PRELIMINARY CONSIDERATIONS FOR FRESHWATER MUSSEL REPRODUCTION AND POSSIBLE APPLICATION FOR EXTENSIVE REARING IN ITALY

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*Abstract The present work is aimed to investigate the physiology of reproduction of the freshwater mussel (*Anodonta anatina*) for future extensive farming. In this paper the knowledge of life history traits and reproductive aspects is considered as the first step for obtaining juveniles for rearing mussels in captivity. In the first part of this research 2,285 specimens of* A. anatina *were placed in 5 sites in the Avigliana Lakes (NW Italy) and reared for a 2 years period. A sample of 180 specimens was collected in the Lake in November 2003 and 57% of mussels were gravid. The brooding period started from late August to the next spring. Histology performed on 11 individuals collected in April and late June 2004 clearly showed either females or hermaphrodites, confirming that* A. anatina *sexual strategy is characterised by a high plasticity. In order to obtain juveniles in captivity, a correct assessment of glochidial maturity was necessary. In April hooks of glochidia were completely developed and the adductor muscle was clearly visible. A partial extrusion of the mantle before glochidial release can be considered a good indicator of glochidial maturity, while water temperature and glochidial snapping activity are less reliable. Four Pumpkinseed (*Lepomis gibbosus*), 8 Bleaks* (Alburnus alburnus*) and an European Perch (*Perca fluviatilis*), were infested with glochidia in aquarium, confirming that* A. anatina *is a low selective host fish species and that host fish parasitism can be easily performed in artificial conditions with fish species commonly found in Avigliana Lakes.*

A case of direct development to a juvenile A. anatina *without the parasitic stage on fish was also observed: a metamorphosed glochidium (juvenile) was found in the genital duct together with sub-triangular glochidia of smaller size. This is the first observation of direct development reported for an European unionid.*

Freshwater mussel farming could be interesting activity for the future for bacteria filtration in integrate productions and as alternative feedstuffs for farmed fish nutrition. This preliminary work is the first step for larval rearing and potential future of European freshwater mussel farming.

Key words Freshwater bivalves, mussels farming, unionids, Anodonta anatina.

INTRODUCTION

Together with macrophytes (Thiebaut & Muller, 2004), freshwater mussels play a major role in reducing eutrophication in aquatic systems (Strayer, Hunter, Smith & Borg, 1994; Vaughn & Hakenkamp, 2001), actively filtering phytoplankton, bacteria and organic debris (Nichols & Garling, 2000; Stuart, Eversole & Brune, 2001; Ward, Sanford, Newell & Macdonlad, 1998). Ravera & Sprocati (1997) found encouraging evidence of conversion on soft tissues of algae filtered together with bacteria and organic suspended matter. Freshwater mussels have been used for filtering water from commercial aquaculture farm (Soto & Mena, 1999) and partitioned aquaculture systems (Stuart et al., 2001). Unionids are freshwater bivalves present in Europe; considering their large size they are potential candidate for aquaculture. Freshwater mussel farming is currently developed in far east, principally for freshwater pearls production, while in other countries, like North America, this activity is aimed to conservation biology. In this study freshwater mussel farming is oriented to improve the water quality through bacteria filtration. The presence of pathogen bacteria in water prevents bathing activities in the lakes; the cages with freshwater mussels could decrease the amount of bacteria and consequently allow the use of the lake for recreational activities. Furthermore, a recent paper (Sicuro, Panini, Palmegiano & Forneris, 2005) indicates the potential utilisation of freshwater mussel meal as potential alternative feedstuff for fish nutrition in aquaculture. The main difference with marine bivalves biology is larval development, since Unionids larvae are parasitic, not planktonic as marine bivalves; the *A. anatina* larva is called glochidium and it pass over a parasitic stage on fish host during early development.

Observations of the main features of *A. anatina* biology, i.e. population sex ratio, glochidial release and host fish parasitism characteristics, have been performed as preliminary studies useful to design plans aimed at efficiently rearing juveniles in captivity. A great deal of attention has been focused on threatened Unionoids species from North America (Jones, 1950; Neves,

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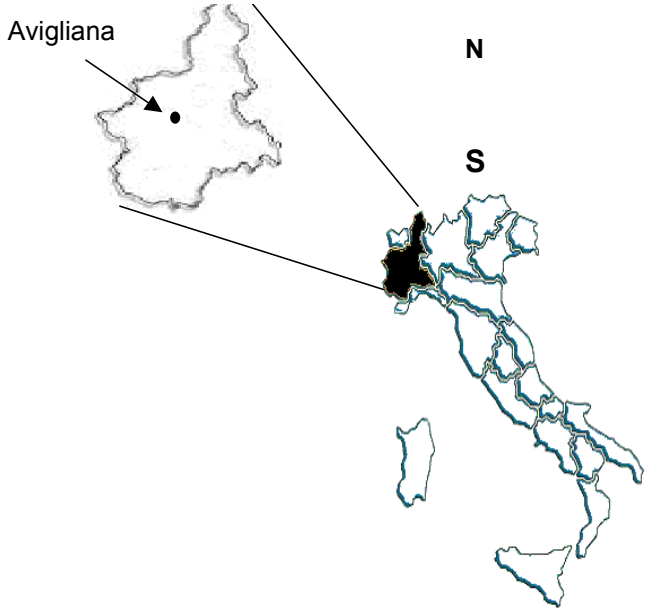

Fig. 1 Study area

1999; Williams, Warren, Cummings, Harris & Neves, 1993) and Europe (Bouchet, Falkner & Seddon, 1999). Although *A. anatina* is not considered a threatened resource, many studies on its reproduction, morphology and population dynamics have been reported in the past decades (Aldridge, 1999; Giusti, 1973; Kat, 1983; Kat, 1984; Negus, 1966; Pekkarinen & Hasten, 1998). The culture of juveniles bivalves starts from mature glochidia collected from gravid females (Hove & Neves, 1994; Jones & Neves, 2002; O'Dee & Watters, 1998; Yeager & Neves, 1986), by inserting a water - filled syringe into the gills and flushing their contents.

The first step in our study was to collect information about the abundance of gravid females in our site, Avigliana Lakes (NW Italy). We also studied the main features of developing and mature glochidia (Wächtler, Dreher -Mansur & Richter, 2001) and hence the correct timing of glochidial collection, since immature glochidia may result in a low infestation of host fish (Barnhart, 2002).

In similar researches on reproductive biology of Unionids, after glochidia collection, suitable fish species are usually infested (O'Dee et al., 1998). We evaluated the feasibility of using typical fish species in Avigliana Lakes, although *A. anatina* is known as a low host selective species (Pekkarinen et al, 1998). We also considered water temperature as a factor affecting the maturation of glochidia and the timing of natural glochidial

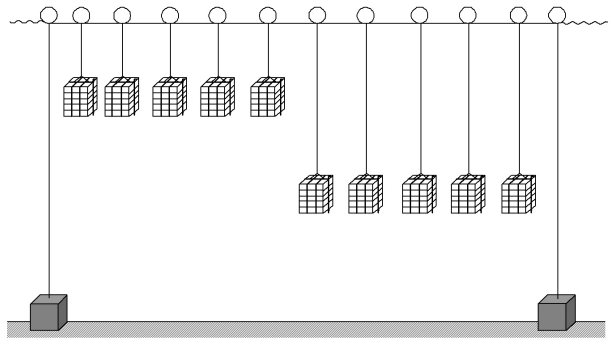

Fig. 2 Rearing lines conceived for cage culture of *A. anatina*. Cages are suspended at 1.5 m and 5 m of depth.

release. The sexual strategy of *A. anatina* in Avigliana Lakes was also investigated. It is common knowledge that this species is characterised by a high sexual strategy plasticity (both monoecious and dioecious populations), depending on population density (Aldridge, 1999), habitat (Kat, 1983) or infestation by trematodes (Kat, 1984). The aim of this work is consider the artificial reproduction for *A. anatina* which is the first step for future freshwater mussel farming.

Materials and Methods

In November 2003, a total of 2,285 adult mussels (*A. anatina*) were collected in shallow waters along the perimeter of the Big Avigliana Lake (N-W Italy) (fig. 1). These mussels were collected and placed in 5 sites in the lake using a floating cages system (Panini, Sicuro & Forneris, 2005) similar to marine bivalve farming methods (fig. 2). The mussels were stored for a period of 2 years in the lake, suspended at 1.5 m and 5 m of depth. Considering the positive results obtained from cage culture of adult mussels, the second step for freshwater mussel farming was the artificial reproduction. The maximum shell length, the height at the umbo and the maximum width were measured on 180 bivalves with a Vernier calliper accurate to 0.5 mm. Mussels were opened by cutting posterior and anterior adductor muscles and the number of gravid females was recorded. Gravid (G) and non - gravid (NG) mussels were distinguished by observing outer gills, highly developed as a marsupia in gravid females (Bauer, 2001a). The viscera and the genital duct were examined for sex determination.

The presence of oocytes in the viscera and in the genital duct (longitudinally dissected) within NG mussels was evaluated with a light microscope. Twenty - three NG specimens were taken to the laboratory and stored at -20° C. Since hermaphroditism in *A. anatina* population was suspected, 11 bivalves were collected in April 2004 (n=3) and in late June 2004 (n=8). They were fixed either in Bouin's solution or 10% formalin and preserved in 70% alcohol for histological examination. Soft tissues of fixed mussels were dissected in the mid - sagittal plane and embedded in paraffin. Slices of 10 µm of thickness were cut and stained with the Mayer haematoxylin-eosin method.

LARVAL MATURATION AND HOST FISH SELECTION

During the brooding period, glochidial maturation was studied by collecting gravid mussels in November 2003, March and April 2004. During previous observations in February 2003, a small amount of glochidia was withdrawn with a Pasteur pipette from the posterior part of the outer gills, by gently opening the valves, and the snapping activity was observed. The size (length and height following Hoggarth, 1999) and the shape of glochidia collected from outer gills of each female were measured to evaluate their maturation stage (Giusti, Castagnolo, Moretti Farina & Renzoni, 1975; Wächtler et al., 2001).

The time of year for glochidial release from gravid females was observed in a closed circulation aquarium. In November 2003, twenty-three gravid females were collected, they were placed in a 225 l aquarium, at a density of 30 kg/m^3, and they were reared until the glochidial release occurred. The aquarium was filled with unfiltered water from the lake and the water was changed every two weeks. Water was oxygenated with air stones and two 220 l/h flow rate pumps circulated the water. Aquarium was maintained at natural thermo period and water temperature was monitored every two weeks from January to March and every 3-4 days from March until the end of the experiment. The occurrence of glochidiosis on three fish species commonly found in the Lakes was tested. Four Pumpkinseed (*Lepomis gibbosus* L.), eight Bleaks (*Alburnus alburnus* L.) and one European Perch (*Perca fluviatilis* L.) were collected with a net and placed in the aquarium during glochidial release. Parasitism was confirmed by visually observing the attachment of glochidia on the fins and gills of the fishes.

RESULTS

Of the 180 specimens sampled in the Lake in November 2003, 57% were gravid. *A. anatina* gravid females were easily distinguished from non-gravid individuals, since they showed external gills fully filled with glochidia. The occurrence of hermaphroditic tissue was observed in three specimens collected in June 2004 (Fig. 3a). Female acini in early active stage (Fig. 3b) and male acini in late active stage (Fig. 3c) were sparsely distributed in the visceral mass among gut loops. No specimens were collected that only had male gonad. The size distribution of the population showed that the shell length was between 7.5 cm and 15.2 cm, with an average of 10.4 ± 1.4 cm. No smaller specimens were found. The shell height and the shell width had a mean value of 5.6 ± 0.7 cm and 3.3 ± 0.6 cm, respectively. The shell length, the shell height and the shell width have a typical normal distribution.

GLOCHIDIAL MATURATION

The brooding period was expected to be from late August to the next spring, as reported by Aldridge (1999). In November glochidia were completing their development, reaching a mean size of 327 µm in length and 347 µm in height (Fig. 4a). They had rudimentary hooks, in order to attach themselves to host fish (Wächtler et al., 2001). Glochidia collected from gills in February quickly snapped for about 60 seconds without addition of sodium chloride crystals. In March 2004 glochidia were 348 µm and 350 µm in height and length, respectively (Fig. 4b). One month late, their size had increased to an average of 388 µm for both the height and length, with a maximum size of 400 µm x 410 µm (Fig. 4c). Hooks were completely developed, the adductor muscle was clearly visible and the colour of the valves was reddish orange. Interestingly, a glochidium of 370 µm x 370 µm in size (Fig. 4d) was found in the genital duct together with two specimens showing the typical juvenile aspect, having lost the glochidial sub-triangular shape and measuring 310 µm x 330 µm (Fig. 4e).

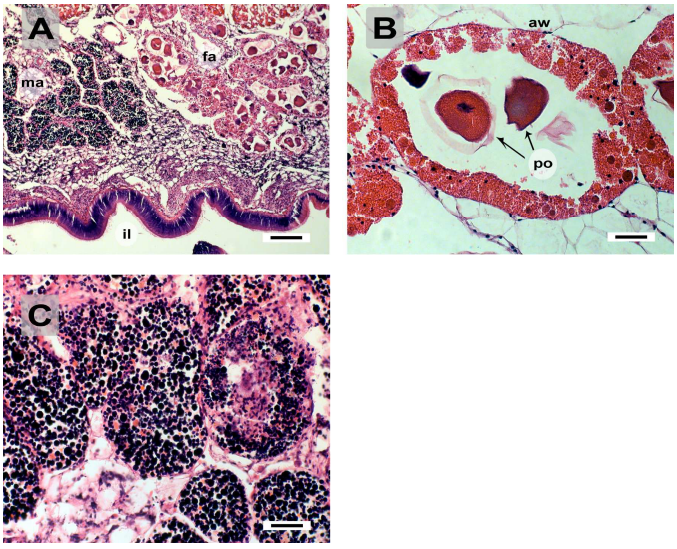

Fig. 3 **A** Occurrence of hermaphroditism in *A. anatina* collected in June 2004 in the Big Avigliana Lake. Bar equals 200 µm. **B** Female acinus in early active stage in a hermaphrodite specimen. Note thick acinar wall. Bar equals 50 µm. **C** Male acinus in late active stage in the same hermaphrodite specimen. Bar equals 50 µm. Abbreviations **aw**, acinar wall; **fa**, female acini; **ma**, male acini; **il**, intestinal lumen; **po**, primary oocyte.

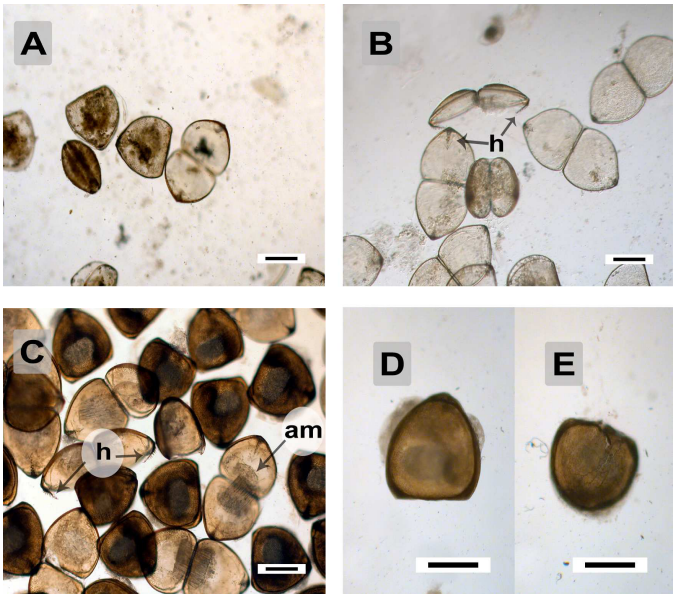

Fig. 4 Glochidial maturation during the brooding period. **A** Glochidia in November 2003. **B** Developing glochidia in March 2004. **C** Completely developed glochidia in April 2004. **D** A developed glochidium compared with a juvenile mussel (e) found in the genital duct of a same specimen in April 2004. Bars equal 200 µm. Abbreviations **am**, adductor muscle; **h**, hooks.

Release of glochidia and host fish infection

The aquarium experiment begun in November 2003 showed that gravid females started to discharge glochidia on the 21st of April, when water temperature was 15° C (Fig. 5). During the glochidial release, gravid mussels partially extruded the mantle (Fig. 6a). Glochidia were released from the mantle apertures with pumping movements either individually or in groups, agglutinated by a mucous secretion (Fig. 6b). The main glochidial release was observed in the females on April the 21st, but other smaller events were detected in the following 12 days. During this period, several females were found with half - empty outer gills. The occurrence of glochidiosis was observed on the fins and gills of all the fishes reared in the aquarium.

Discussion

This experimentation showed that in Avigliana Lakes the rate of gravid females was higher (57%) when compared to other *Unio* and *Anodonta* species (Bauer, 1998). This could primarily suggest that environmental conditions are favourable for *A. anatina* reproduction. This finding is encouraging for successful mussel farming in Avigliana Lakes in artificial conditions. From the beginning of the research project, some thousand of bivalves were collected, showing that *A. anatina* population is mostly composed by adult specimens (no individuals < 7 cm were collected); however our observation does not necessarily indicate that the population is in decline, since juvenile mussels live almost completely burrowed into the sediment during the first years of life (Wächtler et al., 2001), making their collection difficult. Considering the low natural occurrence of juveniles, first objective of freshwater mussel farming is juvenile specimens production. The improvement of number of juveniles is crucial step for artificial reproduction.

Histological sections of gonads showed that three out of 11 specimens were hermaphrodites, with female and male acini in early active and in late active stage, respectively. Life history and sexual phenomena among the Unionidae are characterised by a great plasticity (Henley, 2002). For instance, only dioecious *A. anatina* specimens were detected in Wicken Lode (Aldridge, 1999), while Kat (1983) found that hermaphroditism

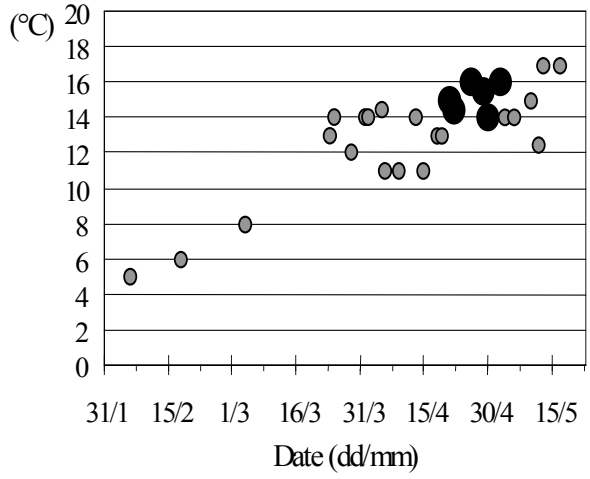

Fig. 5 Temperature in the aquarium during the rearing of gravid mussels (in grey) and the glochidial release (in black).

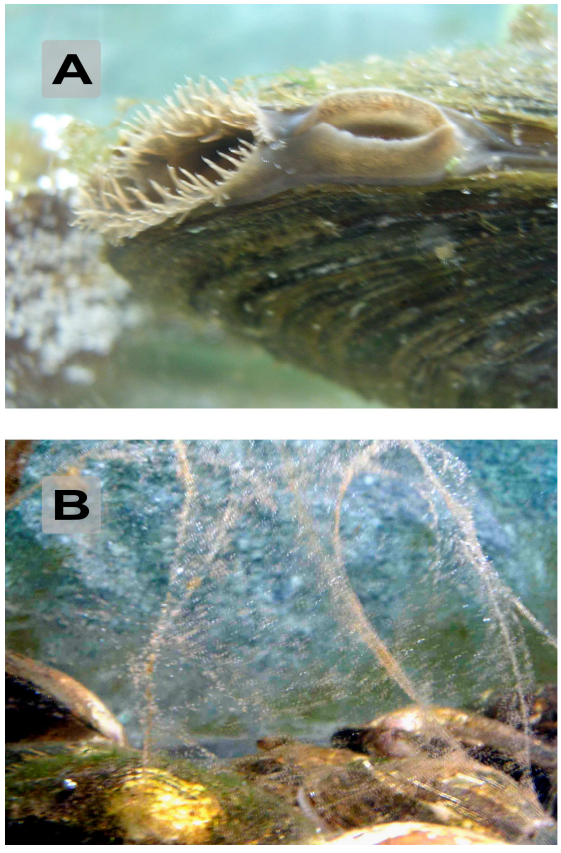

Fig. 6 Glochidial release in aquarium on April the 21st. **A** Partial mantle extrusion during the discharge of glochidia. **B** Simultaneous release of glochidia in females in aquaria. Note the glochidia agglutinated by a mucous secretion.

occurred among *A.*(now *Utterbackia) imbecillis* specimens infested by trematodes. *A. anatina* is considered an occasional hermaphrodite (Pekkarinen & Hasten, 1998); in this kind of sexual strategy, male and female gonads are mixed in the visceral mass without a precise organisation (Grande, Araujo & Ramos, 2001; Henley, 2002), as it was observed in this study. The occurrence of occasional hermaphroditism can vary across populations in *Anodonta peggyae* (Heard, 1975) and *Margaritifera margaritifera* (Grande et al., 2001). Hermaphroditism occurs especially in lentic systems such as Avigliana Lakes, where current dispersal of sperm to females is less successful (Kat, 1983) thus increasing the probability of fertilisation.

From the applicative point of view, glochidial maturity is an important aspect in life cycle of freshwater mussels, since an incorrect timing of glochidial collection may negatively affect the attachment to host fish (Barnhart, 2002). Part of this work is aimed at finding some reliable indicators of glochidial maturity. *A. anatina* has a long breeding period (bradytictic), carrying mature glochidia from late summer until the following spring when they are released. The brooding period lasted until early April, as was found by Negus (1966), but contrary to the results of Heard (1975).

Within the Unionidae, glochidial discharge may be induced by the presence of the host fish (Haag & Warren, 1999). In the present experiment, *A. anatina* females started to release glochidia before fish were introduced in the aquarium, contrary to Bauer (2001b) results. This suggests that gravid females may not necessarily discharge glochidia in presence of a host fish, thus reducing the chance of glochidia to complete their development. The mucous structure in which the most of glochidia are grouped increases the time of suspension in the water column, thus increasing the chance to attach on host fish. Furthermore, the observation of half-empty gills confirms that *A. anatina* females do not discharge all their glochidia in one time, as similarly observed for *Lampsilis cardium* (Haag & Warren, 1999). In the wild, this reproductive strategy may assure higher success of glochidiosis; but in captivity this can be a restrictive condition, since glochidia could be artificially collected when they have not yet completed their maturation.

It is important to correctly estimate the timing for glochidial collection, in order to diminish the collection of immature glochidia. Another factor affecting glochidial release is the presence of stress-inducing conditions, such as hypoxia (Aldridge & McIvor, 2003). Under stress, females tend to evacuate their glochidia prematurely, reducing the chance of glochidiosis to occur.

Mantle-display is described in literature for Lampsilinae and it can be considered an adaptive mechanism to draw fishes (Haag & Warren, 1999). Although mantle-display in Anodontinae subfamily has never been observed, before discharging glochidia in the water, females partially extruded the mantle. This phenomenon can be considered a good indicator for the assessment of the correct time for glochidial collection in a facility setting. Release of glochidia in the aquarium was synchronised within the specimens. Synchronisation of release of glochidia has been well documented for *M. margaritifera* (Hastie & Young, 2001). The simultaneous release of glochidia in many females means that *A. anatina* glochidia in the gills are likely to reach maturity at the same time. Then a high number of glochidia can be obtained simultaneously and juvenile rearing can be performed in a small number of tanks or aquaria in which there are no differences in glochidial developmental stage. This fact can be considered an interesting result for future larval rearing of freshwater mussels.

The maturation and the release of glochidia are also strictly dependent on water temperature (Giusti et al. 1975; Hastie & Young, 2003). Gravid *A. anatina* females held in the aquarium started to discharge glochidia in early April, when the water temperature was 15° C. Water temperature should not be considered the only parameter affecting glochidial maturity, since geographical variations also occur; for instance *A. anatina* gravid females from the region of Wichen Lode released glochidia in March (Aldridge, 1999).

One of the ripeness characters is the snapping activity of glochidia before the attachment to the host fish (Wächtler et al., 2001). *U. pictorum* glochidia are considered mature when they have spined hooks and snap actively (Aldridge & McIvor, 2003). Observations on glochidia collected from a gravid female gills in February 2003 showed they were already actively snapping two months before their release. Glochidia collected in November 2003 already had spined hooks, although they still had a rudimentary shape, confirming that glochidia were not ripe; conversely, hooks looked well developed in April 2004. The size of glochidia reached an average of 388 µm, with the maximum value of 410 µm in height. This observations are in disagreement with those presented by Wächtler et al. (2001), who reported 340 µm x 360 µm in size for mature glochidia. Since glochidial size increases in the last brooding period, this may be a reliable indicator of glochidial maturity.

An interesting observation was the presence of two metamorphosed glochidia, i.e. juveniles, in the genital duct of one female collected in April 2004. The shape and the size of the juvenile, compared to the surrounding glochidia, were dramatically different and they were supposed to be the most important features indicating that metamorphosis was reached. This suggests that the juvenile stage was reached bypassing the parasitic period on host fish, as reported for some North - American species (Wächtler et al., 2001). The presence of metamorphosed glochidia either in the gills or in the genital duct has not previously been reported for an European Unionid. The metamorphosed glochidia are interesting for future larval rearing because they don't necessitate of host fishes to complete their development. However at the rate observed in this study, the parasitic stage could not be replaced.

Due to the high amount of organic debris in the bottom of the tank, data on the rate of successfully metamorphosed glochidia are not available. On the other hand *L. gibbosus*, *A. alburnus* and *P. fluviatilis* are commonly considered host fish species for *A. anatina*, as widely reported by Pekkarinen & Hastén (1998); since these three species are very common in Avigliana Lakes, host fish parasitism could be easily performed in captivity. Until this moment in our experimentations the rearing activities with freshwater mussels have been focused only on adult stocking in cage; these preliminary results indicate a possible methodology to get juveniles mussels for future rearing.

The rearing of freshwater mussels could be interesting for bacteria filtration and consequently improving recreational activities in the Lakes. After 2 years of researches with a freshwater mussel pilot rearing system, the Regional Agency for Environmental Protection permitted to restore bathing activities in the Avigliana Lake

that was not allowed for 15 years. At present, there is no complete scientific demonstration of effect of *A. anatina* on pathogen bacteria, but a study on *Unio mancus* and *A. cygnea* has shown that bacteria and organic suspended matter are filtered together with algae and converted in soft tissue (Ravera & Sprocati, 1997). The bivalve filtering activity was demonstrated in eutrophic lagoon waters (Nakamura & Kerciku, 2000) and in different models of bioremediation of eutrophication due to shrimp culture (Hopkins, Hamilton, Sandifer & Browdy, 1993; Jones, Preston & Dennison, 2002) and salmon culture (Soto & Mena, 1999).

Biological filtration of polluted sites such as integrated production could be an interesting biological reason for freshwater mussel rearing. Integrated farming of salmon in cage and blue mussels is already developed in Norway. The soft tissues of mussel could be also used in fish nutrition (Sicuro et al., 2005) and the mussel shell in laying hens (and/or other monogastric animals) nutrition as calcium source.

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