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

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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 using scanning electron microscopy of newly formed juveniles of the freshwater species Margaritifera margaritifera (L.) (Margaritiferidae) and Unio mancus Lamarck (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 hypothesize that suspension feeding in these two species improves due to a gradual development of 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 likely perish.

KEYWORDS
biodiversity conservation, development, juvenile mortality, metamorphosis

1 | INTRODUCTION
The majority of bivalves are rhythmic filter and/or deposit feeders (Morton, 1973). Adult freshwater bivalves of the Unionoida are generally considered suspension feeders (phytoplankton, zooplankton and particulate detritus) or deposit feeders (particulate detritus in sediment); however, some authors have stated that deposit-feeding bivalves are never found in fresh water (Nicol, 1984). Various authors have reported that the diet of some freshwater mussels consisted of suspended bacteria, fungal spores, dissolved or sediment organic matter (Vaughn & Hakenkamp, 2001), or clearing coliform bacteria (Silverman et al., 1997). River and lake unionids always ingest bacterial carbon instead of algal carbon, and are not always primary consumers or omnivores (Nichols & Garling, 2000). Nevertheless, the importance of algae in the diet is derived from the phytosterol, and cholesterol and vitamin B12 are also key dietary items (Nichols & Garling, 2000). Some unionids feed directly on the living microbial and algal components of fine organic material on the sediment surface (Raikow & Hamilton, 2001). Other authors have hypothesized that the food required by two species of Margaritifera comes from a healthy rhizosphere, whereas eutrophication of the immediate environment was responsible for the absence of food (Howard, Cuffey, & Solomon, 2005; Hruska, 1999; Negishi, Katsuki, Kume, Nagayama, & Kayaba, 2014). Indeed, seasonal differences in feeding and metabolism have also been reported for different mussel species (Baker & Hornbach, 2001; Strayer, 2008; Vaughn, Nichols, & Spooner, 2008; Vaughn, Spooner, & Galbraith, 2007; Yasuno et al., 2014). Despite having much information, a good understanding of the food sources and feeding mechanisms of freshwater mussels (Nichols & Garling, 2002; Raikow & Hamilton, 2001; Strayer, 2008; Vaughn & Hakenkamp, 2001; Vaughn et al., 2008; Welker & Walz, 1998) is still lacking, hindered by the abstract concept of detritus (Coker, Shira, Clark, & Howard, 1921; Howard, 1922;
Lefevre & Curtis, 1912), a seemingly ‘catch all’ term for less clearly defined food sources.

Little is generally known about adult feeding mode of the Sphaeridiidae, which may share common size and feeding mechanisms with juvenile freshwater mussels of other families (Yeager, Cherry, & Neves, 1994). Several adult sphaeriid species have been maintained with bacterial suspensions. However, whether they are deposit feeders, using the foot for particle collection, or suspension feeders, filtering the overlying water, is unclear. Sphaeridiids are highly mobile, and adopt an infaunal position with the dorsal surface facing downwards, from just below the sediment surface to a depth of several centimetres (Holopainen, 1985; Meier-Brook, 1969), indicating that the overlying water drawn into the mantle cavity by the mantle aperture is not inhaled (Lopez & Holopainen, 1987). Indeed, in these species, the short siphon does not reach the sediment surface and pumping of the overlying water is low (Efford & Tsumura, 1973), indicating they are infaunal suspension feeders siphoning interstitial water and/or filtering microbes from the sediment (Holopainen & Hanski, 1979). Glucose uptake only accounts for a small proportion of the metabolic demands of Pisidium castoratum (Poll); therefore, utilization of dissolved organic matter from interstitial water is likely a less important feeding mode in sphaerids (Efford & Tsumura, 1973). Nevertheless, some authors have claimed sphaeriids deposit feed, using the ciliated foot to create inhalant currents that collect food, including microbes (Burky, 1983; Hornbach, Way, Wissing, & Burky, 1984; Mackie & Qadri, 1978; Mitropolsski, 1966). In contrast, other authors suggest this mechanism is not an important feeding mode for sphaerids (Lopez & Holopainen, 1987), concluding that interstitial suspension feeding on bacteria is the primary mode and that the small size of these bivalves is likely the result of selection on this feeding mode. Finally, food choice may also differ between genera: plant detritus or herbivory appears to be more important for Sphaerium, and microbes for Pisidium (Holopainen & Lopez, 1989).

Freshwater mussels (Unionoida) have a complex life cycle which involves a temporary but obligatory parasitic stage in which the larvae (glochidia) attach to the external surface or gill filaments of their vertebrate hosts (mainly fish). 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 glochidial 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 fragile and experience high mortality, although the reasons (winter survival, ecological problems, absence of food) are unclear (Archambault, Cope, & Kwak, 2014 ASTM, 2013; Augspurger, Dwyer, Ingersoll, & Kane, 2007; Strayer & Malcom, 2012). We hypothesize that this critical juvenile stage can be related to a second metamorphosis event that occurs just after emergence as juveniles. Due that deposit feeders use the foot for particle collection, but suspension feeders filter the overlying water, this second metamorphosis results in drastic morphological changes that, regardless of the presence of available food, substitutes deposit feeding for suspension feeding (Kovitvadhi, Kovitvadhi, Sawangwong, Thongpan, & Machado, 2006, 2007; Lasee, 1991; Lima, Kovitvadhi, Kovitvadhi, & Machado, 2006; Schartum, Mortensen, Pittman, & Jakobsen, 2016; Trump, 2010). This process would be similar to the metamorphosis observed during settlement in marine juvenile bivalves (Cannuel, Beninger, McCombie, & Boudry, 2009; Helm, Bourne, & Lovattelli, 2004; O’Foighil, Kingzett, O’Foighil, & Bourne, 1990; Reid, McMahon, O’Foighil, & Finnigan, 1992).

The little information we know about feeding modes in juvenile freshwater mussels of the families Unionidae and Margaritiferidae stems from rearing captive cultures. Recently emerged juveniles appear to first pedal feed, then switch to deposit and suspension feeding (e.g., bacteria, bacteria-sized particles, and algae in interstitial water) (Yeager et al., 1994) or pedal deposit feeding (e.g., algae and detritus but not bacteria) before finally becoming algae filter feeders (Beck & Neves, 2003; Gatenby, Neves, & Parker, 1996; Gatenby, Parker, & Neves 1997). However, captive breeding of freshwater mussels for conservation are still hindered by the lack of detailed knowledge of the diet and feeding mode of juveniles, with few studies available describing changes in their feeding morphology during breeding (Kovitvadhi et al., 2007; Lasee, 1991; Lima et al., 2006; Schartum et al., 2016; Trump, 2010).

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 (Araujo, Quirós, & Ramos, 2003; Bamhart, 2006; Beck & Neves, 2003; Eversole, Stuart, & Brume, 2008; Eybe, Thielen, Bohn, & Sures, 2013; Gatenby et al., 1996, 1997; Gatenby et al., 2003; Gum, Lange, & Geist, 2011; Guyot, 2005; Henley, Zimmerman, Neves, & Kidd, 2003; Hudson & Isom, 1984; Jones, Mair, & Neves, 2005; Kovitvadhi et al., 2006, 2007; Kovitvadhi, Kovitvadhi, Sawangwong, & Machado 2008; Liberty, 2004; Liberty, Ostby, & Neves, 2007; Malo, 2012; OBeirn, Neves, & Steg, 1998; Schmidt & Vandré, 2010; Thielen, 2011).

In this study, using information from the literature and our personal experience in freshwater mussel captive care, the anatomical feeding organs of M. margaritifera and U. marcus juveniles were studied. We investigated the high level of early juvenile mortality observed in natural (and captive) habitats, in which both pedal and filter feeding occur (Araujo, Feo, Pou, & Campos, 2015). Using juveniles of two species of endangered freshwater mussels belonging to two different families, Margaritiferidae and Unionidae, we aimed to test two hypotheses. Our first hypothesis is to check if young juveniles experience another metamorphosis that causes a change from deposit feeding by the ciliated foot to suspension feeding. Our second hypothesis is that, regardless of the availability of food, many juveniles at this stage are unable to successfully transition feeding modes, thus resulting in high mortality.

2 | MATERIAL AND METHODS

Juveniles of Margaritifera margaritifera (Linnaeus, 1758) were collected from infections of 0+ Salmo salar L., in a mussel rearing facility in Galicia (NW Spain) with glochidia specimens from the Arnego River (Ulla basin). The metamorphosis process in M. margaritifera lasts
between six and nine months. After six months, the water temperature of the infected fish was increased incrementally from 10 to 18 °C (Eybe, Thilen, Bohn, & Sures, 2015), and juveniles emerged between 29th March and 8th April 2015. Four hundred juveniles were maintained in a box filled with 475 ml of river water and 25 ml of detritus without substrate. Juveniles were fed algae once weekly during a water exchange. The algae consisted of 120 µl of Shellfish Diet 1800 (Isochrysis sp., Pavlova sp., Thalassiosira weissflogii (Grunow) and Tetraselmis sp., with a diameter of 4–20 µm) and 200 µl of Nanochloropsis sp. (1.5–2 µm) suspended in 10 L of river water (Eybe et al., 2013; Scheder, Lercheegger, Jung, Csar, & Gumpinger, 2014). The boxes were kept in a conditioning cabinet at a constant temperature of 17 °C. The algal diet was doubled after the first month and tripled after six months.

Juveniles of Unio mancus (Lamarck, 1819) were collected from infections of Barbus meridionalis Risso, with glochidia specimens from Banyoles Lake (Spain); they emerged on 12 June 2015. The metamorphosis process in U. mancus lasts between 7 and 28 days (Araujo et al., 2015). Juveniles were then maintained in a PVC cylinder measuring 16 cm in diameter and 17 cm in height (total volume capacity of 2.2 L) without sediment but with a 200 µm mesh at the bottom. This cylinder was suspended within another receptacle (33 × 46 × 44 cm) filled with 70 L of water from Banyoles Lake (Spain). This culture was fed three times weekly with 2 mg/L of frozen Easy Reefs (1/3 Butcher 12–14 µm + 1/3 Phaeodactylum tricornutum Bohlin 2–6 µm). The outer receptacle was artificially aerated, and daily, the inner cylinder was moved up and down five times first to empty the water through the mesh and then to homogenize the inner cylinder water with the surrounding water. Water exchanges of 100% were performed every two days.

Between 5 and 10 juveniles of each species were sacrificed every 10 days until day 90 and then once a month until day 360. Prior to fixation in 2.5% glutaraldehyde (2–24 hr), specimens were first relaxed by slowly adding MgCl2 until the valves opened. If the valves did not open, one valve was broken before dehydration. Specimens were then cleaned with PBS buffer and dehydrated through a graded ethanol series (30–60 min each in 30%, 50%, 70%, 90%, 96%, 100%, and 100% ethanol). Specimens were stored in 100% ethanol until graded into solutions of 100% ethanol and hexamethyldisilazane (HMDS) (15–30 min each in 2:1; 1:1; 1:2 ethanolHMDS).

Scanning electron microscopy analyses focused on the following features: (a) Gill buds and inner gill growth. (b) Inner gill reflection and ventral groove. (c) Mouth. (d) Labial palps. (e) Mantle cilia (border and inner side). (f) Cilia and ventral groove on the foot. (g) Gill buds and the appearance of the external gill.

Additional observations were made in living specimens using a dissecting scope.

3 | RESULTS

After one year of culture, the juvenile mortality of Margaritifera margaritifera was about 90%, while that of Unio mancus was about 40%.

3.1 | Margaritifera margaritifera

The mean length of newly emerged juveniles was 350 µm (Figure 1a and Tables 1 and 2). The most conspicuous characters were the ciliated foot, with a marked ventral groove, the ciliated border of the mantle (Figure 1b–d) and the presence of three filaments and one posterior developing gill bud (Figure 1a). The ventral pedal groove is the byssus groove with the byssus pit (the posterior) and the byssus disc pit (the anterior) at either end (Figure 1b). 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 (Figure 1e); however, they did not bend before day 130, in specimens greater than 1 mm. There were also two diagonal rows of long cilia on the inner side of the posterior mantle wall (Figure 1f).

At 10 days (430 µm) 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 (Figure 1g). Diagonal rows of long cilia on the inner mantle wall were present.

By 30 days (580 µm), there were five gill filaments covered with cilia and ciri (Figure 1h) and one developing posterior gill bud. We observed the anterior adductor muscle and a ciliated mouth (Figure 2a), 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 µm), although there were ciliary tufts on the margin (Figure 2b). 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; Figure 2c). At 180 days, 13 filaments were present on each side of the gill, but the ventral gill groove was still not present (Figure 2d). 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 (Figure 2e,f) and the labial palps were highly discernible (Figure 2f,g). The palps were folded, and the inner sides ciliated (Figure 2g). The cilia and the ventral groove of the foot were shorter than at 100 days (Figure 2e). Two pseudodiaphragms were well-developed at the posterior mantle margin, increasing the separation between the gill chambers (Figure 2h). The area around the inhalant aperture was darkly pigmented in live animals at 200 days. More cilia were present on the mantle margin near the pseudodiaphragm that in other places of the mantle border. The first gill bud of the external gill was present at 210 days.

At day 240 (2 mm), the cilia of the mouth were shorter than at younger stages. Cilia were present on the pseudodiaphragm (Figure 3a) as were external gill filaments (Figure 3b). In adults, these filaments, along with the pseudodiaphragm, will form the gill chambers. At 300 days (2.1 mm), the developing external gill was apparent and possessed bent filaments (Figure 3c). The byssal pit on the ventral foot and ciliary tufts on the posterior roof of the mantle were both present (Figure 3d),
and the papillae of the inhalant aperture were discernible. The pseudo-diaphragm and the borders of the inhalant and exhalant apertures were both ciliated. Ciliary tufts on the mantle border were evident, leaving circular marks (Figure 3e). At day 360 (2.2 mm), the folded labial palps were covered with cilia (Figure 3f), the external gill was better developed and the papillae on the inhalant aperture were more visible. At no time point were muscular connections between gill filaments observed.

**FIGURE 1** *Margaritifera margaritifera*, scanning electron micrographs images of different ontogenetical stages. (a) Ventral view. Anterior part is down (0 days). (b) Ciliated foot with the byssal ventral groove and the two byssus pits (arrows). Anterior part is down (0 days). (c) Cilia at the mantle border (0 days). (d) Detail of the cilia at the mantle border (0 days). (e) Gill filaments (0 days). (f) Diagonal row of cilia at the inner mantle wall (0 days). (g) Inner gill filaments, gill buds (arrow) and long cilia at the posterior mantle margins. Anterior part is to the left (10 days). (h) Cilia and cirri in the inner gill filaments (30 days)
3.2 | Unio mancus

The mean length of newly emerged juveniles was 260 μm (Tables 1 and 2). The ciliated mouth of *U. mancus* was much larger than that of *M. margaritifera*; however, the foot, mantle margin (Figure 4a–c), and gills were very similar in the two species at this initial time point.

At 60 days (1.1 mm), the outer demibranch was reflected, and the ventral groove of the inner gill had formed, and at 70 days, primordial labial palps with cilia were present (Figure 4d). The mantle border was also covered with cilia, although these were shorter than those observed at younger stages.

At 120 days (1.6 mm), the inner gill had 18 filaments. The external gill became evident at 180 days (1.9 mm) and was associated with the posterior part of the inner gill, where a separation between the upper and lower gill chambers will eventually form on the inner wall of the mantle (Figure 4e), rather than a pseudodiaphragm as in margaritiferids. The developing external gill also already had bent filaments.

The papillae of the inhalant siphon were clearly recognizable at 345 days (2.8–3 mm; Figure 4f), resembling spheres on the inner mantle border (Figure 4g). By this time, the overall appearance of the juvenile was similar to that of the adult, although with unfused mantle borders (Figure 4h). The inner sides of the labial palps had ciliary folds (Figure 5a) and a flat external surface with a ciliary flange (Figure 5b). Also at 345 days, 45 inner gill filaments with complete cilia and cirri were present and connected by horizontal muscular bridges (Figure 5c, d). The foot was covered with short cilia, and at 395 days (3.8 mm), the ventral groove only remained in the posterior part (Figure 5e). The exhalant siphon was completely developed (Figure 5f), but the ventral borders of the inhalant siphon were not yet connected.

### Table 1

|                  | 1 day | 10 days | 30 days | 60 days | 80–100 days | 150 days | 180 days | 210 days | 300 days | 360 days |
|------------------|-------|---------|---------|---------|-------------|----------|----------|----------|----------|----------|
| *M. margaritifera* | 350   | 430     | 580     | 780     | 0.8–1       | 1.4      | 1.6      | 1.8      | 2.1      | 2.2      |
| *M. margaritifera* | –     | 460     | –       | –       | –           | –        | –        | –        | –        | –        |
| *M. margaritifera* | –     | –       | –       | –       | –           | –        | –        | –        | –        | –        |
| *M. margaritifera* | –     | –       | –       | –       | 0.4–0.9     | –        | –        | –        | –        | –        |
| *M. margaritifera* | –     | –       | –       | –       | 0.7–0.9     | –        | –        | –        | 0.7–0.9  | 1.1–1.2  |
| *M. margaritifera* | 300–480| –       | –       | –       | –           | –        | –        | –        | –        | –        |
| *U. mancus*      | 260   | 450     | 750     | 1100    | 1.2–1.4     | 1.7      | 1.9      | 2        | 2.2      | 3        |
| *V. iris*        | 300–400| –       | –       | –       | 0.8–1       | 1.7–2.3  | –        | –        | –        | –        |
| *L. ventricosa*  | 227   | 350     | 530     | 889     | –           | –        | –        | –        | –        | –        |
| *U. imbecillis*  | 320   | –       | –       | 800     | 2.1         | –        | –        | –        | –        | –        |
| *H. myersiana*   | 150   | 200     | –       | 1800    | 2.5         | 24       | –        | –        | –        | 85       |

This article.

Malo (2012).

Schmidt and Vandre (2010).

Lavictoire et al. (2016).

Schartum et al. (2016).

Gatenby et al. (1997).

Lasee (1991).

Trump (2010).

Kovitvadhi et al. (2007, 2008).

### Table 2

|                  | Inner gill cirri | Basket | Inner gill bent | Ventral groove | Labial palps | Outer gill | Inhalant papillae |
|------------------|------------------|--------|-----------------|----------------|--------------|------------|------------------|
| *M. margaritifera* | 0                | 10     | 130             | 180            | 180          | 240        | 300              |
| *M. margaritifera* | –                | –      | 1.1–2.2 mm*     | 2.2–4.5 mm*    | –            | 4.5 mm*    | –                |
| *U. mancus*       | 0                | 10     | 40–60           | 60             | 70           | 180        | 300              |
| *V. iris*         | –                | 14     | –               | –              | –            | –          | After 272         |
| *L. ventricosa*   | 0                | –      | After 56        | –              | 2            | –          | –                |
| *U. imbecillis*   | 0                | 7–28   | 113             | –              | 3            | 130        | 175              |
| *H. myersiana*    | 0                | 10     | 30              | After 50       | –            | 90         | 50               |

For references, see in Table 1.

*No data about age.*
Pedal feeding is likely the first method used in young juvenile-staged benthic bivalves as an evolutionary constraint of a plesiomorphic character (Reid et al., 1992; Stasek, 1963). This feeding method has been observed in some juvenile and small adult marine and freshwater bivalves [e.g., *Macoma balthica* (L.), *Corbicula fluminea* (Muller) and *Kurttiella bidentata* (Montagu)] (Caddy, 1969; Reid et al., 1992; Schartum...
et al., 2016). The transition to suspension feeding, at least in phytoplankton fed juvenile freshwater mussels or in the wild, seems to be a gradual process (Beck & Neves, 2003; Lasee, 1991; Trump, 2010; Yeager et al., 1994) related to the development of new organs, particularly of two basic bivalve feeding organs, the gills and the labial palps (Galbraith, Frazier, Allison, & Vaughn, 2009; Kellogg, 1915; Schartum et al., 2016; Stasek, 1963). Once these two structures are well-developed, definitive filter feeding is initiated. Our results are in agreement with other authors (Schartum et al., 2016; Trump, 2010): the anatomical development of feeding structures is a result of an overall increase in an animal’s size rather than strictly as a function of age (Tables 1 and 2).

Based on the observation of the living specimens, newly emerged juveniles of both *M. margaritifera* and *U. mancus* first feed by pedal feeding using the cilia of the foot and of the mantle margin, similar to what has been observed in other post-metamorphosed juveniles (Kovitvadhi et al., 2007; Lasee, 1991; Trump, 2010; Uthaiwan, Chatchavalvanich, Noparatmaraporn, & Machado, 2001). These observations, using dissection scopes and analyzing scanning electron micrographs, suggest that the initiation of suspension feeding begins when the cilia of the posterior parts of the inner gill filaments join to form a virtual hole for water current. In effect, the gill filaments build a basket with a dorsal and a ventral chamber with a ciliated posterior opening. This opening allows water to enter, facilitated by the mantle and gill cilia,

![FIGURE 3 Margaritifera margaritifera, scanning electron micrographs images of different ontogenetical stages. (a) Ciliated pseudodiaphragm (240 days). (b) Inner right gill and the beginning of the outer gill (arrow). Anterior part is to the right (240 days). (c) Lateral view. See the labial palps (arrow) and the mouth everted. Anterior part is to the left (300 days). (d) Ciliary tufts at the posterior mantle roof (300 days). (e) Cilia and ciliary marks at the mantle margin (330 days). (f) Ciliary folds at the inner surface of the labial palps (360 days)](image-url)
similar to the ulterior inhalant aperture (or siphon) in the adult. In this new mode of suspension feeding, a posterior-anterior current, opposite to the one used for pedal feeding (i.e., anterior-posterior), is generated. Furthermore, the function of this basket improves after the formation of the labial palps and the corresponding ventral groove between the two-inner gill demibranchs, which direct food into the mouth, and as suspension feeding improves, the cilia on the foot become shorter and less numerous.

In *M. margaritifera* and *U. mancus*, as in *Lampsilis ventricosa* (Barnes) (Lasee, 1991), we did not find evidence of an elaboration of the byssal...
thread; nevertheless, in both species, the two byssus pits on the ventral pedal groove are present in newly metamorphosed juveniles, whereas in L. ventricosa, the byssal complex forms between 4 and 8 weeks post-metamorphosis (Lasee, 1991). Although pedal feeding is facilitated by the ciliated foot and mouth, initial suspension feeding is initiated by currents made by the lateral filaments on the posterior mantle margin and by the cilia from the left and right gills forming the gill basket (Gatenby et al., 1996; Lasee, 1991; Schartum et al., 2016; Trump, 2010). Rejected food likely reaches the outside current by the cilia forming diagonal rows on the inner mantle wall and those on the posterior mantle roof and anterior mantle margin (Beninger, Veniot, & Poussart, 1999). In Villosa iris (Lea) juveniles 14 days post-metamorphosis, pedal and filtering feeding are both utilized but always using the pedal cilia and not the posterior apertures (Yeager et al., 1994). These authors concluded that the cilia of the foot create the filtering current, although in this case, the foot does not extend as it

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**FIGURE 5** *Unio mancus*, scanning electron micrographs images of different ontogenetical stages. (a) Ciliated folds in the inner side of the labial palps (345 days). (b) Outer side of a labial palp. See the cilia at the margin (arrow). (c) Filaments in the inner gill. See the connections between filaments (395 days). (d) Filaments in the inner gill. See the connections between filaments (395 days). (e) Foot, inner gills and labial palps. Anterior part is to the right (395 days). (f) Exhalant (above) and inhalant siphons (395 days).
does during pedal (deposit) feeding (Yeager et al., 1994). However, as observed in 500–800 μm larvae of the marine bivalve Pecten maximus (L.), particle capture is likely inefficient (Beninger, Dwiono, & Le Pennec, 1994). In 60-day-old Hiatropsis myersiana (Lea) juveniles, both the ciliated foot and the cilia on the mantle and gills are used to transport phytoplankton (Kovitvadhi et al., 2006, 2008). The development of inner gill frontal cilia and laterofrontal ciri is important to form the gill basket for initial filter feeding (Schartum et al., 2016). In L. ventricosa, H. myersiana and Utterbackia imbecillis (Say), the laterofrontal ciri were always present in recently emerged juveniles, (Kovitvadhi et al., 2007; Lasee, 1991; Trump, 2010; Wright, 1995). The broadening of the distal ends of the gill filaments between 7 and 28 days in U. imbecillis (Trump, 2010) and at 56 days (890 μm) in L. ventricosa (Lasee, 1991) sufficiently forms a basket for particle capture during gill bud and reflected gill stages. During metamorphosis in P. maximus juveniles (300–900 μm of shell length), a similar process of gill basket formation occurs while the velum (larval feeding organ) disappears; but in this case, the inner gill has not yet reflected (Beninger et al., 1994). Nevertheless, formation of both the inner demibranch ventral groove and the pedal papillae is required for successful filter feeding (Kellogg, 1915). Once these structures are well developed, and filter feeding becomes the main (or only) feeding mechanism, juvenile mortality likely decreases. Based on our observations here, filter feeding occurs around 180 days (1.6 mm) in M. margaritifera with the formation of the labial palp ridges and cilia and the ventral groove of the inner gill. However, other authors have reported this feeding change occurs later (2.2 mm) in this species (Schartum et al., 2016). In U. mancus, the palp primordia and the transition to filter feeding occurs at 70 days (1 mm). The initial small folds of the labial palps were present at two days in L. ventricosa (Lasee, 1991) and three days in U. imbecillis (Trump, 2010), but in the latter species, the ridges did not appear until 130 days (Trump, 2010).

We have observed the reflection of the outer demibranch of the inner gill at 130 days (1.2 mm) in M. margaritifera and between 40 and 60 days (0.8–1 mm) in U. mancus. In these species, there is a clear relationship between effective filtering mechanisms and inner gill reflection, as has also been hypothesized in other freshwater (Herbers, 1914; Kovitvadhi et al., 2007; Trump, 2010) and marine (Beninger et al., 1994; Cannuel et al., 2009) species. In M. margaritifera, the inner gills of juveniles between 0.8 and 1.1 mm in length initially have I-shaped filaments, bending to a final V-shaped form in juveniles between 1.1 and 4.5 mm, whereas the outer gill filaments are already bent from initial growth (Schartum et al., 2016). Inner gill reflection occurs at 7 weeks in Anodonta cygnea (L.) (Herbers, 1914), 30 days in H. myersiana (Kovitvadhi et al., 2007) and 113 days in U. imbecillis (consisting of 15–18 filaments; Trump, 2010). However, by 56 days in L. ventricosa, gill reflection had still not been observed (Lasee, 1991). The increase in the number of gill filaments during growth improves filter feeding. In our study, M. margaritifera juveniles had 4–6 filaments 50 days post-emergence, 8–12 up to 120 days, and more than 20 at 210 days. One filament is added for every 123 μm of shell length (Schartum et al., 2016). In U. mancus, 18 filaments were present at 120 days and 45 at 345 days. In L. ventricosa, there were four pairs of filaments at 21 days and 5–10 pairs at 28 days (Lasee, 1991). For U. imbecillis, the data are unclear: one study reported 23 pairs of filaments in a 74-day old, 5.1 mm juvenile (Hudson & Isom, 1984), while another reported 23 filaments in an 158-day old, 2.1 mm juvenile (Trump, 2010). The outer demibranch, which appears to be the last structure to form prior to definitive filter feeding, forms when species reach approximately 2 mm in shell length. Different species reach this length at different times: 90 days in H. myersiana (Kovitvadhi et al., 2007), 130 days in U. imbecillis (Trump, 2010), 240 days in M. margaritifera (this study) [although it has also been reported to occur at 4.5 mm (Schartum et al., 2016)], and 180 days in U. mancus (this study). However, in another M. margaritifera study, it has been reported that the inner gill begins to bend at 16 months and the complete gills (inner and outer) are formed at three years (Lavictoire, Moorkens, Ramsey, Sinclair, & Sweeting, 2015).

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

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

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

Freshwater mussels are one of the most imperilled animals in the world, and one of the most fragile stage in a mussel’s life is as recently emerged juveniles (Archambault et al., 2014; ASTM, 2013; Augspurger et al., 2007; Strayer & Malcom, 2012). Based on these facts, and to improve artificial rearing of these molluscs, a better understanding of the natural diet and the development of the feeding organs of newly emerged juveniles is crucial (Kovitvadhi et al., 2006, 2007; Lasee, 1991; Lima et al., 2006; Schartum et al., 2016; Trump, 2010; Tucker, 1927; Uthaiwan et al., 2001). Given the high level of juvenile mortality reported for U. mancus and M. margaritifera in natural habitats with no food issues (Araujo et al., 2015; Eybe et al., 2015; Hastie & Young,
2003), early juvenile mortality can be 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.

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