The Giant Freshwater Pearl Mussel *(Margaritifera auricularia)* Handbook

Volume 1 – Synopsis on the current scientific literature

by Joaquin Soler, Rafael Araujo, and Karl M. Wantzen


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Frontispiece: *Margaritifera auricularia*, photographed (c) by Philippe Jugé

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Outline:

The Giant Freshwater Pearl Mussel (*Margaritifera auricularia*) is one of the rarest invertebrate species worldwide. This two-volume book aims to bring together all the so far available information on the species. Both volumes are independent books, although they can be seen as complimentary, giving scientific and technical information. They result from work by the authors in the context of the LIFE+ project 13BIO/FR/001162 „Conservation of the Giant Freshwater Pearl Mussel (*Margaritifera auricularia*) in Europe“ and include additional work by the contributors from other conservation and research projects.

Volume 1 is dedicated to a synopsis of the current knowledge about the species, their biology and ecology, environmental impacts that have led to the reduction of the populations, as well suggestions to save this and other unionoid species in the Anthropocene. For a review on conservation and rearing techniques, please refer to Volume 2.

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Preface: Why study freshwater mussels, specifically *M. auricularia*?

Freshwater mussels are important elements of aquatic ecosystems. They can form aggregations of more than 100 animals per m^2 known as mussel beds where they can dominate the benthic biomass. As filter feeders they are important in nutrient cycling, influencing the water chemistry and clarity (e.g., Vaughn & Hakenkamp, 2001, Pusch et al. 2001). Dense mussel beds have a substantial influence on the organic matter budget and benthic-pelagic coupling of freshwater ecosystems, as they transform suspended seston (algae, bacteria) into faecal pellets, which are then available for benthic gatherer-collectors. Their excretions can enhance local populations of algae (Vaughn et al., 2007) and macroinvertebrates (Vaughn & Spooner, 2006, Gergs et al. 2009) and their shells provide habitat for other benthic organisms (Spooner & Vaughn, 2006; Vaughn & Spooner, 2006, Werner & Rothhaupt 2007).

Freshwater mussels also provide important direct services to humans, such as water purification In addition, they have been harvested as a source of pearls, mother-of-pearl, and human food since prehistoric times (e.g., Kunz, 1898, Morrison, 1942, Claassen, 1994; Walker et al., 2001).

Despite having survived for hundreds of millions of years, they are now being decimated globally due to human activities and today they are one of the most threatened animal groups on the planet (Bogan, 1993; Young, Cosgrove & Hastie, 2001; Lydeard et al., 2004; Strayer et al., 2004; Strayer, 2008, and see chapter on Human impacts, below). The decline of freshwater biodiversity is much faster than that of any other biome. This is specifically true for *Margaritifera auricularia*, which is considered one of the rarest and the most endangered bivalve species in Europe (Araujo & Ramos, 2001a; Prié, 2010a).

Besides the ecosystem services they provide, due to their ecological and economic importance, the study of freshwater mussels is fascinating because of their complex and unique life-cycle. In addition, a better understanding of their biology is essential to successfully address their conservation.

The scientific genus name *Margaritifera* translates into „pearl-bearing“, thus most people think at the pearl mussel *M. margaritifera* when the name is mentioned. The Giant Freshwater Pearl Mussel, *Margaritifera auricularia*, is much less common, and it is much less visible, as it only occurs in deep and often turbid waters of larger streams and rivers (see chapter on habitat characteristics). Accordingly, it is much less known than *M. margaritifera.*

The Giant Freshwater Pearl Mussel has previously occurred all over Europe, but today it is scarce. In some countries, e.g., Germany, it is not even known when precisely the species has disappeared (Tittizer et al. 1991).

This work aims at summarizing the current knowledge on the biology of *M. auricularia* in order to provide tools for its conservation considering published literature until mid-2018. As this species is poorly known, we include information on the unionoid order or from *M. margaritifera* to allow inferences if no data are available on the Giant Freshwater Pearl Mussel.

1. History of studies on *M. auricularia*

As evidenced by the finding of shell remains of *M. auricularia* in Chalcolithic archaeological sites, the existence of the species has been known by humans from prehistoric times (Araujo & Moreno, 1999; Llorente et al., 2015). However, despite the improvements achieved in recent decades, the scientific knowledge of the species is still relatively scarce specially if compared with the well- studied *M. margaritifera*.

From its description by Spengler in 1793 until the beginning of the 20th century, the literature devoted to the species was restricted to taxonomic descriptions and records on its distribution. However, some citations of the species in the early literature were misidentifications confused with *M. margaritifera* (Valledor & Araujo, 2006). Thanks to the papers of the German malacologist Fritz Haas, who found a population of *M. auricularia i*n Spain in 1916, we know that the species was very abundant in the River Ebro on a 70-120 metre- wide section of the river among stones and boulders with a predilection for deep bottoms (5-7 m), as well as in one of its channels, the Canal Imperial de Aragón, and that the nacre of the empty valves was collected to manufacture knife hilts (Haas, 1916, 1917). Haas summarized the knowledge that was held about the species until that moment with his own words as follows,

"*Until after the first half of the nineteenth century, it was known only in France. It was found there in many rivers, in some of which it was so frequent that nacre was industrially exploited for buttons and similar objects. Its anatomy and habits are not known and only some data on the colour of the soft part and its marked predilection for the deepest places of the rivers is available. Then, around the year 80, news of its appearance in some Italian rivers and in the Ebro and Tagus reached the ears of zoologists. Data on the current distribution of the species were completed in the last five years with its fossil discovery in West and Central Germany and in England”* (Haas, 1916).

According to the references of Haas, the Spanish naturalist Azpeitia (1933) cites the presence of the species in the Canal Imperial de Aragon, which was the last reference of live specimens in Spain. In France, the last documented finding of a living animal before this date is from 1952, from a small tributary of the Loire River between Blois and Tours (Moolenbeek, 2000).

After a long period without new references, some dead specimens of the species were first rediscovered in Spain in 1985 (Altaba, 1990) and in France in 1989 (Nesemann & Nagel, 1989). The first living individuals were rediscovered in 1996 in Spain (Araujo & Ramos, 1996) and in 1998 in France (Nienhuis, 2003). Since 1998, the biology, distribution and lifecycle of the Giant Freshwater Pearl Mussel in Spain have been described (Araujo & Ramos, 1998a,b; Araujo & Ramos, 2000a,b; Araujo, Bragado & Ramos, 2000; Grande, Araujo & Ramos, 2001; Araujo, Bragado & Ramos, 2001; Araujo, Cámara & Ramos, 2002, Araujo, Quirós & Ramos, 2003; Gómez & Araujo, 2008). Since then, very few reports of the species have been released in national and international congresses (e.g. Nakamura et al., 2015), but, apart from Araujo & Álvarez-Cobelas (2016), Prié et al. (2017), and Nakamura et al. (2018), there are no new scientific results published since 2008. In France, focused surveys have led to the rediscovery of many populations since 2007, but most of these results are unpublished or available only as grey literature.

Due to the fact that the populations of the species in the main course of the River Ebro have been declining for many years, the specimens currently living in the Canal Imperial (Araujo & Ramos, 1996, 1998a, b), in the medium (pers. obs.) and lower river (Altaba, 1997) and in the Loire basin (Cochet, 1996; Nienhuis, pers. com.; Bacchi and Gilbert, pers. com.), the Charente and other french rivers (Prié et al. 2018), are the last representatives of a species that may disappear even before its biology is fully known.

2. Taxonomic position of *M. auricularia* in the context of evolution of Unionoida

2.1 Evolution and global diversity of Unionoida

Among the approximately 20,000 living species of bivalves, only about 1,000 species live strictly in fresh waters (Haszprunar et al., 2008). Freshwater representatives occur in most major bivalve groups, indicating that there have been multiple independent bivalve invasions of fresh waters around the world. However, the greatest freshwater radiation has occurred in the order Unionoida, which comprises nearly 85% of the freshwater bivalve diversity.

Freshwater mussels (order Unionoida) belong to the bivalve sub-class Palaeoheterodonta, together with their marine sister-group Neotrigonia (order Trigonioida). The Trigonoida is an ancient lineage that was diverse and widespread in the Mesozoic (250-65 MYA) but is represented today by only six or seven surviving species restricted to marine waters of Tasmania and Australia (Giribet, 2008). Based on DNA analyses, Curole and Kocher (2002) estimated that the subclass Paleoheterodonta diverged from the rest of Bivalvia at approximately 500 MYA (Middle Cambrian). Unionids first appeared in the Triassic (250-200 MYA), and by the Cretaceous (145-65 MYA) the group attained morphological and taxonomic diversity comparable to the recent fauna (Haas, 1969; Good, 1998; Watters, 2001).

The circumstances that led to the invasion of freshwaters by unionoids are unknown but their success is likely related to two aspects of their life histories: parental care and parasitism. Freshwater mussels retain their eggs in brooding chambers located in their gills, developing obligatory parasite larvae that attach to fish while metamorphosing into juvenile bivalves. This strategy prevents their offspring from being “flushed” downstream, and is a very effective mean of dispersal and colonization of upstream areas of river basins.

Figure 1: Distribution map of Margaritiferidae (Lopes-Lima et al., 2018)

Freshwater mussels are represented by approximately 900 species distributed worldwide except in the Antarctica and the Pacific Oceanic Islands (Bogan, 2008). Six families make up the order Unionoida: Unionidae, Margaritiferidae, Hyriidae, Etheriidae, Mycetopodidae and Iridinidae. By far, the most diverse family is Unionidae, with 707 described species distributed throughout North America, Europe, Africa and Asia (Graf & Cummings 2007). The closely related family Margaritiferidae is considered an ancestral group of freshwater mussels and apparently diverged from Unionidae at a minimum of 230 MYA (Curole & Kocher, 2002). Margaritiferidae present one of the lowest diversities within Unionoida, comprised by only 16 currently recognized species which are found in Asia, Europe, North Africa and North America (Figure 3) (Lopes-Lima et al., 2018). However, their distribution is localized and apparently a relic of a previous wider range (Smith, 2001). The remaining families of the Unionoida are restricted to only one or two biogeographical regions, and none of these families occur in Europe. Indeed, this continent possess one of the lowest freshwater mussel diversity with only 16 recognized species (Figure 1), 11 of which currently live in France (Lopes-Lima et al., 2017).

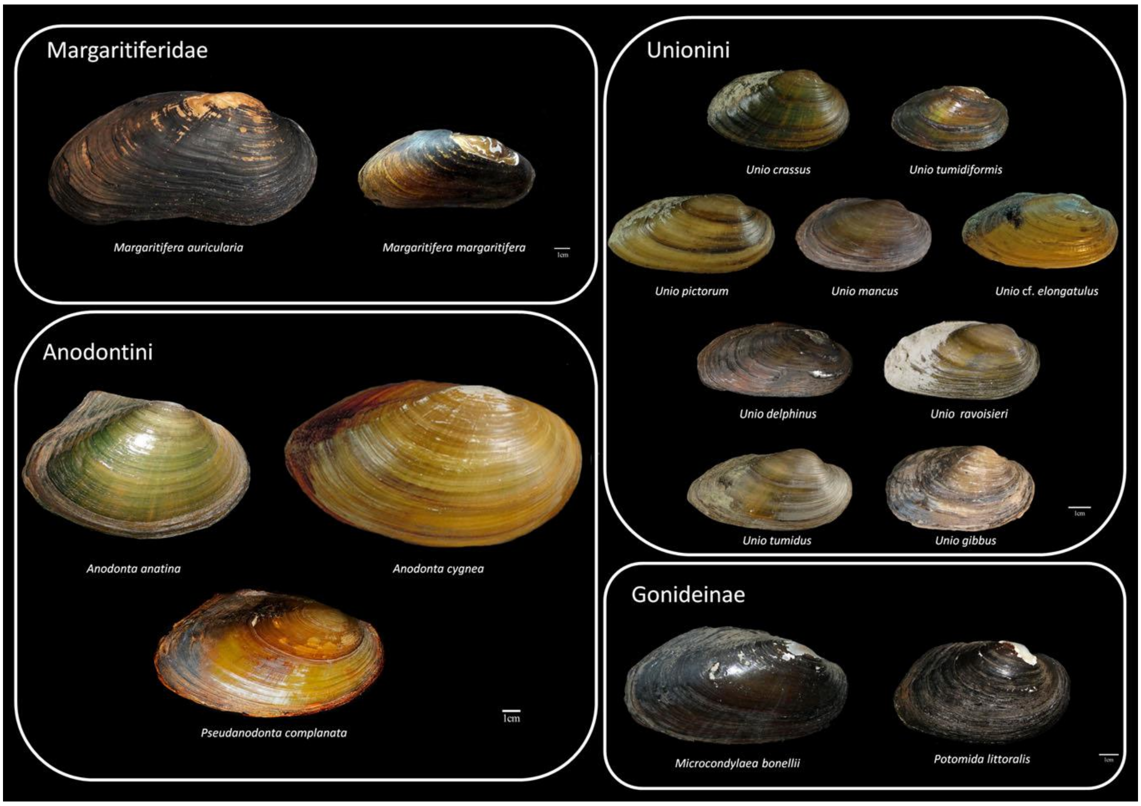

Figure 2: Shells of representative freshwater mussel taxa in Europe. (Lopes-Lima et al., 2017)

2.1.1 Systematics of *M. auricularia*

Margaritifera auricularia was first described by Spengler in 1793 as *Unio auricularius*. Nevertheless, Spengler erroneously cited the locality of the type specimen from East India. In 1819 Lamarck redescribed the same species with European specimens from French rivers as *Unio sinuata*. Haas (1910) introduced the subgenus *Pseudunio* to differentiate this species from *Margaritifera margaritifera* but one year later, *Pseudunio* was synonymized as *Margaritana* (=*Margaritifera*) by Ortmann (1911). *Pseudunio* was used later by Haas (1969) in order to separate *M. auricularia* and *M. marocana* (Pallary 1918) from the rest of species belonging to the genus *Margaritifera*.

Since the beginning of this century, several phylogenetic studies have presented the family Margaritiferidae as monophyletic divided into three to four major clades although keeping

Margaritifera as the single genus (Huff et al., 2004; Graf and Cummings, 2007; Araujo et al., 2017). Nevertheless, other authors have pointed out the necessity of splitting the family in several subgenus, including *Pseudunio* (Bolotov et al., 2016; Lopes-Lima et al., 2018). Since a consensus regarding the nomenclature has not still been reached, we use the genus name *Margaritifera* Schumacher 1816 rather than *Pseudunio* Haas 1910, as *Margaritifera* is more commonly used.

- Phylum Mollusca Cuvier 1795
  - Classis Bivalvia Linnaeus 1758
    - Subclassis Palaeheterodonta Newell 1965
      - Ordo Unionoida Stoliczka 1870
        - Superfamilia Unionoidea Rafinesque 1820
          - Familia Margaritiferidae Henderson 1929
            - Genus *Margaritifera* Schumacher 1816

Synonymes :

Unio margaritifera (Draparnaud, 1801)

Unio sinuatus (Rossmässler 1844)

Unio crassissimus (Ferrussac 1844)

Pseudunio auricularius (Spengler 1913)

Unio sinuata Lamarck, 1819

Unio margaritanopsis Locard, 1889

Common names:

- French: Grande mulette
- English: Giant Freshwater Pearl Mussel, Spengler's freshwater mussel
- Spanish: Almeja perlífera gigante de río, Perla de río, Margaritona, Náyade auriculada
- German: Riesen-Flußperlmuschel

In homology to the abbreviation of *M. margaritifera* as "River Pearl Mussel" (RPM, Gum et al. 2011), we sometimes use the abbreviation of Giant Freshwater Pearl Mussel (GFPM) in the following.

3. Morphology of Unionoida, specifically *M. auricularia*

3.1 Unionoida terminology and basic anatomy

In Greek mythology, Naiads were nymphs who inhabited and gave life to fresh waters. Naiads are bivalve mollusks and share the same basic anatomical plan with their marine counterparts. Here we briefly describe their basic anatomy for a better understanding of the following sections.

Despite their great diversity, the general anatomical pattern of this group of mollusks is very simple and homogeneous. The shell is formed by two valves that encompass the body of the animal and are joined by a protein ligament located near the umbo or apex. Under the umbo runs the hinge that usually has teeth on its inner face fitting between those of the opposite valve (Figure 1). In its internal part, the shells have marks or scars. The insertion of the adductor muscles, whose action allows closing the valves, leaves characteristic rounded or oval marks. The paleal line is the mark formed by the mantle margin of the mollusk on the inner side of the valves.

As a rule of basic orientation for Unionoids, it should be taken into account that the anterior region of a bivalve is the one that is closest to the umbo or apex of the shell, and therefore the posterior region is the opposite. On the other hand, the dorsal part is that in which the vertex is located, and the ventral part is the opposite one (Figure 3).

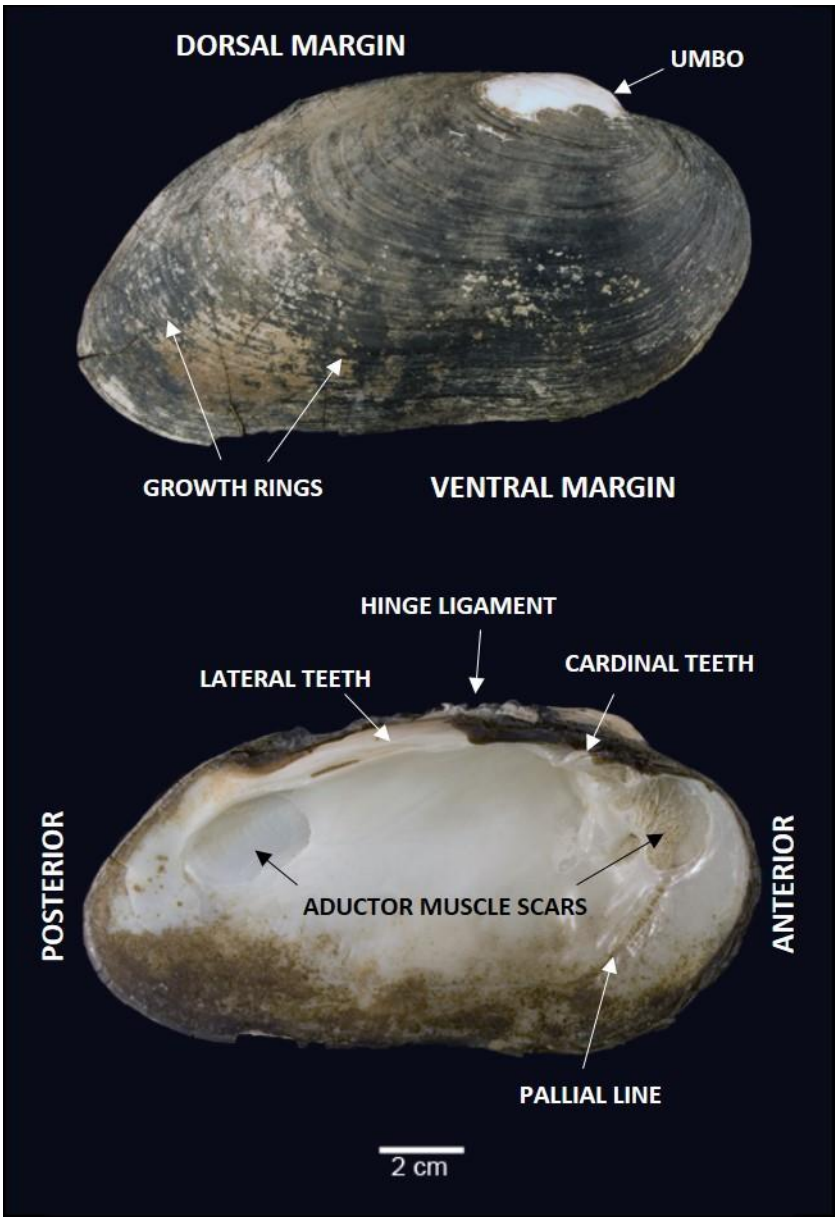

Figure 3: External and internal view of the right (upper) and left (below) valves of *M. auricularia* (Modified from Araujo et al., 2009)

Under the valves, and covered by these, are the lobes of the mantle, which form a kind of bag including the rest of the animal body (Figure 4). The cells of the outer face of the mantle release the calcium carbonate that produces the shell. The posterior edges where the two lobes of the mantle meet has two openings that form the siphons; one superior (excurrent) and another inferior, (incurrent) and a third through which the foot protrudes.

The foot is a very muscular and extensible organ, which allows the animals to bury themselves and move on the substrate by peristaltic movements. It is, together with the gills, the largest organ of the naiads. In young specimens of some species the foot presents a gland called byssus that forms adherent filaments with which the specimens are attached to the substrate (this byssus is easily noticeable in marine mussels of the genus *Mytilus*).

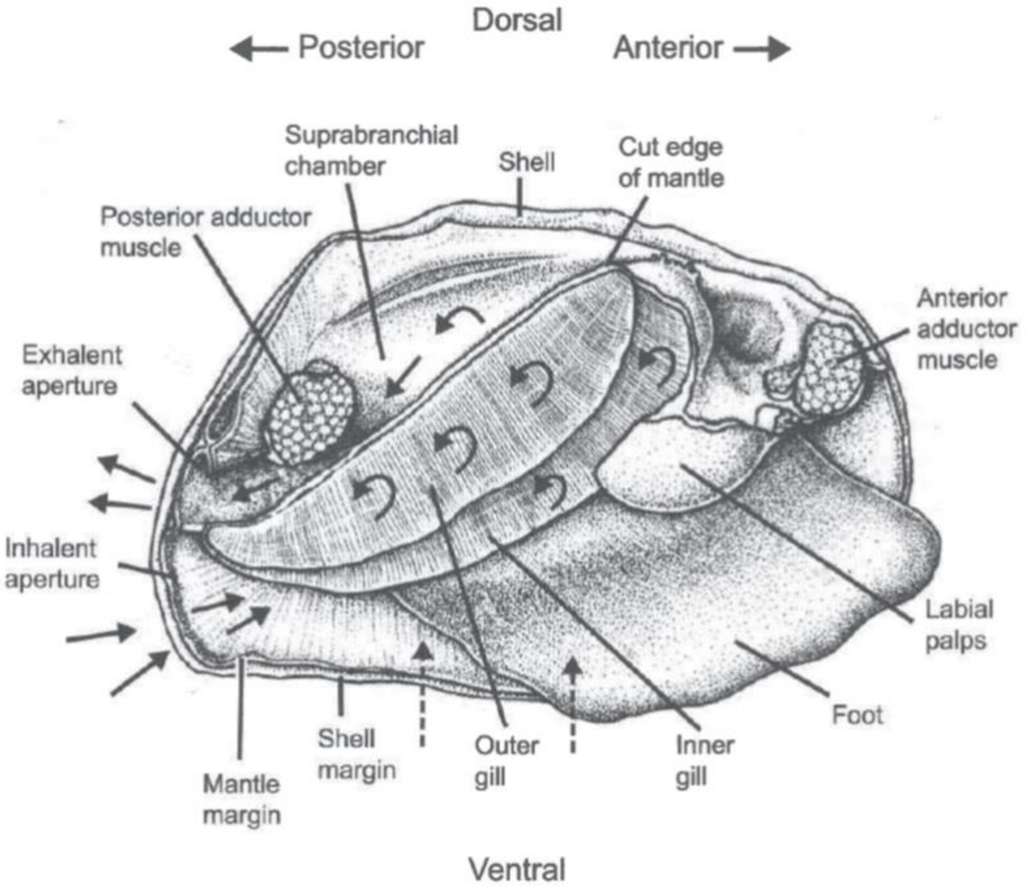

Figure 4: Internal anatomy of a freshwater mussel. Arrows show the direction of water within the animal; arrows whit circular stems show water flowing through and into the gills; arrows with dashed stems indicate interstitial sediment water entering the shell gape (Modified from Haag, 2012)

Between the mantle and the foot the gills are placed, two on each side of the foot (Figure 4). The gills of suspension feeding bivalves are extraordinary organs that are involved in feeding, gas exchange and ion transport although, in some marine and many freshwater species, they are also used to brood larvae during the reproductive period. Gills of freshwater mussels are elongated sheets extending into the mantle cavity and are composed of inner and outer demibranchs. Each demibranch is composed of ascending and descending lamellae, which are organized as filaments surrounding central water channels. The lamellae are joined by connective tissue and in most cases, these connections are developed as continuous septa, running parallel to the gill filaments towards the edge of the gill that form the water tubes. However, in the Margaritiferidae there are no water tubes, and the lamellae of each demibranch are intermittently united by irregularly scattered junctions (for further details on gills anatomy see Ortmann, 1911; Cox, 1969; Kays et al., 1990; McElwain & Bullard, 2014, Soler et al., 2018b.

The gills present numerous cilia and their movement originates the current that allows the filter-feeding mode of the bivalves. The water enters the mantle cavity through the incurrent orifice, passes through the gills until the suprabranchial chamber and exits through the excurrent orifice (Figure 4). The food particles present in the water are retained by the gills and driven to the anterior area where they are selected by the labial palps that surround the mouth.

3.2 Morphology of *M. auricularia*

Although its anatomy has not been very well studied, it shares the common pattern of the Unionoida order (Figure 3). Nevertheless, as in the rest of Margaritiferids, it is characterized by some anatomical features different from the Unionidae family (Araujo et al 2017; Lopes-Lima et al., 2018): 1) the incurrent siphon papillae are small and branched; 2) they lack a supra-anal aperture to the excurrent siphon; 3) the diaphragm dividing the infra and supra-branchial cavities is incomplete, and 4) in the gills, the lamellae of each demibranch are intermittently united by irregularly scattered junctions, rather than by continuous septa.

As indicated by its common name, the Giant Freshwater Pearl Mussel is a very big naiad attaining a maximum length of 20 cm. The shell has a black periostracum and flattened umbones (Figure

3). The valves are elongated posteriorly and shortened and very thick in the anterior part. Growth lines are visible in the external part of the shell, although in aged specimens they become densely packed near its edge, making it very difficult to count them for age estimations. In adults, the shell border is usually sinuated giving an ear shape to the shell, which has inspired its Latin specific name "*auricularia*". The internal part of the valves are white due to the presence of a thick layer of nacre. They have well marked adductor muscle scars and pallial lines. Left valves have two cardinal pyramidal teeth under the umbo and two long lateral teeth behind it. The right valves present one small cardinal teeth and a lateral one that fits between the two lateral teeth of the opposite valves.

4. Life cycle of Unionoida, specifically *M. auricularia*

4.1 Growth and longevity

A generalized depiction of the life histories of freshwater mussels describes this group as long-lived animals, with slow growth, high fecundity and late maturity. This characterization is likely derived from the best-studied freshwater mussel in the world, *M. margaritifera* (Haag, 2012). However, naiads are able to adapt to the conditions of nearly all freshwater habitats requiring adaptability and thus variability of their life-history traits.

M. margaritifera may live for about 200 years (Bauer, 1992; Ziuganov et al., 2001) and is considered to have one of the longest life spans reported for any non-colonial animal (Hurlbert et al., 2007). However, the longevity of freshwater mussels vary widely among and within species and even *M. margaritifera* may live less than 40 years in some places (San Miguel et al., 2004). Other naiad species live more than 50 years, although the majority of them commonly live between 15 and 40 years (Haag, 2012).

Life span is a life-history trait with potentially important ecological costs and benefits, and therefore should be under strong selective pressure (Bauer, 1992). It has been observed that long-lived freshwater mussel species such as *M. margaritifera*, have lower growth rates than short-lived species such as *Anodonta* and *Unio* species (Bauer, 1992; Aldridge, 1999; Hochwald, 2001). It is often assumed that growing faster is better, presenting a series of benefits that include earlier age at maturity and lower risk of predation at smaller sizes (Arendt, 1997; Metcalfe & Monaghan, 2003). However, most organisms do not grow as fast as they can, even if conditions are favourable (Metcalfe & Monaghan 2003). This is because growing faster implies costs, reducing the fitness of adults and leading to lower life spans (Bauer, 1992; Arendt, 1997; Metcalfe & Monaghan, 2003; Rose et al., 2009).

Freshwater mussels grow along their whole life span but the rate at which growth is accomplished varies along their lives. Like most organisms, their growth is usually fast during the first few years of life slowing down with age, as energetic resources are diverted to reproduction and maintenance. However, the amount of energy allocated to growth versus other functions and the timing of this allocation may vary among species (Haag, 2012).

In all bivalves, the shell is secreted by the mantle and seasonal variation in shell deposition produces rings which are observable in both the external surface and cross sections of the shell (Figure 5). These annual rings provide a detailed growth record similar to those found in trees or in fish otoliths and scales and have been utilized for age and growth estimation in bivalves (e.g., Schöne et al., 2004; Haag & Commens-Carson, 2008).

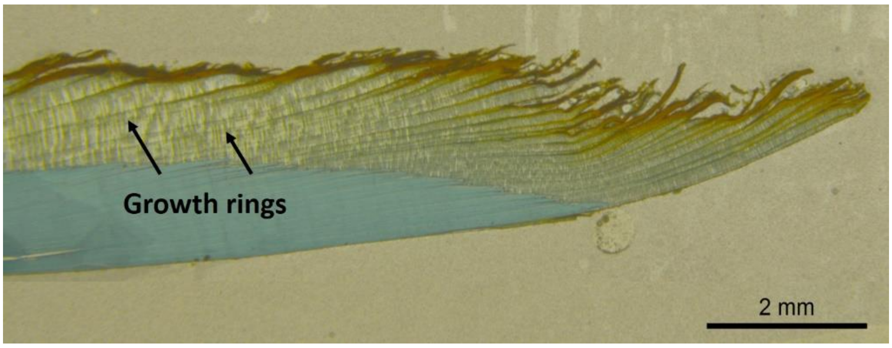

Figure 5: Growth rings observed in a cross section of *M. auricularia* shell treated with Mutvei's solution

4.2 Reproductive Strategies

The reproductive cycle of naiads is quite unique in the Animal Kingdom, and one of the keys to their success colonization of fresh waters, but, in some cases, it may be the cause for the vulnerable situation that most species are currently experiencing. All freshwater mussels (Unionoida) share a complex life cycle, requiring a vertebrate host, usually a fish, during their parasitic larval stage. The cycle starts when the sperm that was released by males in the water becomes absorbed by females with the respiration. The fertilization of the eggs takes place in brooding chambers formed by the gills. The eggs develop into parasitic larvae (in most families called *glochidia*, singular: *glochidium*) which are released into the water by the female. These microscopic, thin-shelled larvae were presumed to be a parasitic species of fish (*Glochidium parasiticum*) in the nineteenth century. The valves of the glochidia usually have hooks to attach themselves to the body of the fish (fins or gills, see next paragraph), where they become encapsulated for a variable period (from weeks to months), before metamorphosing into free-living juveniles. The juveniles excyst from the gill tissue of the fish, and float in the river water for a short time before they settle on (and into) the river substratum, where they grow to adult size and start another cycle (Figure 6). Juveniles spend a considerable time in the hyporheic interstitial of the river sediments before they colonize the substrate surface.

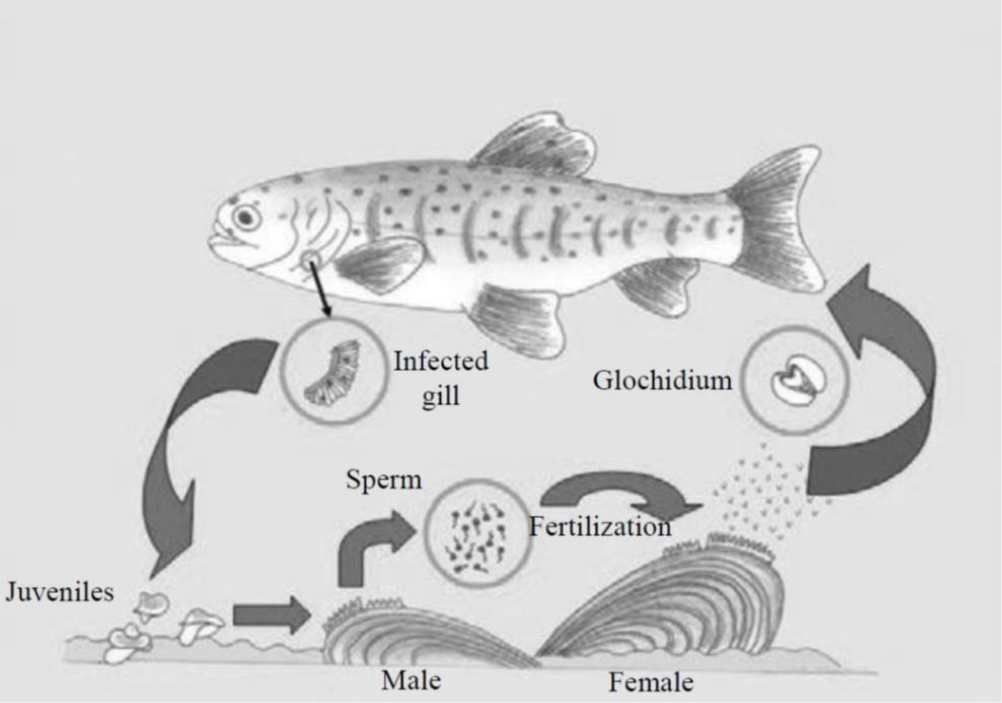

Figure 6: Basic life cycle of a unionoid mussel (adapted from Reis, 2006).

However, numerous variations to this general cycle have been reported among freshwater mussel diversity. Although most species are dioecious, others are simultaneous hermaphrodites (Schalie, 1970; Kat, 1983) and occasionally, hermaphrodite individuals are found in several predominantly dioecious species (Schalie 1970; Kat 1983). Bauer (1987) reported that *Margaritifera margaritifera* individuals are able to change from dioecious to hermaphrodite in response to decreasing population density.

In addition to the previous statements on Unionoida and Margaritiferidae, the available detailed information on the reproduction of the GFPM is given below. In the only population where the sex ratio has been studied (Canal Imperial de Aragón), *M. auricularia* is mainly hermaphrodite, with only some individuals being dioecious (Grande et al., 2001). Female and male gonads are mixed within the visceral mass without a specific location (Figure 7). In this population, the gametogenesis occurs from December to March (Grande et al., 2001) but the period when the ova are transferred to the gills and the duration of brooding are still unknown.

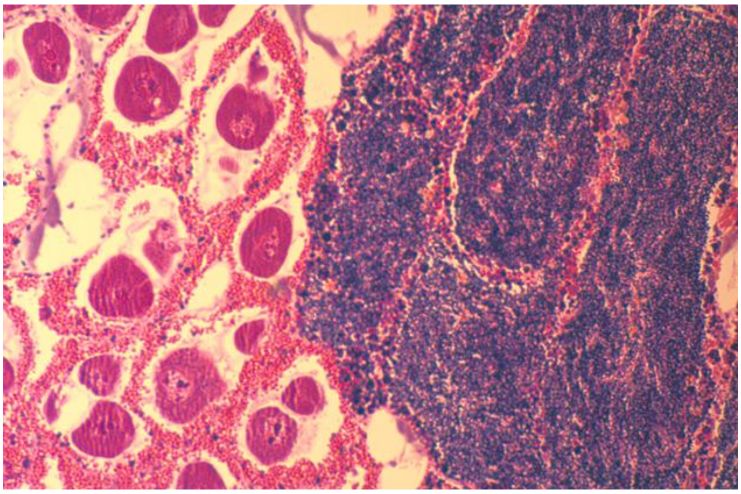

Figure 7: General aspect of the gonad of *M. auricularia* showing mixed female (left) and male (right) tissues (R. Araujo, unpublished picture)

Previous knowledge of the reproductive season of *M. auricularia* is based on Ebro Basin populations. Haas (1917) reported that in the Ebro River, the species does not incubate between mid-July and early September. Results from a drift-net study by Araujo et al. (2000) indicated that in the Canal Imperial, *M. auricularia* has only one reproductive period per year. In this population, gravid individuals have been found in February and the glochidia release has been reported to take place between February and March-April, with a peak in mid-March (Araujo et al., 2000; Araujo & Ramos 2001b). The glochidia release period in French populations is about one month later (see preliminary data in Vol. 2). Further studies are need to determine how glochidia are released, if there are specific cues by the fish for glochidia release (and how mussels perceive them), and if and how the GFPM is able to attract host fish.

The number of eggs that an individual mussel can produce is usually correlated with its shell length (Bauer, 1998; Hochwald, 2001; McIvor & Aldridge, 2007). This allometric constraint is probably a factor contributing to higher growth rates. In addition, for similarly-sized mussels, more glochidia can be produced with the same energetic investment if glochidia are smaller (Bauer, 1994). The morphological brooding pattern is also variable among naiads. The eggs can be brooded in all four demibranchs (tetrageny), only in the inner ones (endobranchy) or in the outer demibranchs (ectobranchy) (Heard, 1998). Furthermore, some species such as the European *Anodonta anatina* usually broods in the outer demibranchs although tetragenous populations have been observed (Lopes-Lima et al, 2016). Variations in brooding patterns seem to be intrinsic and species-specific, and have been used as an important characteristics in the study of unionoid evolution and systematics (Graf & Cummings, 2006).

Freshwater mussels have different strategies in relation to the length of the brooding period, being either short-term (tachytictic) or longterm (bradytictic) brooders. Tachytictic species spawn their gametes in spring and brood the embryos only until glochidia have fully developed, whereas bradytictic mussels spawn in late summer and brood their glochidia over winter, releasing them in early spring (Graf & Ó Foighil, 2000). Climate (Sterki, 1903; Ortmann, 1909; Graf, 1997) and synchronization with seasonal host activity (Zale & Neves, 1982; Young & Williams, 1984a) are considered important factors that determine brooding patterns.

Finally, the way how larvae are released is probably one of the most extraordinary features in unionoid evolution. Many strategies have been developed by freshwater mussels in order to maximize the probability by which their larvae to find an appropriate host. *M. margaritifera* females release millions of larvae, and a typical healthy population may contain hundreds of thousands of mussels, so that the number of glochidia in the water during the reproductive period can be extremely elevated (Bauer, 1991). The behaviour of *Unio crassus* constitutes an unusual modification of simple broadcast: gravid females move into shallows and spurts a stream of water with glochidia that spatters on the surface and, presumably, attracts host fish that are looking for falling insects (Vicentini, 2005). However, the most spectacular host-attracting strategies are known from North American species of the sub-family Lampsilinae, which have developed mantle modifications that resemble the shape of a fish and are used as lures (Figure 8) (Haag et al., 1999; Barnhart, Haag, & Roston, 2008; Haag, 2012). Other species "pack" their larvae in well-visible structures resembling invertebrates that are actively taken up by the fish.

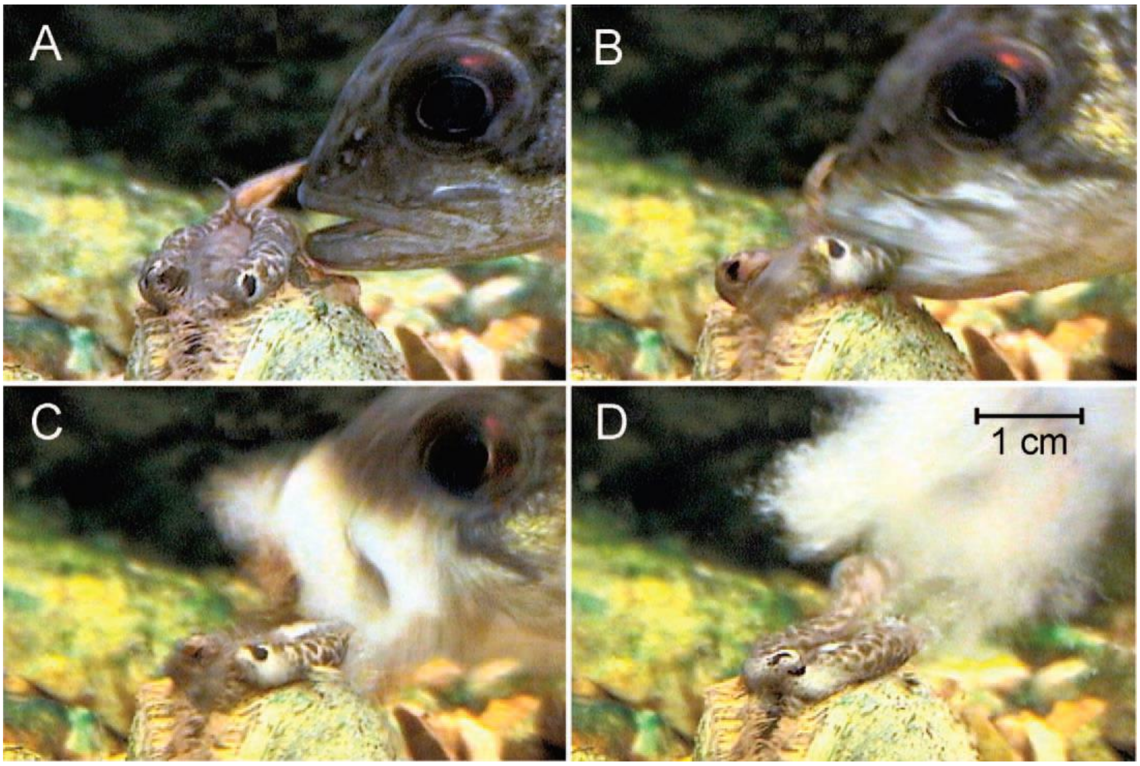

Figure 8: Infestation strategy *by Lampsilis reeveiana*. The fish approaches (A) and bites the lure (B), then abruptly opens its mouth and expands its buccal cavity to inhale the ''minnow'' (C). Suction created by the fish ruptures the marsupium and extracts a cloud of glochidia (C, D) (from Barnhart et al., 2008).

4.3 Parasitic Stage

Once the parent mussel releases the glochidia, the availability of a suitable host becomes critical. If glochidia lack storage tissues, they die within a several days if unable to attach to an appropriate host (Murphy 1942; Hochwald, 1988; Jansen et al., 2001). Although some species have developed highly specialized infestation strategies that maximizes infestation, the tremendous mortality rates of glochidia found in most species, makes this the most critical stage in freshwater mussel life cycle (Jansen et al., 2001).

After attachment, the glochidium becomes encapsulated by the host tissue, forming a cyst in which it metamorphoses into the free-living juvenile stage (Figure 9). The parasitic stage of unionoid mussels performs several functions, including protection, nutrition and dispersal. Most freshwater mussel species parasitize fish (Watters & O'Dee 1998), which carry the glochidia upstream and potentially far away from their progenitors. This is particularly important since adult mussels are almost sessile, and because of the unidirectional nature of the flow in rivers and streams.

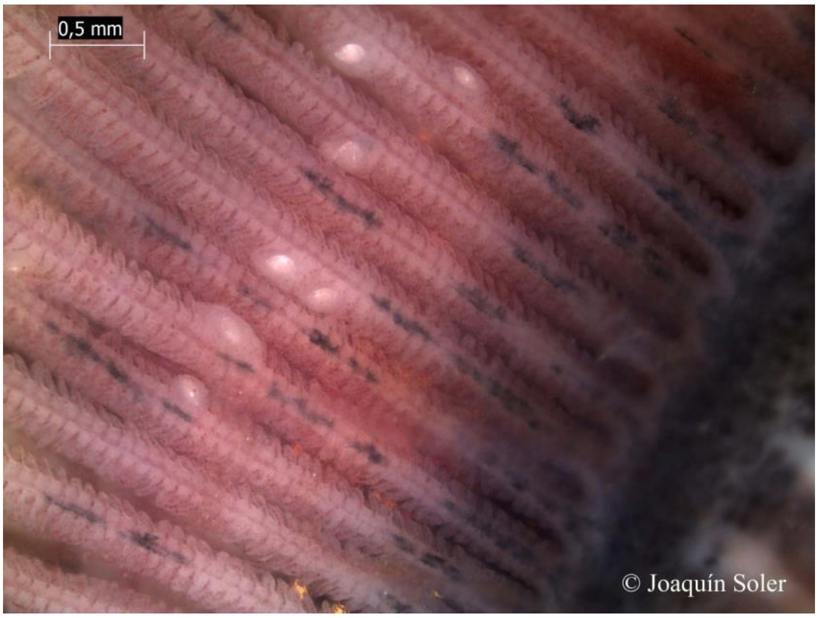

Figure 9: Encysted glochidia of *M. auricularia* in *Acipenser baerii* gill filaments.

Once glochidia are released by the mussels, theoretically any kind of fish could be infested by them. However, fishes develop immunologic responses that glochidia must be able to overcome in order to be physiologically compatible with their host (Bauer, 1997; Bauer & Wächtler, 2001; Haag, 2012). In addition, even adequate fish species develop an immunological memory, i.e. previously glochidia-infected fish cannot be successfully infected any more (Kat, 1984; Barnhart et al., 2008). Regardless of the exact cause of parasite rejection and death, the few available quantitative data show that even on known host fish with no or little previous exposure, glochidial mortality during the parasitic stage may differ between mussel species and can be very high (Jansen, Bauer & Zahner-Meike, 2001). Moreover, not all physiological hosts may be infested under natural conditions since an ecological compatibility between the mussel and the host is also required. Ecological hosts need to be physically available in terms of glochidia-host geographic co-occurrence and phenological match (i.e., synchronous occurrence of the fish and the glochidia in one habitat) (Levine, Lang, & Berg 2012).

While the glochidia of most freshwater mussels develop successfully until the juvenile stage only on a limited number host species, some generalist mussel species are capable of metamorphosing on numerous hosts (Jansen et al., 2001; Barnhart et al., 2008; Strayer, 2008). This is a particularly important characteristic of species because it can determine their degree of susceptibility to changes in ecosystems: a narrow range of host species usually implies a larger risk of extinction.

Although the underlying basis for host specificity is largely unknown, it has been proposed that the preference for hosts has a heritable component resulting from co-evolutionary processes (Cummings & Mayer, 1993; Roe, Simons & Hartfield, 1997; Graf & Cummings, 2016). Thus, a better knowledge of host-mussel relationships could be useful for improving our understanding of the evolutionary history of unionoids.

Nowadays the need to improve this knowledge has become urgent given the worrying conservation status of most freshwater mussels worldwide (Strayer et al., 2004; Lopes-Lima et al., 2017). The identification of host species is essential for conservation programs focused on the reinforcement of populations with low or absent recruitment by the reintroduction of juvenile mussels into the wild (Araujo et al. 2015, Brady & Gatenby, 2018, Soler et al. 2018a and under revision)

The glochidium of *M. auricularia* was described by Araujo & Ramos (1998a) as the biggest of the family Margaritiferidae (length: 127-144 µm; height: 120-142; width: 54-71). It is of the hookless type, but presents very minute teeth at the ventral border, that allow successfully attaching only to fish gill filaments (Figure 9).

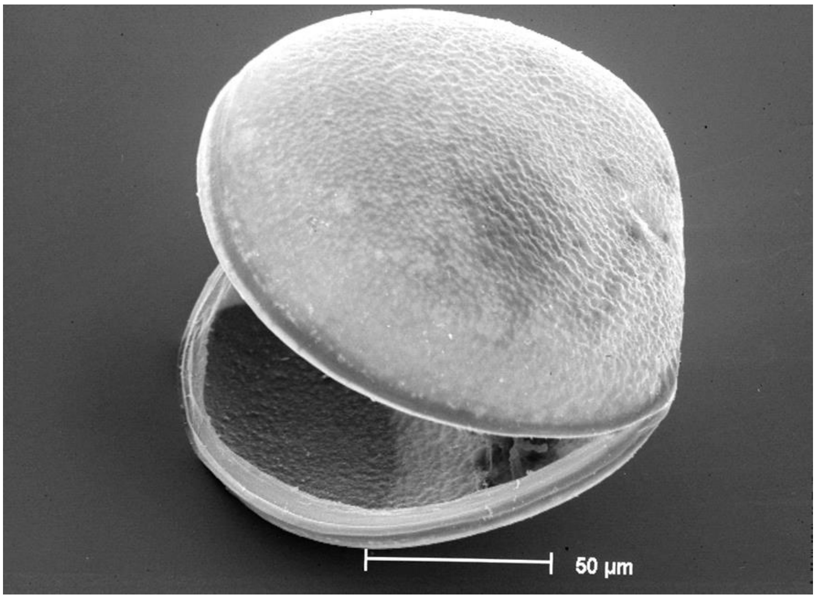

Figure 9: Glochidium of *M. auricularia* observed under scanning electron microscopy (Araujo & Ramos, 2001b)

The duration of the encystment period of *M. auricularia* glochidia in the fish gills varies depending on temperature. It usually takes 4 weeks (30 days at 23–24°C), although longer periods have been reported with lower temperatures (50 days at 18-22°C or even 65 days at 16-17°C) (Araujo & Ramos, 2000a; Araujo et al., 2002).

4.4 Post-parasitic stage (with some details about feeding ecology)

Once the metamorphosis is completed, excysted juveniles can only survive if they had been dropped from their hosts in suitable habitats. Juveniles penetrate the interstitial spaces of well-aerated sediment some centimetres deep and remain there for several years until they grow to a size of 1 or 2 cm that permit them to counter the drag of the flow. In most species, benthic life starts before the gills are fully developed, and thus, filter-feeding is not possible at the first stages of life. The active food uptake occurs by water flow mainly caused by the cilia of their highly developed foot. Pedal-feeding by juvenile mussels may extend to 140 days post-metamorphosis (Gatenby, Parker & Neves, 1997), although it depends on the growth rate of the concerned species. With increasing differentiation of the gills the adult mode of feeding is gradually adopted (Wachtler, Dreher-Mansur & Richter, 2001).

Despite the high losses before reaching this stage, juveniles are still fragile and experience high mortality. Young and Williams (1984b) calculated mortality rates of 95% per year for juvenile *M. margaritifera* in Scotland. The high mortality during juvenile stage is believed to result from the mussels falling into unfavourable habitats, predation or caused by the inability to successfully adapt to the transition of feeding modes.

As a result of the high mortalities experienced during their early life stages, less than 1% of the individuals reach the adult stage (Jansen et al., 2001). Despite the very low survival of their offspring, freshwater mussel used to be found in extremely dense populations, suggesting that reproductive rate did not limit local population sizes. It has been proposed, that naiads have evolved as organisms that are generally long-lived and highly fecund which confers them a very high life-time fecundity, in order to compensate for the high moralities of their early life stages (Jansen et al., 2001).

The released juveniles of *M. auricularia* have spherical shells (Figure 16F) with a finely ciliated foot. Mean measurements of these juveniles are: length = 190 µm (n = 1), width (from the umbo to the ventral border) = 193 µm (n = 4), and height = 210 µm (n = 4). The growth rate of *M. auricularia* glochidia during metamorphosis (length, 41%; width, 53%; and height, 238%) is the smallest reported for the genus (Araujo et al., 2002).

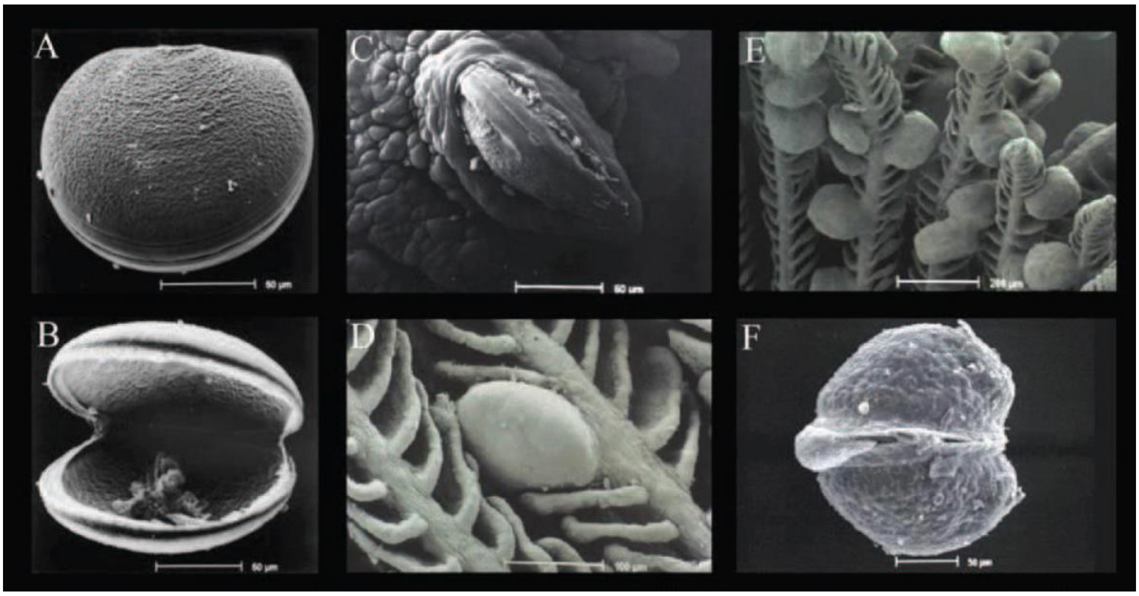

Figure 11. Development of *M. auricularia* glochidia observed by scanning electron microscopy A and B :Glochidium; C: Encysted glochidia on the gills of Acipenser baeri 4 hours after infestation; D and E: Completed encystment; F: Juvenile of *M. auricularia* (Adapted from Araujo & Ramos, 1998a; Araujo et al., 2002).

An early study on the growth of *M. auricularia* juveniles in captivity is that of Araujo et al. (2003). The authors tested the survival capacity of about 2,500 recently released juveniles in a nutrient laden media recreated in tanks filled with well and green water, sediment from the Canal Imperial, soil and vegetation from the Ebro River. Juveniles were kept alive for at least 4 weeks, although the highest mortalities were observed during the first 2 weeks. In the most successful tank, 13 juveniles were found after 39 days of culture, with maximum dimensions of 325 µm in length and 350 µm in height (Figures 12 and 13).

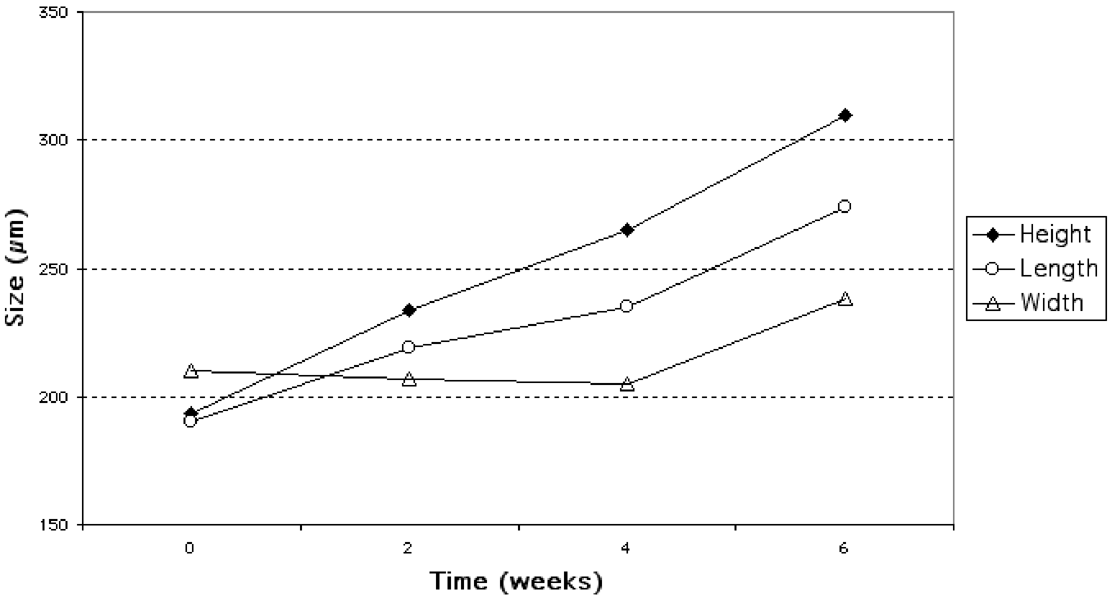

Figure 12: Mean *M. auricularia* juvenile shell size during a 6-week culture (Araujo et al., 2003)

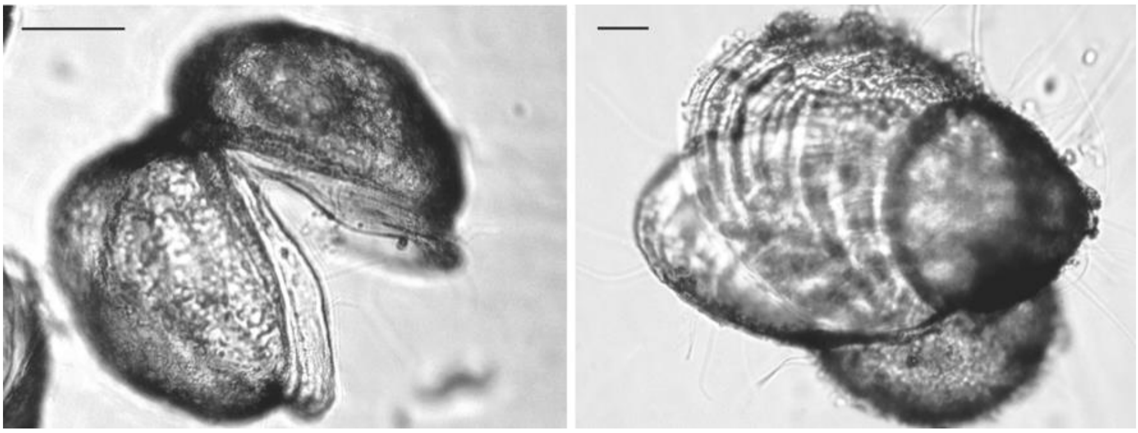

Figure 13: Juvenile shells of *M. auricularia* after release (left) and at 6 weeks old (Araujo et al., 2003).

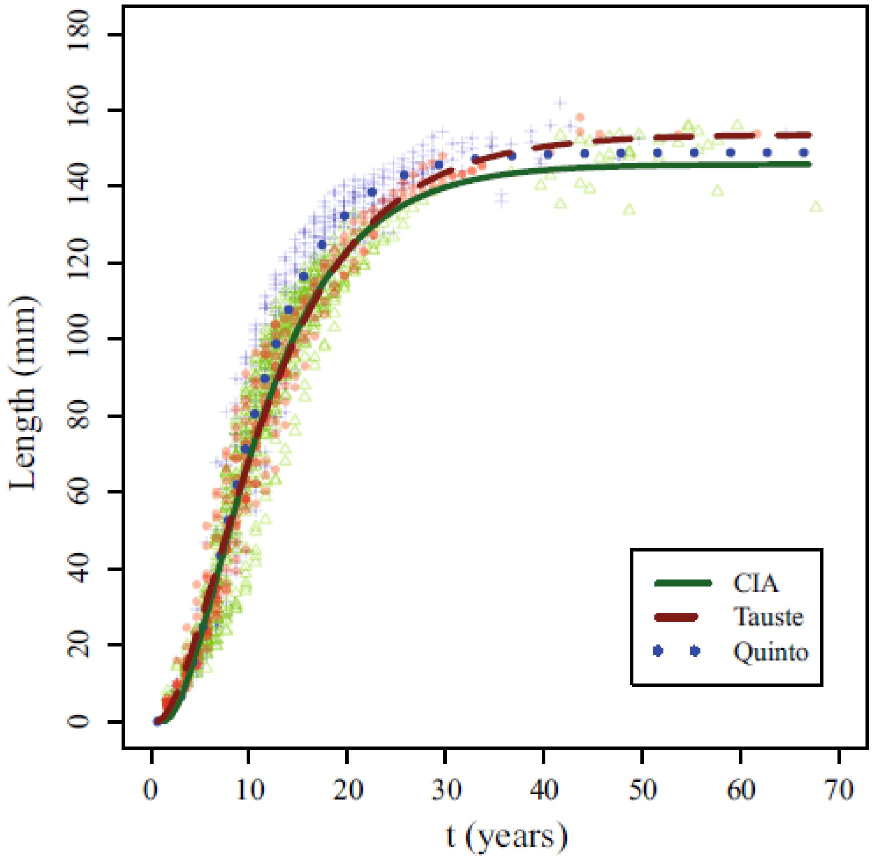

Figure 14 Fitted curves of the generalized Bertalanffy growth model for the three populations of freshwater mussel *Margaritifera auricularia* from the Eastern Iberian Peninsula. CIA (green line and triangles) Canal Imperial de Aragón, Quinto (blue dotted line and crosses) Acequia de Quinto, Tauste (red dashed line and dots) Canal de Tauste (Nakamura et al., 2018)

Recently, Nakamura et al. (2018a,b) published new results on the artificial rearing and growth rates of juvenile *M. auricularia* (Figure 14). The authors tested different feeding treatments culturing the juveniles in plastic boxes. At 120 days a survival rate of 20-70% was achieved by feeding the juveniles with phytoplankton and detritus. When the experiment time was doubled to 30 weeks, the survival rate declined dramatically but some individuals were kept alive for one year reaching a maximal length of 3.5 mm.

5. Biogeography and current distribution of *M. auricularia*

5.1 Prehistorical, historical, and extant distributions of *M. auricularia*

The Giant Freshwater Pearl Mussel is difficult to observe: it lives in the lower sections of large rivers, a habitat that is difficult to survey due to deepness, turbidity, current and often navigation. Hence, not surprisingly, it has been overlooked by malacologists of the twentieth century.

Margaritifera auricularia was once present on most major rivers in Western Europe. In the 19th century, naturalists cited the species on many French rivers from the Seine, Loire, Garonne, Adour and Rhone basins. On the other hand, the species was known on the Rhine in Germany, on the Tagus in Spain (Araujo & Ramos 2000b) and in the Po basin in Italy until the 19th century (Bourguignat, 1883; Araujo & Ramos, 2000b). In the Thames in England its presence is known only from fossil individuals (Figure 15).

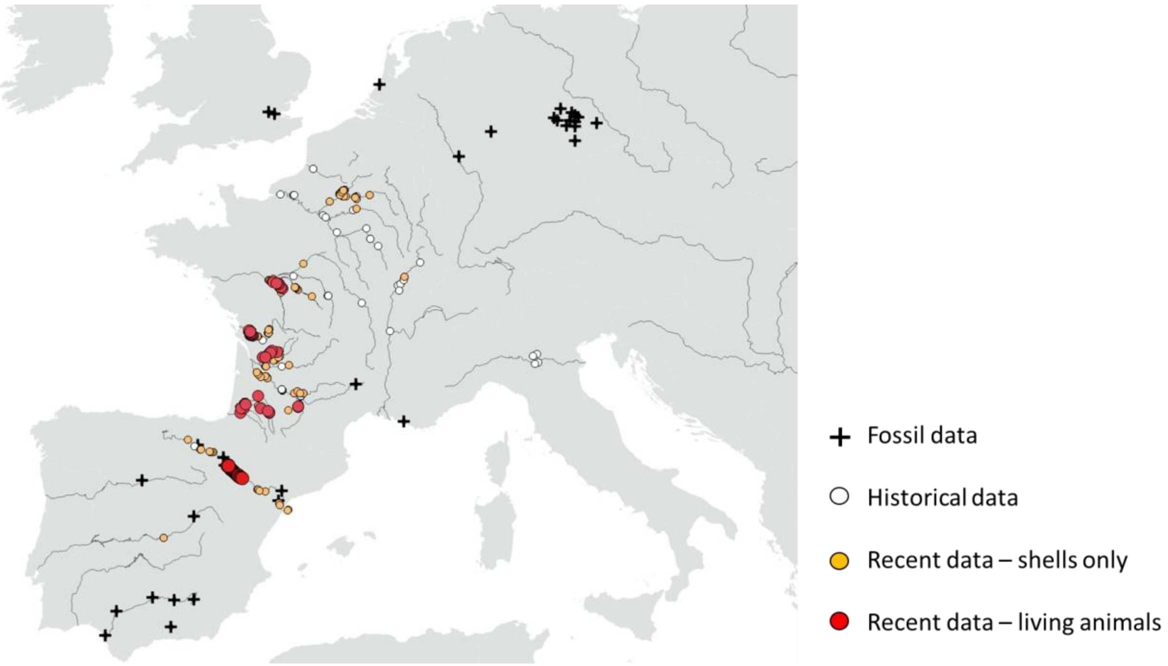

Figure 15: Historical (black dots) and current (shells = grey dots; living populations = red dots) distribution of *M. auricularia* (Prié et al., 2017)

The last living populations are currently found only in France and Spain. Until the beginning of this thesis, the known distribution of the species was synthetized by Prié et al. (2011) as follows:

In Spain, the Giant Pearl Mussel is only present in the Ebro basin, from the La Rioja region to the Ebro delta (Altaba, 1997; Araujo & Ramos, 1998b; Araujo et al., 2000a; Araujo & Ramos, 2000b; Altaba, 2001; Gomez & Araujo, 2008). Nevertheless, the only numerous populations as of today live in the channels "Canal Imperial" and "Canal de Tauste".

In France, the known living populations are located in Vienne and Creuse (Cochet, 2001a, b; Cochet, 2002), in the Charente (Nienhuis, 2003; Prié et al., 2008a), Dronne, Adour, Luy and the Save (Prié et al., 2010). Empty but fresh shells have been found in the Oise (Prié, Philippe & Cochet, 2007; Prié et al., 2008b, which consider it as recently extinct in the Oise), Louts (Cochet, 2004), in the Vesle, the Aisne, the Loire, the Dordogne, the Aveyron, the Isle, (Prié et al., 2010). Fragments of shells were also harvested in the Indre by Nagel, then by Cochet, then by Dohogne

(2008) and at several places in the Dordogne-Garonne basins (Prié & Bousquet, 2010, Prié et al. 2017) (Figure 15).

This state of knowledge on the distribution and habitat structure of *M. auricularia* in France has recently improved by Prié et al. (2017). It has resulted in the discovery of a previously unknown population in the Adour watershed, and has confirmed the constant decline of most of the populations.

1.2.5. Population structure

In Spain, the size of the living populations are estimated to around 5,000 specimens in the Canal Imperial, and 80 in the Canal de Tauste (Araujo & Ramos, 2001a; Gómez & Araujo, 2008; Araujo & Álvarez-Cobelas, 2016). In France, Cochet (2001a) estimated that the population of the Vienne and Creuse rivers should be of about 1,000 individuals. Prié and co-workers estimated population sizes in France of a few hundred individuals between the Dronne and Adour rivers and about 100,000 in the Charente (Prié, 2010b, Prié et al. 2017). Following these estimations, about 90% of the world population is found in France, containing most of the living populations and the largest one known in the Charente.

Araujo & Ramos (2000a), studied the size distribution of 438 individuals of the population of the Canal Imperial population and reported that it was dominated by individuals between 15 and 17 cm and classes less than 12 cm were non-existent, indicating a recruitment failure (Figure 16). Nevertheless, few young specimens of about 10 cm have been recently found in this population (Nakamura et al., 2018). In the Vienne River, Nienhuis (2003) found that the 19 living individuals were between 11 and 16 cm. Cochet (2004) reported that 80% of 292 individuals measured were over 13 cm but found 9 individuals smaller than 11 cm, suggesting that recruitment took place in the Vienne in the relatively recent past. Furthermore, in this river four juveniles (7-8 to 8-10 cm) were discovered in 2009 (Prié et al., 2011). A detailed analysis of the populations of this and other French rivers is under way (Soler et al., unpublished). In the Oise River (Seine Basin), Prié et al. (2008b) harvested and measured 163 dead shells, showing that the former population in this river was ageing (over-representation of adult stages, particularly the size class between 13 and

14 cm). In the Charente River, Nienhuis (2003) reported that the majority of the 42 living animals measured were between 10 and 15 cm except 2 smaller individuals, which measured 7-8 cm. From the same River, Prié et al. (2008a) reported that the dominant size classes of living individuals measured were between 11 and 12 cm.

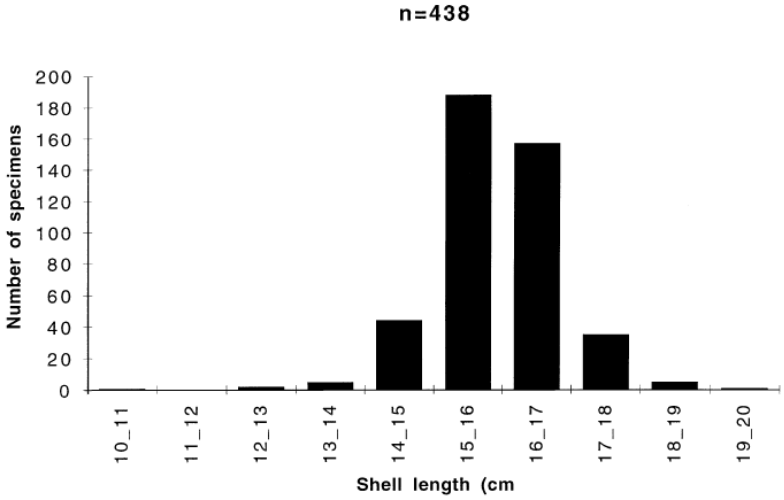

Figure 16. Size frequencies structure of the *Margaritifera auricularia* population on the Ebro Basin (Araujo & Ramos, 2000a).

Size classes reflect age classes. Nienhuis (2003), counting the shell rings on the periostracum, estimated that the larger animals that he found in the Charente (14.4 cm) had a presumed age of 43 years. Moreover, he estimated that most animals in this river with a length of 11-12 cm had an age of about 29 years.

More recently, Nakamura et al. (2018) described the growth pattern of the species in the Ebro basin and found that the Generalized von Bertalanffy and Richards models were the best fitted for the studied populations (Figure 14). They found that preadults with a median shell length of 9.3 cm, corresponded to ages from 11 to 14 years. Adult's shells, with an estimated age of 38–50 years, were measured in most cases between 14.4 and 15.3 cm.

According to these size classes, it seems that the currently known populations in Spain and France are senescent and the recruitment is very scarce or non-existent. It has been proposed that this non-recruitment situation could be explained by a change in the environmental quality (especially water quality) which could be detrimental for juveniles and / or by the extirpation of their host fish (Araujo & Ramos, 2000a; Prié et al., 2011; Araujo & Álvarez-Cobelas, 2016).

6. Habitat structure of sites where *M. auricularia* occurs

Large, almost sessile mussels that live in the middle and lower sections of rivers and large streams encounter manifold problems concerning the habitat structure (Wantzen et al. 2005, 2014). As they are filter-feeders, they require to settle in patches with high plankton flow rates. Patches with fast current, however, are subject to the most intensive substrate turnover during high-flow events, which could wash out the mussels. Riparian areas with high offers of terrestrial organic matter and good light conditions for the development of biofilms (which detach and drift down the river) would also be attractive sites but they bear the risk of drought during the drier phases. Lastly, sites with high sedimentation rates (also for organic seston) are threatened by coverage with inorganic sediments.

An ideal habitat should also be a site with a high probability get into closer contact with the host fish. *Unio crassus* may perform lateral movements in smaller streams to attract host fish with a water jet (see above). For *M. auricularia*, the triggers for glochidial release or the strategies to attract host fish are yet unknown.

While the habitats of other freshwater mussels colonising lower-order streams are relatively well studied (Hastie et al. 2000), very little is known about the distribution of freshwater mussels in larger rivers. Pusch et al. (2001) report on Anodontidae in a lake-river system of the Spree, Germany, where the lakes protect the mussels from excessive sediment load, reduce erosion risk, and provide large amounts of plankton.

Margaritifera auricularia has a remarkable capacity to dig into sediments, which may help it to avoid extreme droughts, and which possibly is a mechanism to remove fowling by dreissenids. However, this retreat is limited by the penetrability of the sediments, and the occurrence of impermeable layers, specifically in canals. Its lateral movement capacity is unknown, so far. If we assume that they are similar to other unionoids (e.g. *Unio pictorum*, KMW, pers. obs.), they are limited to ca. 1 m*day^{-1} in soft bottom substrates. In the case of spates, this would not be sufficient to save the population.

The study of habitat structures of extant populations in France and Spain is hampered by the fact that we are dealing with relic populations, which do not necessarily live under their ideal habitat conditions but rather at places where they have not yet been eradicated. For example, the population in the Canal Imperial in Spain is regularly threatened by drought in summer, low temperatures in winter, and regular dredging to remove excessive sediment. It can only survive due to active help by conservationists who store them periodically *ex situ*. The population in the Luy, France is trapped between two dams and has to cope with the existing sediment structure. In the Charente, the population is threatened by the upwards migration of a sediment layer due to increased sedimentation upstream the St. Savignien dam. Lastly, it is unknown to which extent the population of the Vienne has been impacted by the dam removal and subsequent downstream movement of fine sediments (Rodrigues et al., unpublished). Juveniles smaller than 6 cm (including interstitially living, early stages) have not been found in the wild for several years, their preferential habitat is unknown. Thus hardly any population found at the moment is representative of an “ideal” habitat. From the currently available information we can infer the following.

Margaritifera auricularia is a specific hard water species (but see below), calcium levels in the River Ebro being around 150 mg/l. Several physico-chemical analyses of the water have been taken where the Canal Imperial population lives. Range values of these factors during a complete year indicate that the habitat of *M. auricularia* is basic, well oxygenated, subsaline, beta-alpha meso-saprobic and oligo-hyperthophic water. Temperature in the Imperial Canal ranged between 7.4 (March) and 22.9 °C (August) (Araujo & Ramos, 2000a). The habitat requirements for *M. auricularia* were for Temperature (15,2°), pH (8), dissolved oxygen (9.6 mg/l), conductivity (438µS/cm), total phosphorous (0,07 mg/L), nitrates (12,5 mg/L), nitrite (0,04 mg/L), orthophosphate (o,1 mg/L), ,calcium (64 mg/L), organic carbon (3,4 mg/L), and the sediment structure in more than 70% was sandy-gravellish or gravellish, with moderate flow velocities (Soler et al, unpublished), confirming studies by Araujo & Ramos (2000), describing *M. auricularia* as a very sedentary species living sediment consisting of pebbles, followed by gravel and sand. Freshwater mussels feed by filtering organic material from the water. They particularly dislike siltation, eutrophication and water impoundment. A clean and well oxygenated sediment is very important for juveniles for if the substratum becomes clogged with silt, they can no longer obtain oxygen and die. Nevertheless, data on autecological requirements in the early years of the life of the mussels are still lacking.

The only place where the behaviour of the species has been studied is the Canal Imperial de Aragón, where it lives partially buried in clay-sand and gravel beds, sometimes (especially in the breeding season) nearly vertical with the posterior end exposed and sometimes horizontal, showing only the dorsal margin. However, adults are capable of active mobility and may burrow into the sediment for a few metres, leaving very obvious traces (about 8 cm wide) when the water level is low. Old data from Haas (1916, 1917) situated the species in quiet river pools at depths of up to 6 m. There were flourishing populations in the meandrous areas of the middle course of the Ebro, generally immediately downstream of small waterfalls, where hundreds of specimens were easily collected at low water many years ago (Araujo & Álvarez-Cobelas, 2016).

7. Known and potential host fish of *M. auricularia*

Like most freshwater mussels, *M. auricularia* depend on fish for hosting the metamorphosis of their larvae during their early life stage. Knowledge on host fish and the relationship between mussel and host fish is essential in any attempt to preserve endangered freshwater mussels. On the basis of data by Preece (1988), who found archeologically pieces of *Acipenser sturio* (Linnaeus 1758) and *M. auricularia*, Altaba (1990) hypothesised about the possible specificity between the glochidium of *M. auricularia* and the European sturgeon, a relict fish in European rivers and practically extinct in Spain (Elvira et al. 1991; Blanco & González 1992). Both species have been declining since the first half of this century.

Successful infestations have recently been carried out in aquarium experiments using *M. auricularia* glochidia and specimens of *Acipenser baeri* , an exotic sturgeon (Araujo & Ramos, 1998a, b, 2000a, b). Metamorphosed juveniles have also been obtained with artificial infestations of *Salaria fluviatilis* (the blenny river fish), a native fish from the Ebro basin, also endangered and protected by law (Araujo et al ., 2001).

Although tested in 22 fish species (see summary in Table 2), complete metamorphosis of *M. auricularia* glochidia into juveniles has only been proven to occur in four sturgeon species (*Acipenser sturio*, *A. naccarii*, *A. ruthenus* and *A. baerii*) and the river blenny (*Salaria fluviatilis*) (Figures 17, 18; Araujo & Ramos, 1998b, 2000b; Araujo et al., 2001; Altaba & López, 2001; Araujo et al., 2003; López & Altaba, 2005; López et al., 2007; Nakamura et al., 2012). All these host species have been identified by experimental infestations conducted under laboratory conditions. Araujo et al., (2001) conducted electrofishing surveys in the Canal Imperial but they did not find any naturally infested fish.

Table 2: Artificial infestation in *M. auricularia.* Results prior to the LIFE project. . ? = no value reported.

Species	Number of specimens	Duration of encystment in days	Metamor phosis	References
Acipenser baerii	5	30	Yes	Araujo, & Ramos 2000a
	4	49	?	Lopez & Altaba, 2005
Acipenser naccarii	9	30	Yes	Araujo et al., 2003
Acipenser sturio	2	19-39	Yes	López et al., 2007
Acipenser ruthenus	?	?	Yes	Nakamura et al., 2012
Salaria fluviatilis	2	42-45	Yes	Araujo et al., 2001
	6	31	Yes	Araujo et al., 2003
	11	26-40	Yes	Lopez & Altaba, 2005
Achondrostoma arcasii	1	3	No	Araujo et al., 2001

Species	Number of specimens	Duration of encystment in days	Metamor phosis	References
Alburnus alburnus	10	?	No	Lopez & Altaba, 2005
Anguilla anguilla	3	4	No	Araujo et al., 2001
	8	9	No	Lopez & Altaba, 2005
Barbus haasi	2	?	No	Lopez & Altaba, 2005
Barbus haasi & Luciobarbus graellsii	12	6	No	Araujo et al., 2001
Carassius auratus	10	6	No	Lopez & Altaba, 2005
Chelon auratus	9	?	No	Lopez & Altaba, 2005
Cobitis paludica	1	0	No	Lopez & Altaba, 2005
Cyprinus carpio	7	0	No	Lopez & Altaba, 2005
Gambusia holbrooki	45	15	?	Lopez & Altaba, 2005

Species	Number of specimens	Duration of encystment in days	Metamor phosis	References
	9	8	?	Araujo et al., 2003
Gobio gobio	4	0	No	Lopez & Altaba, 2005
Luciobarbus graellsii	35	2	No	Lopez & Altaba, 2005
Mugil cephalus	4	?	No	Lopez & Altaba, 2005
Parachondrostoma toxostoma	20	6	No	Lopez & Altaba, 2005
Scardinius erythrophthalmus	5	0	No	Lopez & Altaba, 2005
Tinca tinca	1	?	No	Lopez & Altaba, 2005

The European sturgeon, *A. sturio*, is considered its primary natural host. This assumption is based on the following facts: 1) the historic distribution of *A. sturio* matches with the original geographic range of the GFPM (Araujo & Ramos, 2000b, 2001b), 2) both species occur together in Pleistocene deposits (Preece et al., 1983; Preece, 1988) and 3) both species have encountered tremendous population declines in the late 19th and early 20th centuries. Nowadays *A. sturio* is virtually extinct and only one remaining reproductive population in Europe is known to the Garonne River (France) (Gessner et al., 2010). On the other hand, neither *A. naccarii,* nor *A.*

ruthenus or *A. baerii* co-occur with current living *M. auricularia* populations, and the distribution of *S. fluviatilis* only overlaps with that of the mussel in the Ebro Basin.

Figure 17: *M. auricularia* releasing glochidia and developing embryos near an *Acipenser baerii* specimen (R. Araujo, unpublished picture).

Figure 18: *Salaria fluviatilis* on a *M. auricularia* specimen (R. Araujo, unpublished picture).

Recent, albeit weak, natural reproduction of *M. auricularia* , long time after the extirpation of the "classical" host fish, the European sturgeon, indicates that there must be other fish capable of carrying glochidia of the mussel until their full development and excystment. A critical revision of the so-far known host fish is given in Soler et al. (2018a, under revision). There is an essential difference between the “physiological host”, i.e., a fish species that may be successfully infested and carry glochidia until full development under laboratory conditions, and the “ecological host”, i.e., a species that is a physiological host and it also co-occurs at the same habitat and period with the gravid adult mussels. While physiological hosts may be used for catch-infest-and release techniques (only if they naturally occur in the respective river section, to avoid alienation of the local fish community), only ecological hosts may provide a sustainable, natural reproductory success, and should be target of habitat and species protection actions.

We hypothesised that *M. auricularia* has several host fish and that they have a marine origin and or amphidromous migratory fish (Soler et al. unpublished) and suggest to follow this line of investigation (e.g., by testing eel, *A. anguilla* or river lamprey, *Lampetra fluviatilis*) in the future. So far, the best candidates for this technique are *Salaria fluviatilis* for the Ebro, and *Gasterosteus aculeatus* Soler et al. 2018a) and *Petromyzon marinus*, the sea lamprey for the french river systems project (Soler et al. under review). The Wels (*Silurus glanis*) also could be proven to maintain the full development cycle of *M. auricularia* but it does not seem to be an ecological host.

8. Human impacts on and conservation of *M. auricularia*

8.1 Conservation status of *M. auricularia*

Freshwater mussels are amongst the most imperilled invertebrates in the world (Bogan, 1993; Araujo & Ramos, 2000a; Young et al., 2001; Lydeard et al., 2004; Strayer et al., 2004; Strayer, 2008). In North America, 74% of the known 300 species are imperilled and 37 species are considered extinct (Williams et al., 1993; Lydeard et al., 2004). In Europe, of the 16 species recognized, 3 are critically endangered, 2 are vulnerable, and 5 are near threatened (Lopes-Lima et al., 2017). The dramatic decline of *M. auricularia* in Europe has run parallel to that of other naiads in the world. Indeed, both European *Margaritifera* species (*M. margaritifera* and *M. auricularia*) are critically endangered and listed as "fauna requiring special measures to be takes for their protection" under the Bern Convention on the Conservation of European Wildlife and Natural Habitats.

Globally, *M. auricularia* is assessed as Critically Endangered (criterion A2ac), by the IUCN (IUCN 2010, Prié, 2010a). The species is also listed on Appendix IV of the EEC Habitats Directive, which includes animal and plant species of European interest requiring strict protection, and on Appendix II of the Bern Convention, which includes strictly protected animal species and their habitats. It is therefore considered as Critically Endangered A2ac (see regular updates on IUCN webpage http://www.iucnredlist.org/details/12798/0).The Recommendation number 51 the Standing Committee of the Convention on the Conservation of European Wildlife and Natural Habitats (December, 6th, 1996) considered *M. auricularia* as a first priority species requiring an Action Plan (Council of Europe, 1999). In France, it is regionally listed in the „Liste rouge des mollusques de la région Centre (2012)" with criterion B2ab(iv), in Germany, it is listed as „extinct" on the „black list" of extinct species. This has consequences for potential re-introduction, as the species would need to be analysed prior to re-introduction in the same way as a non-native species (Bundesamt für Naturschutz, pers. comm. to KMW, 2012).

Although the conservation of freshwater mussels in other parts of the world is much less well known, the situation seems to be similar (see a review in Patterson, et al, 2018).

Nevertheless, mussels are not the only imperiled group of animals in freshwater ecosystems. Nearly 12,000 species of freshwater invertebrates are either extinct or imperiled globally (Strayer, 2006). Of the approximately 600 species of crayfish worldwide, 32% are in danger of extinction (Richman et al., 2015). Although only about 5,800 of the 15,570 described species of freshwater fish (37%) had been assessed, 30% of them are listed as extinct, extinct in the wild or threatened with extinction (Carrizo et al., 2013). These statistics clearly show that freshwater ecosystems and the species that inhabit them are being decimated globally. In fact, extinction rates in freshwater ecosystems have been estimated to be five times higher than in terrestrial systems (Ricciardi & Rasmussen, 1999).

8.2 Human impacts conducive to the population decrease of *M. auricularia*

Multiple factors derived of human activities have been identified as responsible for the worldwide decline of freshwater mussels, including habitat loss, pollution, host species decline, invasive species and overexploitation (Bauer, 1988; Bogan, 1993; Neves, 1999; Araujo & Ramos, 2000a; Young et al., 2001; Lydeard et al., 2004; Strayer, 2006).

These impacts may reduce the distribution range of species, leading, according to Strayer (2008), to 1) a diminished role of freshwater mussel species in local communities and ecosystems; 2) loss of genetic diversity; 3) increased distance and reduced dispersal among the remaining populations, which may lead to further losses of populations due to metapopulation dynamics; and 4) increased risk of extinction for species.

Habitat destruction and degradation due to dam construction has been identified as one of the most important causes for freshwater mussels decline (Bogan, 1993; Layzer et al., 1993; Neves 1999; Vaughn & Taylor, 1999; Lydeard et. al., 2004; Strayer, 2006). There are more than 45,000 large dams, and one million smaller dams, on the world's streams and rivers today (Jackson et al., 2001, Malmqvist & Rundle, 2002) and the trend in dam construction is still rising (Zarfl et al., 2015). Dams create upstream lentic conditions that are inadequate for lotic species, significantly modify the hydrology of the river downstream of the dam, and constitute a barrier for species dispersal along the river (Layzer et al., 1993; Neves, 1999; Vaughn & Taylor, 1999; Strayer, 2006; Araujo & Álvarez-Cobelas, 2016). Other significant causes of habitat loss are sedimentation, channelization and dredging (Bogan, 1993; Lydeard et al., 2004; Strayer, 2006). Sedimentation often results from other habitat modifications such as dam construction, as well as of poor agricultural practices. It causes reduced oxygen concentrations in the interstitial substrate water, which is fundamental for the survival and development of juvenile mussels, and therefore, is an important factor leading to recruitment failure (Buddensiek et al., 1993; Gatenby et al., 1997). Channelization drastically modifies the habitat and dredging is known to have caused the elimination of whole mussel beds (Killeen et al., 1998; Aldridge, 2000).

Water quality and pollution have become major problems to freshwater mussels (Bauer, 1988; Bogan, 1993; Neves, 1999, Lydeard et al., 2004; Strayer, 2006). Pollution was considered the main cause of decline of *M. margaritifera* in Europe (Bauer, 1988). Strayer and Malcom (2012) found that concentrations of un-ionized ammonia greater than 0.2 mg N/L in interstitial water where correlated with recruitment failure in south-eastern New York rivers.

The disappearance of natural hosts disrupts their reproductive cycle of the mussels, so that recruitment of juveniles is no longer possible. Several studies have shown the importance of the availability of appropriate host fishes during the reproductive period for the dispersal and population status of unionoid mussels (Bauer, Hochwald, & Silkenat, 1991; Watters, 1992; Bogan, 1993; Vaughn & Taylor, 1999; Österling, Greenberg, & Arvidsson, 2008). When host fish disappear, mussel populations decline and finally become extinct (Kat & Davis, 1984, Watters, 1995), whereas reintroduction of host fish species may result in recovery of previously disappeared mussel populations (Smith, 1985). Since identification of suitable hosts is a key element for freshwater mussel conservation, many studies have been devoted to this issue (e.g. Lefevre & Curtis 1912; Stern & Felder 1978; Zale & Neves 1982; Berrie & Boize 1985; Haag & Warren Jr. 1997; O'Dee & Watters 1998; Araujo et al. 2001; Soler et al. 2018a, and unpublished).

Introduction of invasive species is a relatively recent threat to freshwater mussels, but has the potential to become one of the major concerns for their conservation (Bogan 1993; Lydeard et al. 2004). Invasive bivalve species such as the zebra mussel (*Dreissena polymorpha*) and the Asian clam (*Corbicula fluminea*) have been reported to cause the decline or extinction of native freshwater mussel populations (Bogan, 1993; Ricciardi et al., 1998; Neves 1999; Yeager et al. 1999; Burlakova et al. 2000; Lydeard et al. 2004; Strayer 2006). They compete with native mussels for resources and can drastically modify the water quality and substrate. It is yet unknown to what degree invasive mussels may serve as "troyan horses", i.e., carry diseases or parasites that have a negative (or even deleterious) effect on native unionoids.

Introduced fish species may also be an important threat to freshwater mussels by significantly contributing to the disappearance of native fish faunas (Ribeiro et al. 2009), which are essential as hosts for unionoids.

In some areas of the world, overexploitation has been a major cause of decline of some freshwater mussel species with commercial interest. However, in most cases the associated industries have decreased in importance or are now extinct. The freshwater pearl mussel *M. margaritifera* has been heavily harvested in Europe for freshwater pearls, but that activity is now extinct (Young et al., 2001). In the United States, mussels from the Mississippi drainage support a multi-million dollar commercial shell industry for the manufacture of buttons (Neves, 1999) although this activity is now regulated and managed to prevent overexploitation.

The high level of imperilment in global freshwater mussel populations combined with their important ecological function is causing great concern among scientist, prompting the creation of freshwater mussels conservation programs around the world. Artificial propagation has become a key conservation management strategy for restoration and recovery of freshwater mussels. Large numbers of juveniles can be produced in captivity using identified host fishes and then reintroduced to the wild. The release of juveniles raised to larger size is desirable because of the high natural mortality of newly transformed juveniles (Jones et al 2006; Araujo et al., 2018). Because of the difficulty of culturing juveniles, the release of native host fishes infected with glochidia has been shown to be an economic alternative that has been successfully applied in different conservation programs (Altmüller & Dettmer, 2006; Araujo et al., 2015; Carey et al., 2015). The action plans of many species already include artificial propagation as necessary for their recovery (e.g. Araujo & Ramos, 2001a). Nevertheless, these strategies should be regarded as temporal measures while the natural recruitment is recovered by habitat conservation measures (Gum et al., 2011; Patterson et al., 2018).

Many countries or regions of the world have created their own legal instruments to protect species and habitats. In Europe the Habitats Directive (Council Directive 93/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora) was created to this purpose, and freshwater mussel species such as *M. auricularia, M. margaritifera, Unio crassus, U. mancus* (=*U. elongatulus*) and *Microcondyleae bonelli* (=*M. compressa*) were listed as requiring conservation or management actions.

Although there are important knowledge gaps that prevent accurately identifying the causes of the decline of *M. auricularia*, it has been proposed that these are manifold and similar to those of other endangered freshwater mussel species. The following are the main threats that have been related to its current critical situation:

- The almost disappearance of its primary host, *Acipenser sturio* (Altaba, 1990; Araujo & Ramos, 2000a; López et al., 2007) in Europe and the rarefaction of *Salaria fluviatilis* in the Ebro basin (Araujo & Álvarez-Cobelas, 2016).
- The physical degradation of watercourses. In Spain, direct impacts on the populations of *M. auricularia* have been reported due to alterations in the channels where the species is present, mainly due to the dredging and paving of their beds (Gomez & Araujo, 2005). In addition, in the Ebro, local extirpations have been related to flow diversion for hydroelectric power plants (Araujo & Álvarez-Cobelas, 2016). The natural biotope of *M. auricularia*, being moderately rejuvenated benthic habitats of large rivers of Western Europe is clearly disappearing, in rivers such as the Rhine, only few near-natural habitats can be found, and the species has been extinct for centuries (Wantzen et al. 2019).
- Siltation due to human activities such as intensive agriculture and dams has been also identified as an important threat (Araujo & Ramos, 2001a; Prié et al., 2011). Furthermore,

dams constitute obstacles preventing the free movement of migratory fish and altogether with overfishing and water pollution, are related to the decline of diadromous fish species such as *A. sturio* in Europe (Limburg & Waldman, 2009; Mateus et al., 2012).

- Overharvesting from the 18th to the beginning of the 20th century. Despite its common name, *M. auricularia* does not produce pearls. However, it is the thickness of its mother-of-pearl which is very striking and for which it has been exploited commercially. In the Charente and Ebro rivers, extraordinarily high quantities of specimens were extracted for the manufacture of buttons and for the manufacture of knife handles (Bonnemère, 1901; Álvarez-Halcón, 1998; Araujo & Álvarez-Cobelas, 2016).
- Introduction of invasive species. The effects of the Asian Clam (*Corbicula fluminea*) on *M. auricularia* populations have not been well studied but it could be of major concern given the filtering capacities of those small bivalves (Pigneur et al. 2014). On the other hand, the Zebra Mussel (*Dreissena polymorpha*) seem to be harmful by fixing on the valves of the Giant Pearl Mussel, hindering the movement of their valves (Araujo, 2006).
- Water pollution has also been identified as an important threat factor, although tolerances to pollution at the adult stage and especially at the larval and juvenile stages have not been determined by ecotoxicological studies.

8.3 Conservation activities in favour of *M. auricularia*

Spain and France have elaborated National Strategies and Action Plans for its conservation (MARM, 2009; Prié et al., 2011) following the recommendations proposed in the European Action Plan for *Margaritifera auricularia* (Araujo & Ramos 2001a).

As a consequence of this status, it is strictly forbidden to disturb or sample juveniles or adults of the species, or to modify their habitats, without previous permission. The manipulation that involves the death of animals is of course forbidden and the number of individuals that can be manipulated is usually very small. Permits need to be demanded at the regional authorities for environmental conservation, and may be very time-consuming, as the responsible evaluation teams often do not decide ad hoc, but wait for regular meetings. This has important consequences for the study of a species with which it is already difficult to work due to the deep, fast-flowing and turbid nature of the habitats in which it is found, which in many cases requires the use of scuba diving techniques for its location.

Nevertheless, despite the difficulties and given its critical situation, several conservation programs have been devoted to the species. Among these, the LIFE projects stand out for their importance. LIFE is the European Union’s financial instrument supporting environmental, nature conservation and climate actions projects thought-out the EU. In Spain, two LIFE projects have been devoted to the conservation of *M. auricularia*: the project LIFE04 NAT/ES/000033 leaded by the Regional Government of Catalonia between 2001 and 2004 and the project LIFE04 NAT/ES/000033 leaded by the Regional Government of Aragón between 2004 and 2007. After the end of the project, the Regional Government of Aragón have maintained the financial support and nowadays this is the longest conservation program for *M. auricularia* with more than 13 years of experience.

In France, the LIFE+ project “LIFE13 BIO/FR/001162: Conservation of the Giant Pearl Mussel in Europe” is the most comprehensive conservational effort deployed on the species up to date. Lead by the University of Tours and the Conseil Départemental Charente-Maritime, it was started in 2014. The overall goal of this project is the conservation and the reinforcement of populations of this critically endangered species in France and more widely in Europe. For a detailed manual on restoration and reintroduction techniques, please, refer to Volume 2 – Technical Manual: Monitoring, artificial reproduction, rearing techniques, and suggestions for habitat conservation produced by the LIFE project.

9. Conclusions – (How) can *Margaritifera auricularia* be saved in the Anthropocene?

The fate of the Giant Freshwater Pearl Mussel is uncertain. The number of threats and their impact strength are very high, the cumulative or synergetic effects of multiple stressors (Tockner et al., 2010, Statzner & Beche, 2010, Hering et al. 2015) on the species is completely unknown. The restoration of hydromorphological deficits due to centuries of riverine mismanagement are much more cost-intensive and politically less feasible than that of water pollution, which is showing positive trends in Europe (Wantzen et al., 2019), and we can assume that hardly any of the extant population still resides under ideal hydromorphological conditions. We are completely blind concerning the effects of diseases, pesticides (Malaj et al., 2014) emerging substances, and specifically of endocrine disruptors (Gavrilescu et al., 2015). Climate change and recurrent heat-waves and prolonged drought periods in European rivers (Arora et al., 2016 and citations therein) increase the chances of exotic invaders and reduce the survival chances of the native Fauna in Central-Western Europe (Hesselschwerdt & Wantzen, 2018). The deplorable status of the European Sturgeon as one of the most important host fishes is a fact that will not change soon, in spite of efforts to reintroduce the species (Carrera-Garcia et al. 2016). All studied populations of *M. auricularia* are shrinking at an amazing pace (Prié et al., 2017), including recent observations on the Spanish populations (Soler & Araujo, unpublished data).

Despite these discouraging facts, there are some findings allowing a modest expression of optimism. The genetic variability found in *M. auricularia* was in the usual ranges for unionoids, suggesting the real possibility of species recovery. There is still a large population in the Charente river, and we remain optimistic that the planned dredging action, which was targeted to be executed during the LIFE project, will still take place in the next year. This would reduce the menace by silting up of the habitats of this population. The LIFE project has evidenced three so far unknown alternative host fish species. This does not only show that *M. auricularia* is a host fish generalist (i.e. further host fish species may also be detected), but it also offers the opportunity to perform cost-effective infestation and immediate release of electro-fished host fish. Moreover, the findings by the LIFE project and in the project in Aragon (Nakamura et al.

2018b) show that rearing of juveniles is possible, even though the efficiency of these methods require further refinement.

To end with, we formulate a catalogue of research questions that should be addressed in the next years:

- What are the causes for the recent increase of the population decline (age effects, climate change, (cumulative effects of) chemical pollution, parasites, new diseases, competition by invasive filter-feeders?)
- Which are the ideal conditions for glochidia meeting the host fish? (Hydrology, water chemistry, perceptional cues for host fish, timing vs. climate change, ...)
- How to improve reintroduction of sturgeon specifically in rivers bearing *M. auricularia*?
- How to protect infected fish better?
- What are the ideal habitat parameters for *M. auricularia* juveniles, and how can these be restored? (sediment clogging, anoxic zones, intermediate sediment turnover....)
- What are the most efficient conditions for artificial rearing of *M. auricularia* juveniles, at which period (size class) they have the highest chance to survive when released?
- Which release technique is most efficient, considering that *M. auricularia* juveniles are extremely small and that cage-like structures risk to suffer sediment clogging and development of anoxic zones?
- How to protect habitats for *M. auricularia* adults better?

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