, *H. myersiana* and *Utterbackia imbecillis*, the  
41 laterofrontal cirri were always present in recently emerged juveniles, (Lasee, 1991;  
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3 Wright, 1995; Kovitvadhi *et al.*, 2007; Trump, 2010). The broadening of the distal ends  
4 of the gill filaments between 7 and 28 days in *U. imbecillis* (Trump, 2010) and at 56  
5 days (890  $\mu\text{m}$ ) in *L. ventricosa* (Lasee, 1991) sufficiently forms a basket for particle  
6 capture during gill bud and reflected gill stages. During metamorphosis in *P. maximus*  
7 juveniles (300-900  $\mu\text{m}$  of shell length), a similar process of gill basket formation occurs  
8 while the velum (larval feeding organ) disappears; but in this case, the inner gill has not  
9 yet reflected (Beninger *et al.*, 1994). Nevertheless, formation of both the inner  
10 demibranch ventral groove and the pedal palps is required for successful filter feeding  
11 (Kellogg, 1915). Once these structures are well developed, and filter feeding becomes  
12 the main (or only) feeding mechanism, juvenile mortality decreases. Based on our  
13 observations here, definitive filter feeding occurs around 180 days (1.6 mm) in *M.*  
14 *margaritifera* with the formation of the labial palp ridges and cilia and the ventral  
15 groove of the inner gill. However, other authors have reported this feeding change  
16 occurs later (2.2 mm) in this species (Schartum *et al.*, 2016). In *U. mancus*, the palp  
17 primordia and the transition to filter feeding occurs at 70 days (1 mm). The initial small  
18 folds of the labial palps were present at 2 days in *L. ventricosa* (Lasee, 1991) and 3 days  
19 in *U. imbecillis* (Trump, 2010), but in the latter species, the ridges did not appear until  
20 130 days (Trump, 2010).

21  
22 We have observed the reflection of the outer demibranch of the inner gill at 130 days  
23 (1.2 mm) in *M. margaritifera* and between 40 and 60 days (0.8-1 mm) in *U. mancus*. In  
24 these species, there is a clear relationship between effective filtering mechanisms and  
25 inner gill reflection, as has also been hypothesised in other freshwater [9,10,78] and  
26 marine (Beninger *et al.*, 1994; Cannuel *et al.*, 2009) species. In *M. margaritifera*, the  
27 inner gills of juveniles between 0.8 and 1.1 mm in length initially have I-shaped  
28 filaments, bending to a final V-shaped form in juveniles between 1.1 and 4.5 mm,  
29 whereas the outer gill filaments are already bent from initial growth (Schartum *et al.*,  
30 2016). Inner gill reflection occurs at 7 weeks in *Anodonta cygnea* (Herbers, 1914), 30  
31 days in *H. myersiana* (Kovitvadhi *et al.*, 2007) and 113 days in *U. imbecillis* (consisting  
32 of 15-18 filaments) (Trump, 2010). However, by 56 days in *L. ventricosa*, gill reflection  
33 had still not been observed (Lasee, 1991). The increase in the number of gill filaments  
34 during growth improves filter feeding. In our study, *M. margaritifera* juveniles had 4-6  
35 filaments 50 days post-emergence, 8-12 up to 120 days and more than 20 at 210 days.  
36 One filament is added for every 123  $\mu\text{m}$  of shell length (Schartum *et al.*, 2016). In *U.*  
37 *mancus*, 18 filaments were present at 120 days and 45 at 345 days. In *L. ventricosa*,

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3 there were 4 pairs of filaments at 21 days and 5-10 pairs at 28 days (Lasee, 1991). For  
4 *U. imbecillis*, the data are unclear: one study reported 23 pairs of filaments in a 74-day-  
5 old, 5.1 mm juvenile (Hudson & Isom, 1984), while another reported 23 filaments in an  
6 158-day-old, 2.1 mm juvenile (Trump, 2010). The outer demibranch, which appears to  
7 be the last structure to form prior to definitive filter feeding, forms when species reach  
8 approximately 2 mm in shell length. Different species reach this length at different  
9 times: 90 days in *H. myersiana* (Kovitvadhi *et al.*, 2007), 130 days in *U. imbecillis*  
10 (Trump, 2010), 240 days in *M. margaritifera* (this study) [though it has also been  
11 reported to occur at 4.5 mm (Schartum *et al.*, 2016)], and 180 days in *U. mancus* (this  
12 study). However, in another *M. margaritifera* study, it has been reported that the inner  
13 gill begins to bend at 16 months and the complete gills (inner and outer) are formed at 3  
14 years (Lavictoire *et al.*, 2015).

15  
16 Notably, we observed an important difference in inner gill development between *M.*  
17 *margaritifera* and *U. mancus*, likely explained as a character difference between  
18 families: muscular junctions between filaments were clearly observed at 345 days in *U.*  
19 *mancus* but were completely absent in *M. margaritifera*.

20  
21 The papillae of the inhalant aperture were present at 330 days (2.2 mm) in *M.*  
22 *margaritifera*, although at this time, the flange, which will form the pseudodiaphragm,  
23 was already present in the internal wall of the mantle. The papillae of the inhalant  
24 siphon of *U. mancus* were present at 300 days (2.2 mm). In comparison, papillae were  
25 observed at 100 days in *H. myersiana* (Kovitvadhi *et al.*, 2007), 175 days (3 mm) in *U.*  
26 *imbecillis* (Trump, 2010) and 272 days in *V. iris* (Gatenby *et al.*, 1996).

27  
28 Of the freshwater mussels compared (Table 1), *H. myersiana* is the fastest growing  
29 species, reaching 1.8 mm in 60 days, 24 mm in 150 days and 85 mm in one year. The  
30 feeding organs of this species also develop more rapidly, having well-developed gills  
31 and siphons by 50 days post-emergence (Table 2). However, for the other Unionidae  
32 species mentioned (Table 2), these organs developed at similar times. In the case of *M.*  
33 *margaritifera*, the only member of the Margaritiferidae studied, these organs developed  
34 much more slowly. Interestingly though, cultured *M. margaritifera* juveniles at 360  
35 days had a mean shell length twice that of wild populations (Lavictoire *et al.*, 2016).  
36 During the winter, wild populations stop growing and thus are delayed, whereas our  
37 cultured juveniles were grown at a constant temperature of 17 °C. However, ctenidial  
38 development is not related to temperature (Schartum *et al.*, 2016).

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40 Winter survival, ecological problems [2-5] and the absence of food (Schartum *et al.*,  
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2016) have all been cited as causes for juvenile mortality in freshwater mussels. Given the high level of juvenile mortality reported for *U. mancus* and *M. margaritifera* in natural habitats with no food issues (Hastie & Young, 2003; Araujo *et al.*, 2015; Eybe *et al.*, 2015), we hypothesise that early juvenile mortality is instead due to the inability to successfully transition feeding modes, facilitated by changing anatomical structures. The transition from pedal feeding to filter feeding occurs around 150-200 days post-emergence in *M. margaritifera* and around 70 days in *U. mancus*, after juveniles are greater than 1 mm in length, which coincides with the timing of reported high mortality. Once this feeding metamorphosis is complete, juvenile mortality decreases.

In our cultures of *M. margaritifera* and *U. mancus*, juveniles were maintained for more than one year. Based on the anatomical observations reported here, juveniles of both species first feed with the foot and then by filtering algae or another food source. Future studies will next focus on determining the actual food type ingested during these two distinct feeding stages.

#### Acknowledgements

The culture of *M. margaritifera* was funded by the LIFE-Margal Ulla (LIFE09 NAT/ES/000514) and rearing was achieved at the Veral rearing facility (Consellería de Medio Ambiente, Xunta de Galicia). We are grateful to S. Latas and P. Caballero (Consellería de Medio Ambiente, Xunta de Galicia) and Dr. R. Mascato for constantly supporting the Galician FPM conservation project. We also thank E. Corral, R. Ocharan and P. Castrillo for their active assistance of the rearing work. Furthermore, we wish to thank Dr. F. Thielen and his team for their encouragement and support of mussel culturing.

The rearing of *U. mancus* was carried out at the Laboratory of breeding of freshwater mussels of the lake of Banyoles (Consorci de l'Estany) under the LIFE-Potamo Fauna (LIFE12 NAT/ES/001091). We are grateful to Dr. Q. Pou, I. Camós, R. Casadevall and G. Dalmau for their active assistance of the rearing work.

This study was made possible by the networking of the two aforementioned LIFE projects co-financed by the European Commission.

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## Table caption

Table 1. Mean length ( $\mu\text{m}$  and  $\text{mm}$ ) of juvenile freshwater mussel species at specified days as reported in this study and by other authors.

Table 2. Days of development of feeding structures in juvenile freshwater mussel species reported in this study and by other authors. For references, see in Table 1. \* No data about age.

## Figure captions

Figures 1-8. *M. margaritifera*. 1. Ciliated foot with the byssal ventral groove and the two byssus pits (arrows) (0 days). 2. Cilia at the mantle border (0 days). 3. Detail of the cilia at the mantle border (0 days). 4. Ventral view (0 days). 5. Bent gill filaments (0 days). 6. Diagonal row of cilia at the inner mantle wall (0 days). 7. Inner gill filaments, gill buds and long cilia at the posterior mantle margins (10 days). 8. Cilia and cirri in the inner gill filaments (30 days).

Figure 9-16. *M. margaritifera*. 9. Detail of the ciliated mouth (arrow) (30 days). 10. Cilia at the mantle margin (80 days). 11. The foot and the labial palps (arrows) (150 days). 12. Connection between the left and right inner gill filaments (180 days). 13. Ventral groove at the inner gill (210 days). 14. Ventral view. See the labial palps (210 days). 15. Outer surface of the right labial palps. 16. Ventral view. Pseudodiaphragm and posterior filaments of the inner gill. (210 days).

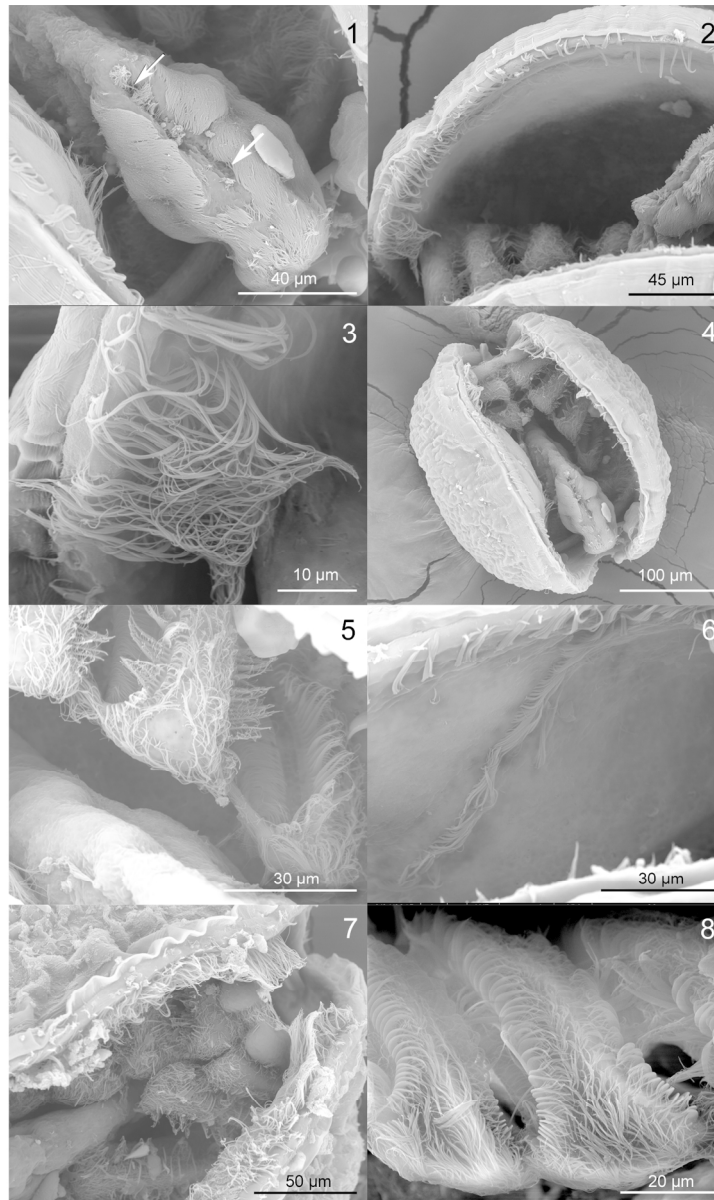
Figure 17-22. *M. margaritifera*. 17. Ciliated pseudodiaphragm (240 days). 18. Inner right gill and the beginning of the outer gill (240 days). 19. Ciliary tufts at the posterior mantle roof (300 days). 20. Lateral view. See the labial palps and the mouth everted (300 days). 21. Cilia and ciliary marks at the mantle margin (330 days). 22. Ciliary folds at the inner surface of the labial palps (360 days).

Figure 23-30. *U. mancus*. 23. Ventral view. See the big mouth (arrow) and the gill filaments and buds (0 days). 24. Cilia at the inner gill filaments. 25. Cilia at the mantle margin. 26. Labial palps in formation (70 days). 27. Formation of the outer gill (arrow) (180 days). 28. Exhalant siphon papillae and posterior inner and outer gill filaments (345 days). 29. Birth of the siphon papillae (345 days). 30. Ventral view (395 days).

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Figure 31-36. *U. mancus*. 31. Ciliated folds in the inner side of the labial palps (345 days). 32. Outer side of a labial palp. See the cilia at the margin. 33. Filaments in the inner gill. See the connections between filaments (395 days). 34. Filaments in the inner gill. See the connections between filaments (395 days). 35. Foot, inner gills and labial palps (395 days). 36. Exhalant (above) and inhalant siphons (395 days).

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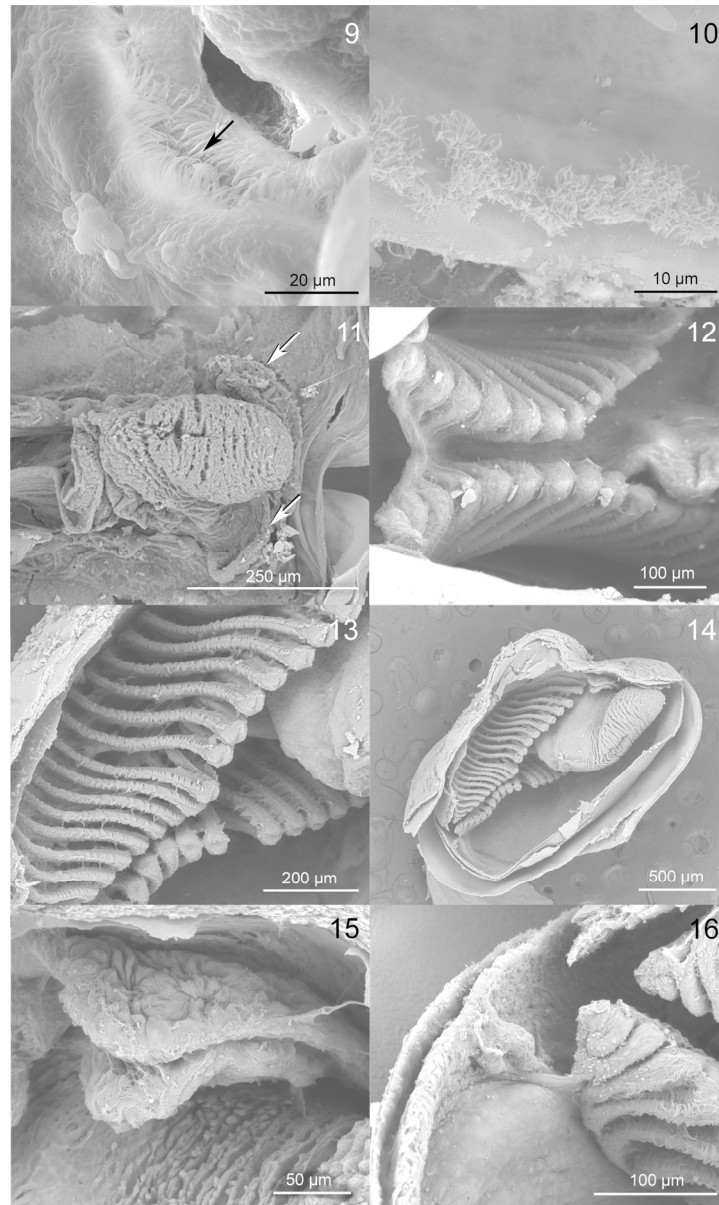


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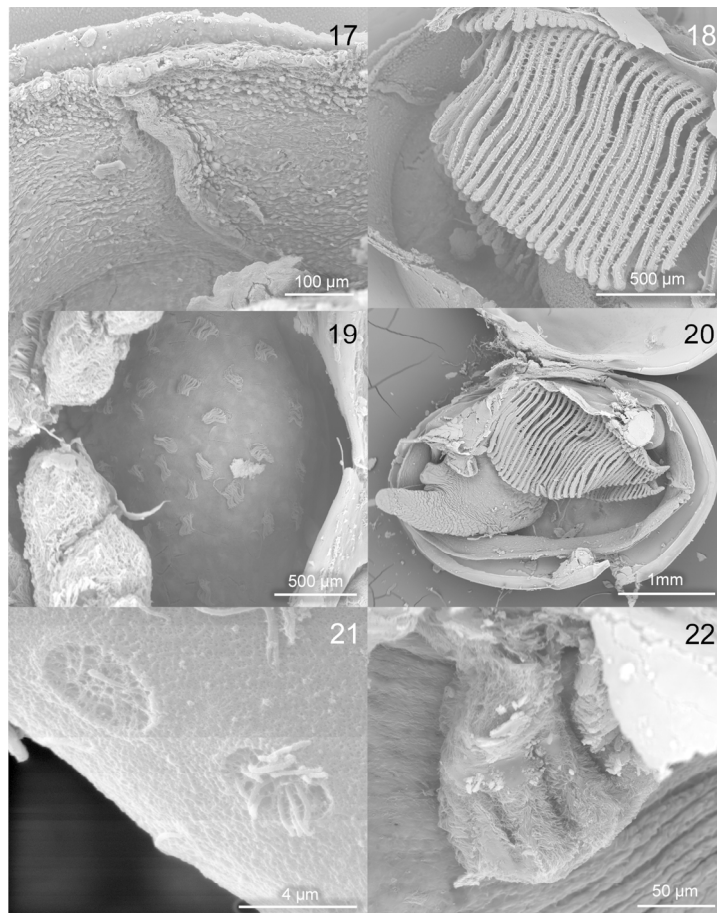


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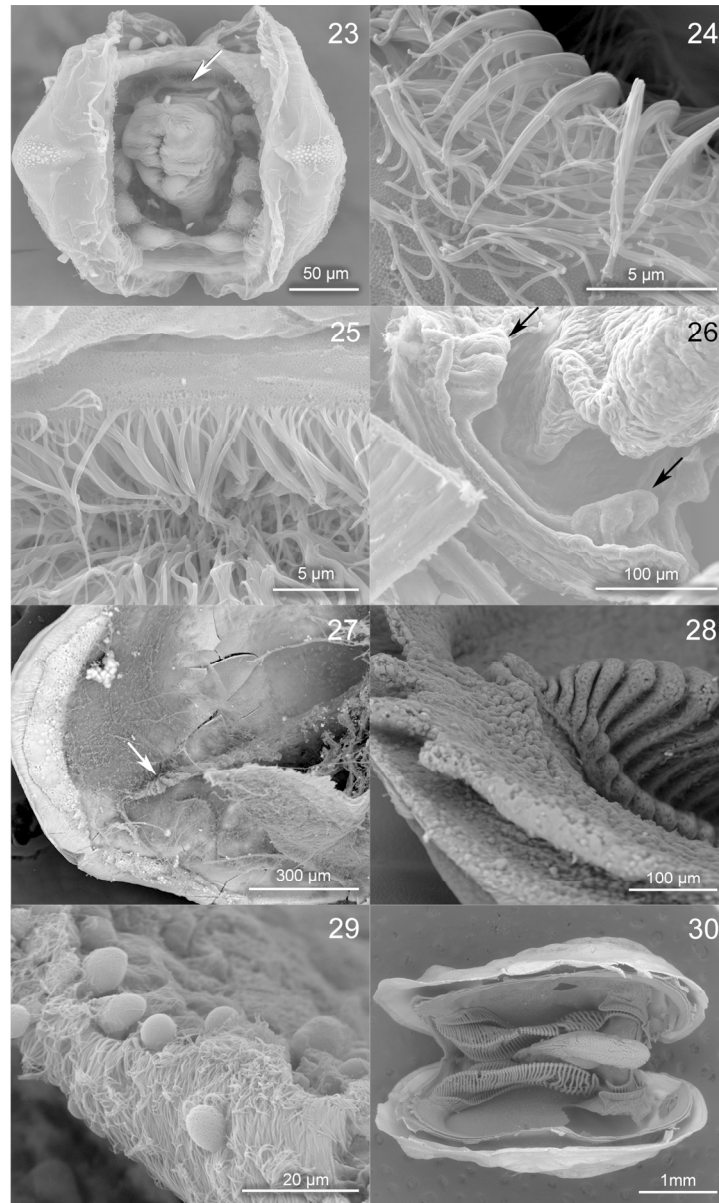


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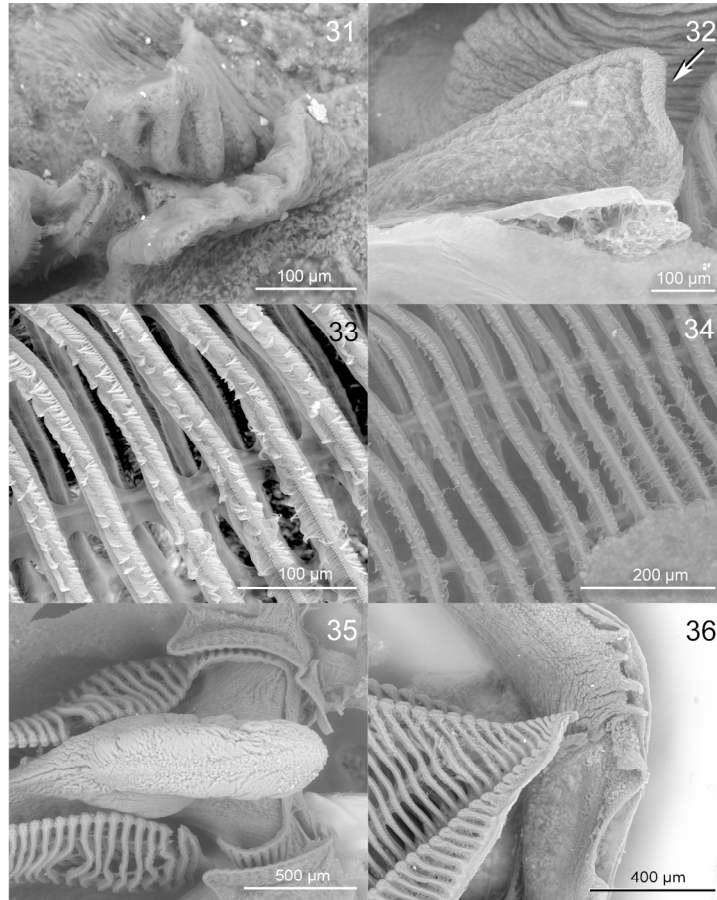


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134x180mm (300 x 300 DPI)

Table 1. Mean length ( $\mu\text{m}$  and  $\text{mm}$ ) of juvenile freshwater mussel species at specified days as reported in this study and by other authors.

	1 d	10 d	30 d	60 d	80-100 d	150 d	180 d	210 d	300 d	360 d
	$\mu\text{m}$	$\mu\text{m}$	$\mu\text{m}$	$\mu\text{m}$	$\text{mm}$	$\text{mm}$	$\text{mm}$	$\text{mm}$	$\text{mm}$	$\text{mm}$
<i>M. margaritifera</i> <sup>1</sup>	350	430	580	780	0.8-1	1.4	1.6	1.8	2.1	2.2
<i>M. margaritifera</i> <sup>2</sup>	-	460	-	-	-	-	-	-	-	-
<i>M. margaritifera</i> <sup>3</sup>	-	-	-	-	-	0.4-0.9	-	-	-	-
<i>M. margaritifera</i> <sup>4</sup>	-	-	-	600-700	0.7-0.9	0.7-0.9	-	-	0.7-0.9	1.1-1.2
<i>M. margaritifera</i> <sup>5</sup>	300-480	-	-	-	-	-	-	-	-	-
<i>U. mancus</i> <sup>1</sup>	260	450	750	1100	1.2-1.4	1.7	1.9	2	2.2	3
<i>V. iris</i> <sup>6</sup>	300-400	-	-	-	0.8-1	1.7-2.3	-	-	-	-
<i>L. ventricosa</i> <sup>7</sup>	227	350	530	889	-	-	-	-	-	-
<i>U. imbecilis</i> <sup>8</sup>	320	-	-	800	-	2.1	-	-	-	-
<i>H. myersiana</i> <sup>9</sup>	150	200	-	1800	2.5	24	-	-	-	85

1 This article

2 Malo (2010)

3 Schmidt &amp; Vandr  (2010)

4 Lavictoire et al., 2016

5 Schartum et al. (2016)

6 Gatenby et al., 1997

7 Lasee, 1991

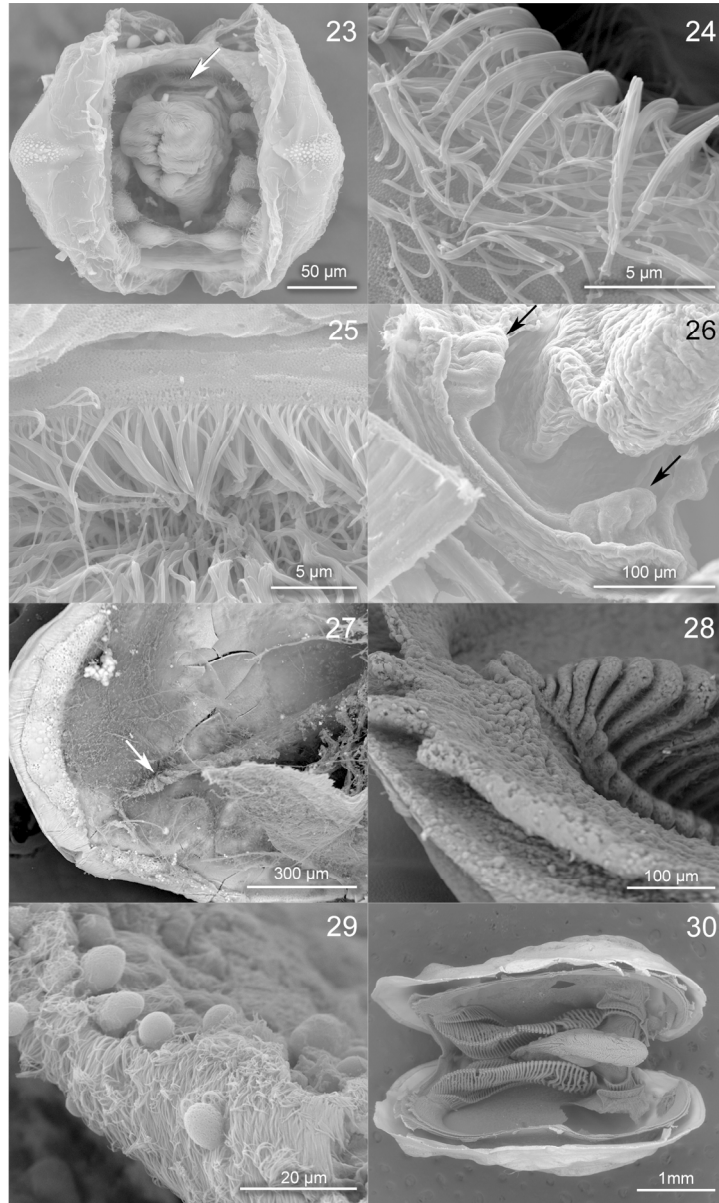
8 Trump, 2010

9 Kovitvadhi et al., 2007, 2008

Table 2. Days of development of feeding structures in juvenile freshwater mussel species reported in this study and by other authors. For references, see in Table 1. \* No data about age

	Inner gill cirri	Basket	Inner gill bent	Ventral groove	Labial palps	Outer gill	Inhalant papillae
<i>M. margaritifera</i> <sup>1</sup>	0	10	130	180	180	240	300
<i>M. margaritifera</i> <sup>5</sup>	-	-	1.1-2.2 mm*	2.2-4.5 mm*	-	4.5 mm*	-
<i>U. mancus</i> <sup>1</sup>	0	10	40-60	60	70	180	300
<i>V. iris</i> <sup>6</sup>	-	14	-	-	-	-	after 272
<i>L. ventricosa</i> <sup>7</sup>	0	-	after 56	-	2	-	-
<i>U. imbecilis</i> <sup>8</sup>	0	7-28	113	-	3	130	175
<i>H. myersiana</i> <sup>9</sup>	0	10	30	after 50	-	90	50

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Graphical Abstract Image

Development of juvenile feeding apparatuses in *Unio mancus* (0 to 395 days).

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The decline of freshwater mussels is attributed to their complex life cycle and mass mortality of juveniles has been reported. We show that a second metamorphosis occurs and juveniles unable to successfully transition perish.

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