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Methods

Freshwater mussel (Bivalvia, Palaeoheterodonta) larvae in natural history collections: an underutilised resource

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Abstract

Natural history collections which store the soft bodies of freshwater mussels can provide information for research and the public in a comparatively simple way that has so far been insufficiently considered. This involves the examination of larval forms, which are often present, unnoticed, in gravid animals. The features of the larvae provide important information about the systematic position of the bivalves and their study in light microscopic or electron microscopic images can illuminate the importance of freshwater mollusc collections. A processing routine and a standard for the presentation of freshwater mussel larvae is proposed. The processing of old samples is challenging, as their condition cannot be predicted, examples of which are discussed. Nevertheless, even samples that are not in optimal condition can provide useful information. In view of the global threat to this group of animals, any additional information is also of great value for applied aspects of species conservation.

Key words: Glochidium, lasidium, methods, presentation, processing, Unionida, valorization

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Introduction

Natural history collections which house freshwater mussel (Unionida) specimens primarily have only the dried shells of these animals. However, if whole animals, i.e. with soft bodies, are also kept in a wet collection (usually in alcohol), there are additional possibilities for the evaluation and presentation of this material. This is the case with specimens that are gravid and have larvae in their gills. The larvae, of which there are two very different types (glochidium, lasidium), remain in the marsupial gill chambers for a period of several weeks to several months and can thus enter a collection unnoticed.

The presence of larvae is generally not included in inventories of global specimen collections (e.g. Ituarte and Mansur 2020). This is unfortunate, because the larval stage of many taxa are unknown. Even in the otherwise well-studied fauna of the USA, with a total of 301 Unionida species, 115 of these species (38%) lack data on the glochidia (Hopper et al. 2023). Moreover, larval characteristics can provide important clues for the taxonomy and

systematics of the species (Inaba 1964; Panha and Eongprakornkeaw 1995; Hoggarth 1999; Sayenko 2006; Sayenko et al. 2021). Glochidium and lasidium type larvae are small (60–400 µm), but their shells nevertheless have some distinctive structures. These are mainly known for the glochidium type ("hooks", "teeth", surface microsculpture) and much less so for the lasidium type. The soft bodies of Unionida can be examined for the presence of larvae with relatively little effort. If gravid specimens are found, it is of great benefit to remove a small proportion of these larvae and display them for future examination and research.

Here, we explain and propose a routine procedure for the examination, removal, and imaging of larvae present in freshwater mussel wet-collections. We address which information experts would need for determining whether they should access the collection to for specific research question purposes. Provisioning this information increases the scientific value of a collection and increases the effectiveness of research. We present findings from the Senckenberg Research Institute collection as examples. We also supplement these with the second author's many years of experience in the study of glochidia.

Methods

The results we present here and the recommendations derived from them were obtained using material from the malacological collection of the Senckenberg Research Institute and Natural History Museum (SMF) (Table 1) and the collection of the Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch of Russian Academy of Sciences (FSCEATB FEB RAS) (Table 2, abbreviated as FSCEATB). The collection numbers for the latter are provisional working numbers. They will be changed once the material has been transferred to a natural history collection. We used a stereo microscope Nikon SMZ1270 with DFK 33UX264 camera and a microscope Leica DM 2000 LED with Flexacam C1 at SMF and a Nikon light microscope and Zeiss stereo microscope with AxioCam MRc at the Biology and Genetic Engineering Center for Collective Use at the FSCEATB FEB RAS. SEM images were taken with a Hitachi TM4000Plus II tabletop scanning electron microscope at SMF.

Results

Findings from the collection of the Senckenberg Research Institute

Fresh material or material that has been well preserved over a short period of time is usually easy to prepare and display. In contrast, samples that have been stored for a longer period of time (around 20 years or more) and possibly under unknown circumstances are often much more difficult to assess. We describe in detail the findings of two such samples, which illustrate some of the problems encountered. Further results on samples that have been stored for different periods of time are presented in Table 1.

Physunio superbus (Lea, 1843), SMF 346003, Malaysia, received 1923 from Museum Hamburg

These glochidia were deep brown-red in color and still within their egg membrane. Little could be seen under the light microscope. The cause of this coloration is unclear, it is probably due to the circumstances of transport from Malaysia or storage (sealing wax for storage jars?). Various means were tried to achieve a brightening of the objects: storage in distilled water for several days, use of detergent solution made from commercially available cleaning tabs, alone or with subsequent ultrasonic cleaning bath, use of sodium hypochlorite solution from commercial bleach. None of these approaches had the desired success, a clear lightening or even discoloration of the larvae was not achieved. To a small extent, the egg membranes could be destroyed and the larvae released from them. The SEM image showed only flat, rather shapeless and obviously unstable formations that could not be interpreted. An illustration of these larvae in the SEM was previously given by Panha and Eongprakornkeaw (1995). It is therefore possible that these larvae were not yet fully developed. In comparison, it can be shown what can still be recognized in the microscope even in the unsatisfactorily preserved old material: type and overall shape (semielliptical), dimensions (length, height, approximate width), presence of a larval thread (not mentioned previously). But the asymmetrical, long-conical appendix on a shell valve was not noticed. As a result, an important feature that would have led to a clear assignment to a tribe (Contradentini) was not recognized.

Acuticosta chinensis (Lea, 1868), SMF 346002, China, received 1912 from M. Kreyenberg

In the binocular under low magnification, only dark objects could be discerned, just a few with a clearly defined shape. Most of them were spherical or opaque, i.e. either immature and still within their egg membrane or else covered by

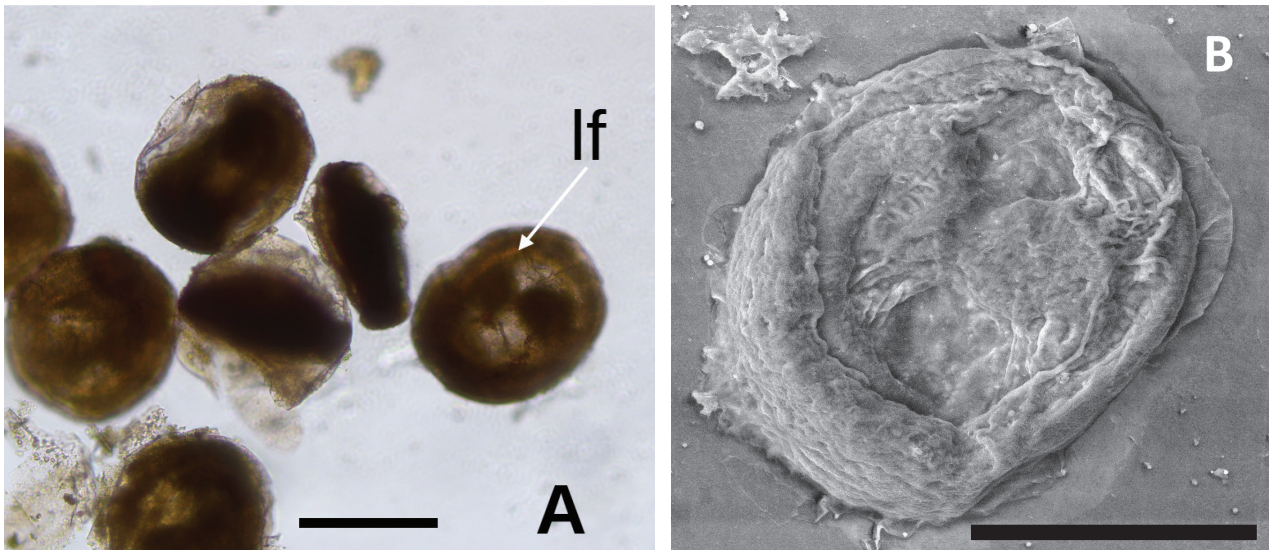

Figure 1. *Physunio superbus* (Lea, 1843) (SMF 346003). **A.** Microscope image; **B.** SEM image. Abbreviation: lf – larval thread. Scale bars: 100 µm.

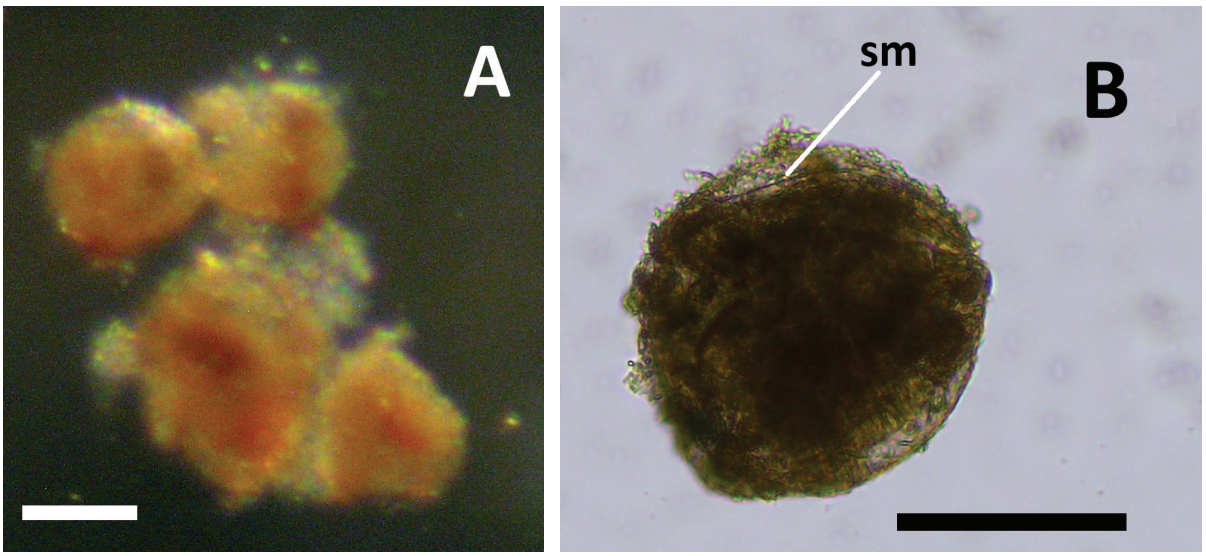

Figure 2. *Acuticosta chinensis* (Lea, 1868) (SMF 346002). **A.** Stereo microscope image; **B.** Microscope image. Abbreviation: sm – shell margin. Scale bars: 100 µm.

tissue. Some showed an outline that matched the SEM images of Wu et al. (1999). The type, dimensions, and approximate outline shape could be recognized and it did not seem useful to prepare a sample of it for the SEM.

Proposal for a processing routine for Unionida collection material with larvae

1. Examination of soft bodies for larvae

If not already done, it is recommended to separate the soft bodies from the adult shells. This allows the shells to be stored in a dry place and, if necessary, preserved to prevent the shell from cracking or the periostracum from flaking off. It also prevents the colors of the periostracum from changing in alcohol. It is good museum practice to label shells and associated soft bodies individually in order to ensure a clear assignment. Gravid specimens containing larvae or their pre-stages can usually be recognised by their thickened, swollen gills (Fig. 3). All four gills, only the outer or inner gills (depending on the bivalve taxon) or only certain areas of the gills, usually the centre ones, may be swollen (Wächtler et al. 2001). With appropriate preservation, the filling may also result in a difference in colouration, when the changed colour of the filled gill, usually darkening, can also be an indicator of the full maturity of the larvae present inside.

2. Determination of the developmental stage of the marsupial gill filling

If the contents have not already leaked out, a very small opening is cut into the gill with a fine needle and forceps or scissors and a small sample of the filling is taken with the needle. Samples should be taken in the middle, thickest part of the marsupial gill because in some unionids, maturation in the gills is uneven and the chance of detecting mature larvae is best in the central portion of the marsupium.

Table 1. Summary table of the glochidia series from the Senckenberg collection examined. In brackets, the type of source material, if not both shells and associated soft bodies.

Collection no. SMFSMF	Species	Sampling date	Results	
			Light microscope	Scanning electron microscope
Unionidae: Unioninae				
940/23	*Unio crassus* Philipsson, 1788	1914	immature stages, no larval shell yet recognizable	empty egg membranes only can be visualized
1026/5 (soft bodies)	*Unio tumidus* Philipsson, 1788	1914	mature, closed, mostly free	most shells cracked, hook not recognizable
346372 (glochidia)	*Unio crassus* Philipsson, 1788	2015	mature, free, already many empty shells	inner surface of some shells still covered with soft body
3088/4 (soft bodies)	*Lanceolaria gladiola* (Heude, 1877)	1911	mostly mature, all still in egg membrane	most shells covered with egg membrane, free shells fragile, broken
3063/18 (soft bodies)	*Acuticosta chinensis* (Lea, 1868)	1911	mostly mature, mostly still in egg membrane	nearly all shells completely covered with egg membrane
346002 (glochidia)	*Acuticosta chinensis* (Lea, 1868)	1911	probably mature, all in egg membrane	not observed
346010 (glochidia)	*Anodonta* sp.	1908?	mature, but many broken (softened) and/or in egg membrane	not observed
1307/10	*Anodonta cygnea* (Linnaeus, 1758)	1914	mature, partly open, free	inner surface of some shells still covered with soft body
3192/5 (soft bodies)	*Pseudanodonta complanata* (Rossmässler, 1835)	27.04.1923	not observed	generally fine, no soft tissue remains, some shells broken
346006 (glochidia)	*Pseudanodonta complanata* (Rossmässler, 1835)	27.01.1910	not observed	generally fine, no soft tissue remains, some shells broken, especially the thickened and angularly offset margin slightly detached from the shell surface (**Figs 6A, B**)
346334/3	*Sinanodonta woodiana* (Lea, 1834)	1989	not observed	inner surface of some shells still covered with soft body
Unionidae: Parreysiinae				
323062/20 (soft bodies)	*Coelatura choziensis* (Preston, 1910)	1931	probably mature, all in egg membrane	glochidia out of membrane, fragile, broken
348826/22	*Lamellidens marginalis* (Lea, 1834)	2014	mature, mostly free, some slightly gaping	generally fine, no soft tissue remains, some shells broken
Unionidae: Rectidentinae				
346003 (glochidia)	*Physunio superbus* (Lea, 1843)	before 1923	probably mature, all in egg membrane	shells covered with egg membrane, contents softened
Hyriidae				
346321/4	*Velesunio ambiguus* (Philippi, 1847)	1989	mature, free, already many empty shells (**Fig. 4C**)	inner surface of some shells still covered with soft body

The sample is inspected under a microscope at different magnifications to check for the presence of well defined, ripe larvae (Fig. 4). Eggs or early developmental stages do not need to be analysed further. Mature, fully formed (and therefore infectious) larvae are usually characterized by a well-defined outline with a clearly visible shell edge. In the case of hook-bearing glochidia (family Hyriidae, subfamily Unioninae), however, the shell edge may be clearly visible even before the ventral shell hook is formed, i.e., before the larvae reach infective maturity.

Table 2. Material from the FSCEATB collection.

FSCEATB no.	Genus, species	Sampling locality (all in Russia)	Sampling date	collectors	shown or used in figure
816/1-3	*Buldowskia* sp.	Primorye, Khanka Lake basin, Studenaya River near Zharikovo village	27.04.1994	L.A. Prozorova	3A, B
816/1					3C
without	*Cristaria plicata* (Leach, 1814)	Primorye, Khanka Lake basin, Nesterovka River	10.2000	M.B. Shedko	4A
1856-м.т.	*Beringiana beringiana* (Middendorff, 1851)	Okhotsk coast, Tauiskaya Bay, Ola River basin, Chistoye Lake	08.08.1997	not given	4B
1-2024/ Sin	*Sinanodonta schrenkii* (Lea, 1870)	Primorye, Ussuriysk urban district, Lotos Lake	24.04.2024	I.A. Rodionov, E.M. Sayenko	8

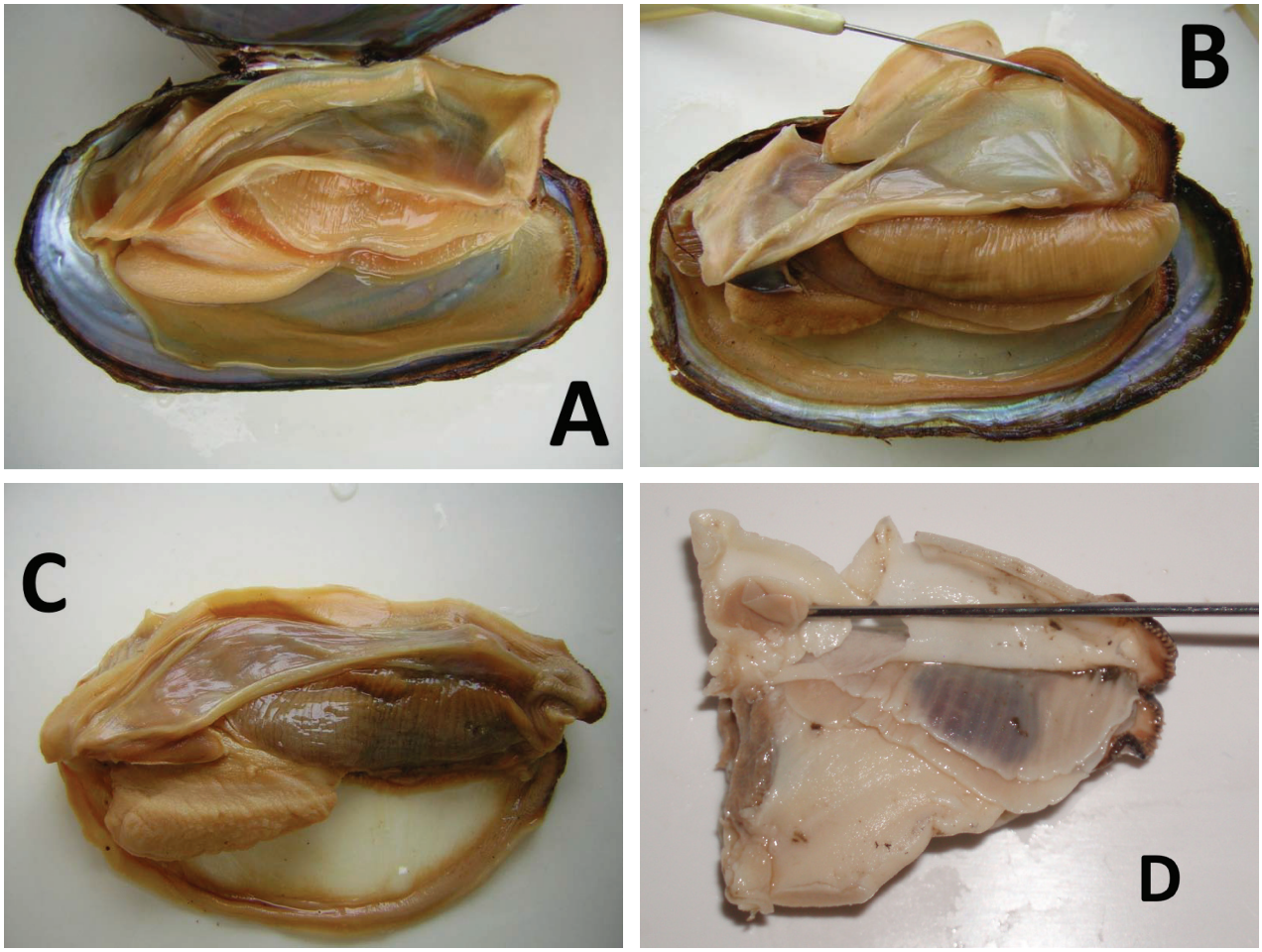

Figure 3. Examination of freshwater mussels for presence of filling of marsupial gills. **A.** Empty gills, light and thin; **B.** Outer gill (= marsupium) swollen but light, here with immature glochidia; **C.** Outer gill swollen and dark, here with mature glochidia; **D.** Outer gill partly filled. **A–C.** FSCEATB collection no. 816/1-3 (**C.** 816/1), *Buldowskia* sp.; **D.** SMF 348826 (specimen 14.1), *Lamellidens marginalis* (Lamarck, 1819).

3. Removal of a piece of a swollen gill

When mature larvae are present, a small piece is cut off from the lower edge of a filled gill. The cut is made as parallel as possible to the gill filaments and then parallel to the lower edge. This allows structural features of the marsupial gills to be recognised and depicted later.

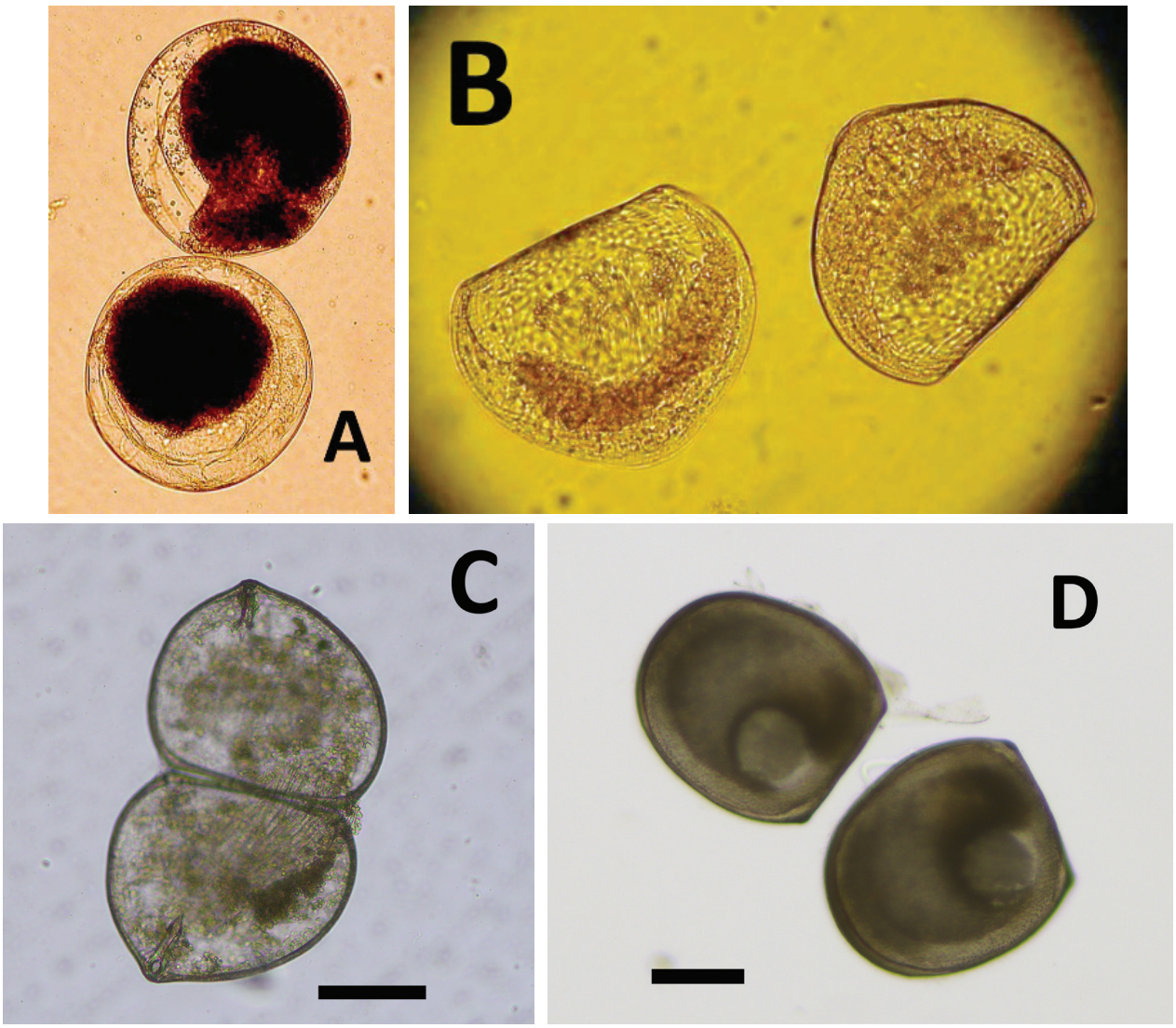

Figure 4. Immature and mature developmental stages of glochidia. **A.** Immature glochidia, *Cristaria plicata* (Leach, 1814) (without FSCEATB collection number); **B.** Immature glochidia with well-defined outline but ventral hook not yet visible, *Beringiana beringiana* (Middendorff, 1851) (FSCEATB collection no. 1856-м.т.); **C.** Mature glochidium with clearly visible shell rim and fully developed ventral hooks, *Velesunio ambiguus* (Philippi, 1847) (SMF 34632/4, from specimen no. 1); **D.** Mature hookless glochdia, *Lamellidens marginalis* (Lamarck, 1819) (SMF 384426/22, from specimen no. 14). Scale bars: 100 µm.

4. Separation of the larvae

The gill piece is best transferred to a micro-reaction vessel with a screw cap. If samples are to be analysed later on the SEM, fill this with distilled water now. If necessary, larvae are removed by shaking. For SEM, a subsample of this is transferred to another reaction vessel.

5. Documentation using the light microscope

Some larvae are photographed under the light microscope without further treatment. Well suited for this is a slide with a hollow section. A cover glass is not necessary. To facilitate the examination of the larvae, a drop of glycerine can be placed on the slide with the larvae. When mixing glycerine and water, the larvae

are rotated by the slow turbulent movement so that they can be examined from different angles.

The following characteristics should be visualised: size, outline shape, presence of a shell hook and/or a larval thread.

6. Optional: Documentation of structural properties of the marsupial gills

Details of the inner structure of the marsupial gills can be recognised under the binocular on the cut-out gill piece. The anterior/posterior cut surface is viewed for this purpose (Fig. 5). This allows the structure of the interlamellar septa to be recognised (complete, fenestrated, reduced).

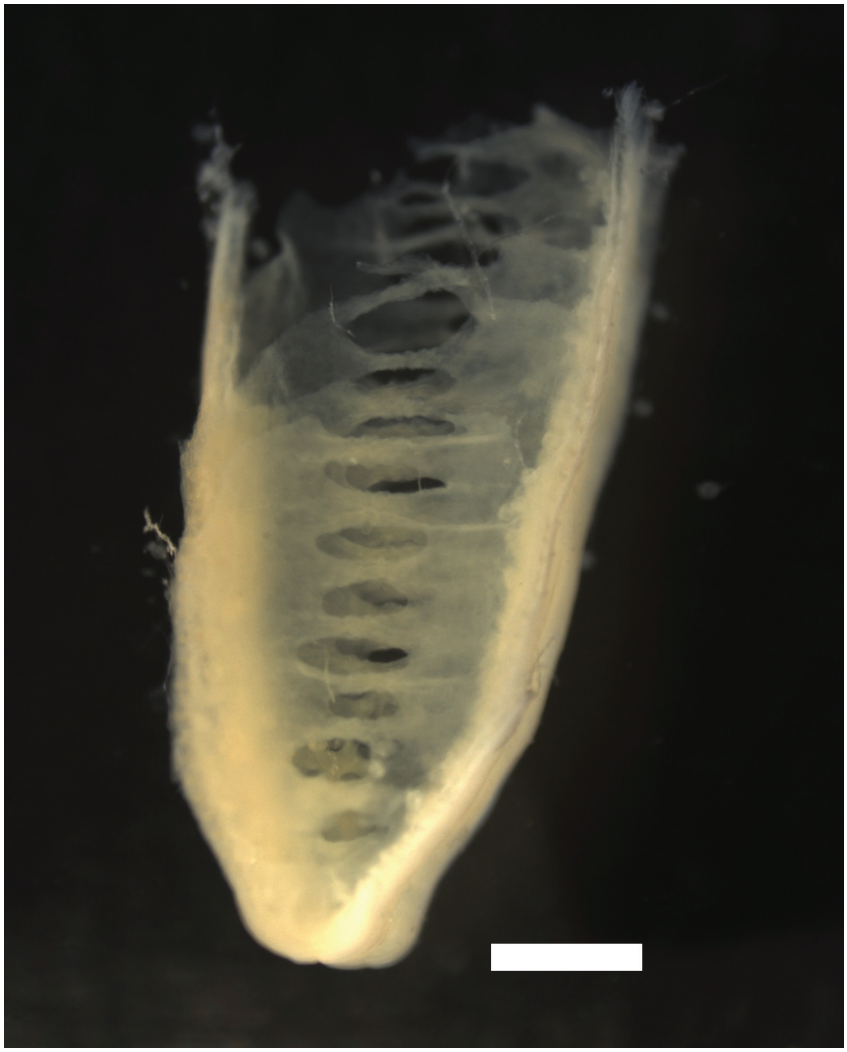

Figure 5. Transverse section through the lower half of the marsupium in frontal view, *Acuticosta chinensis* (Lea, 1868) (SMF 3063/18). Scale bar: 2 mm.

7. Preparation of glochidia for SEM

This procedure is essentially based on recommendations in Sayenko and Kazarin (2022). It dissolves the soft bodies and prepares the larval shells cleanly. Steps that deviate from this and their results are listed below.

Following step 4, the subsample (in reaction vessel with distilled water) is treated as follows:

- at least 5 times water exchange, pipette off the excess at the end;
- addition of 5% KOH solution;
- leave in the solution for 1.5 to 2 hours (gill pieces), larvae only for less time;
- shake several times in the meantime;
- at the end replace the solution with distilled water and shake vigorously;
- allow glochidia to settle, pipette off the excess (with tissue residue);
- repeat until no foam or bubbles remain on the liquid surface.

To avoid unwanted precipitation, the KOH solution should ideally be freshly prepared and stored correctly.

8. Mounting on SEM carrier

A small amount of larvae (10–30) is aspirated with a thin glass pipette and dropped onto the prepared SEM stub in a drop of water. The water is then allowed to evaporate (in a heating cabinet if necessary). N.B. that overdrying leads to disruption of the external microsculpture of the larva. It is possible to align individual objects with a thin needle or a bristle. This is best done when there is still some residual moisture on the substrate. But even after this, the sticky surface still allows the alignment of individual objects to be changed, however, there is an increased chance of damaging the hooks and even the larval shells. If sputtering (with e.g. gold, gold/palladium, platinum) is assumed, then glochidia should be in absolute alcohol. This can be achieved by adding undiluted alcohol to the stubs with glochidia a couple of times.

9. Visualisation in the SEM

The use of a modern table-top SEM is recommended. It allows the objects to be displayed without prior sputter coating.

The following characteristics should be visualised in addition to those already mentioned for the light microscope: Overview of the shell hook (if present), details of dentition (denticles/microstylets) on shell margin and/or shell hook, thickness (curvature) of the shell, external microsculpture of the glochidial valves (if microscope magnification allows).

Discussion

Importance of larval characters

Glochidium-type larvae have characteristics that can be of great importance for the systematic classification of the animals. Table 3 is based on Pfeiffer and Graf (2015). It gives an overview of the “information content”of freshwater mussel larvae types in relation to the systematics of Unionida. In contrast to

Table 3. Structural features of bivalve larvae relevant for systematics.

type	features	rank	taxon
glochidium		superfamily	Unionoidea
	hookless spherical or semi-oval	family	Margaritiferidae
	"modified" (S-shaped or bicuspid hook)		Hyriidae
	hooked subtriangular	subfamily	(Unionidae:) Unioninae
	hookless: elongate oval or subelliptical		(Unionidae:) some Ambleminae
	hookless: subelliptical or subligulate		(Unionidae:) most Ambleminae and some Gonideinae
	bilaterally asymmetrical (with appendage)	tribe	(Unionidae: Rectidentinae:) some Contradentini
	axe-head shape		(Unionidae: Ambleminae:) some Lampsilini
lasidium		superfamily	Etherioidea
	"normal"	family	Etheriidae, Mycetopodidae
	haustorium-type		Iridinidae

the Unionoidea, the glochidium-bearing freshwater mussels, the Etherioidea, which have the lasidium larval type, have few precise descriptions of the larvae of individual species (Wächtler et al. 2001; Graf and Cummings 2006). Therefore, the diagnostic value of particular features cannot yet be assessed. To our knowledge, there are also no studies in which lasidium larvae from specimens already preserved and in a collection have been examined. The classification of the family group used here follows MolluscaBase (2025).

In the field of alpha-taxonomy, there are a number of morphological and fine-structural characteristics of the larvae that allow genera or even in some cases species to be differentiated. These are: Dimensions, outline shape and proportions, thickness, microsculpture of the surface, characteristics of a shell hook, size and distribution of denticles/microstylets on the shell margin and eventually on the hook. From this, forms of visualisation can be derived that are suitable and sufficient to show the features. Most of them only require a microscope, in some cases just a binocular with medium magnification (>10×). Details of the dentition and the shell surface must be visualised under a scanning electron microscope. So far, these are only characteristics of the larval shell. The soft body also has some variable features (e.g. number and position of sensory hair cells, presence of a larval thread), but these have hardly been systematically surveyed and are not yet suitable for routine use as a classification aid.

Challenges with old material

Processing very old material can be difficult because it may not be possible to reliably identify the developmental state of the gill contents or the condition of the objects is not (or no longer) suitable for SEM analysis. The latter especially applies to samples fixed in formalin because it destroys the outer layer of glochidial shells. For a comparison of the exterior microsculpture of glochidia fixed in alcohol and formalin, see Sayenko and Kazarin (2022).

A major problem is to obtain fully developed, mature ("infectious") larvae that are either already free or that can still be removed from the egg membrane without being damaged. The condition and behavior of material that has been preserved for a long time cannot be predicted. Several trials are necessary here, the outcome of which is unfortunately uncertain. Therefore, if the material is not suitable

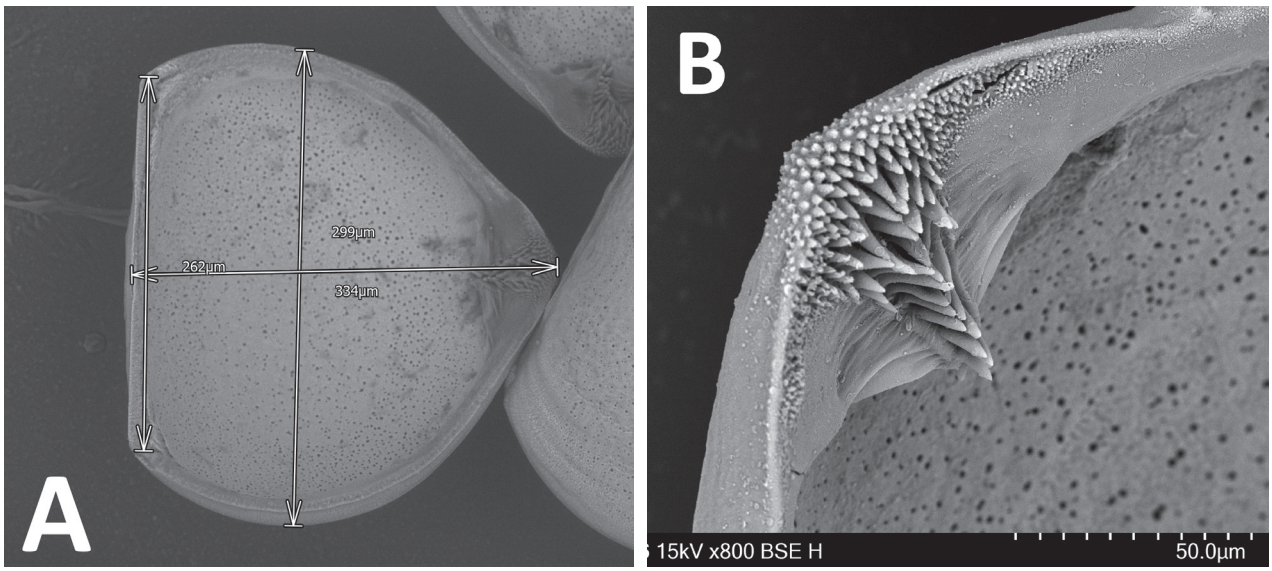

Figure 6. Very well preserved and presentable glochidium, kept in the collection for 115 years, *Pseudanodonta complanata* (Rossmässler, 1835) (SMF 346006). **A.** One shell valve with length information; **B.** Ventral hook with spines.

for visualization in the SEM, the microscope remains an important tool to provide some basic information about the larvae present (dimensions, type) in such cases. Considering that for many species information on larvae is still unknown, any data will be very useful. We can say from our own experience that even if a sample with glochidia is dried out due to improper storage, it is possible to obtain a satisfactory image on a light microscope, at least in regards to the shape and approximate size of the larvae. With proper handling and storage, the results of old collection material are quite comparable to those of freshly collected material (Fig. 6).

Additional findings

When inspecting the gills, it is possible to find commensals or parasites of the freshwater mussels. The latter include, for example, mites of the genus *Unionicola* (family Unionicolidae) (Edwards and Vidrine 2013) (Fig. 7), mussel-associated leeches (family Glossiphoniidae) (Bolotov et al. 2019) and some other groups of invertebrates. Their detection is valuable additional information, as these animals are rarely found during routine examinations.

The mantle cavity and gills of freshwater mussels may also contain eggs and larvae of bitterling fish (Fig. 8) providing additional data on a complex relationship between unionid mussels and bitterling (e.g. Reichard et al. 2007).

Recommendations for the presentation of larvae from collection material

1. Light microscope images: approx. 40× magnification, motifs: shape and size of closed and possibly also open shells, a focus on ventral margin if hooks and/or denticles are visible, larval thread.
2. SEM images: magnification approx. 200×, motifs: shape and size, outer side with external microsculpture, inner side with inner pores, ventral margin (hooks, denticles, folds as attachment structures).

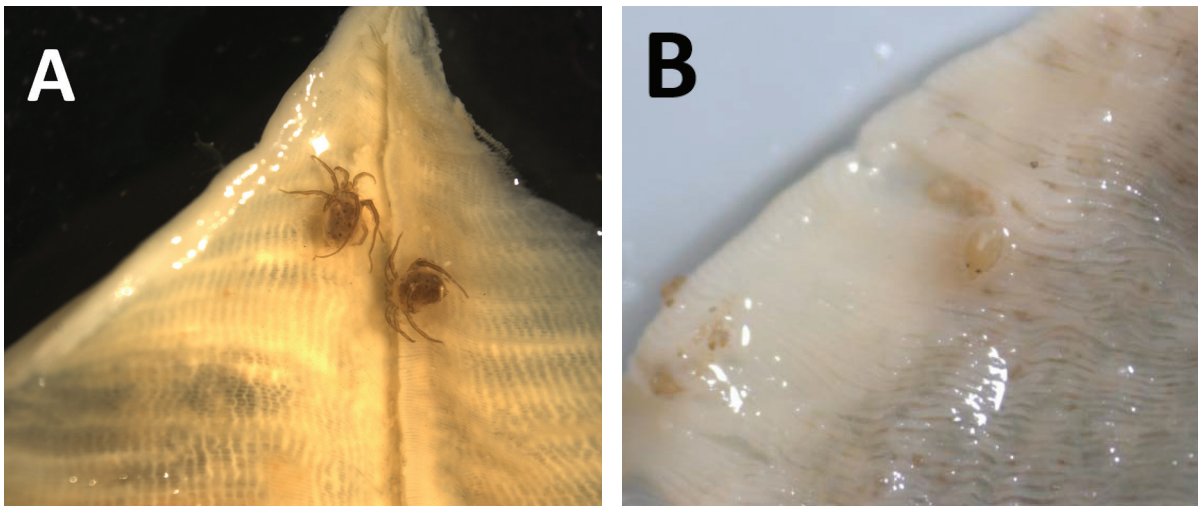

Figure 7. Water mites (family Unionicolidae) as an incidental find. **A.** Two adults of unidentified *Unionicola* sp. mites on the gills of *Acuticosta chinensis* (Lea, 1868) (SMF 3063/18). **B.** Larvae of *Unionicola* (*Pentatax*) *aculeata* (Koenike, 1890) mites on the gills of *Buldowskia shadini* (Moskvicheva, 1973).

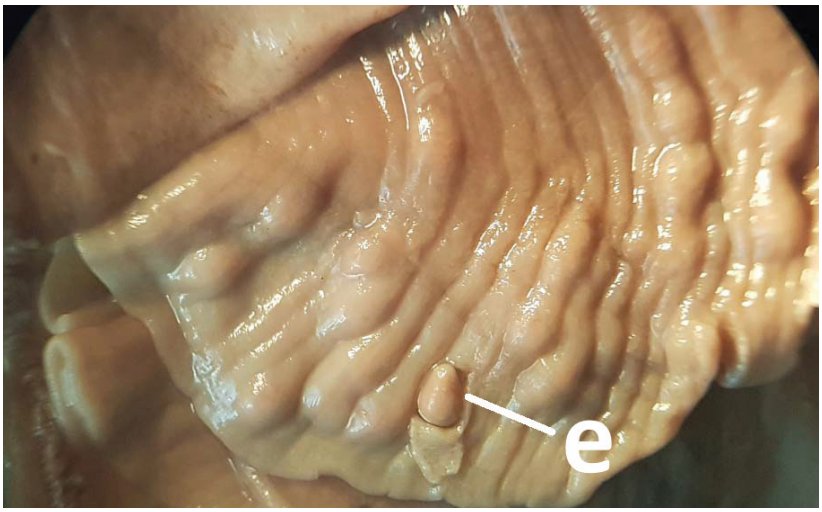

Figure 8. Inner gill of *Sinanodonta schrenkii* (Lea, 1870) with incapsulated bitterling (*Rhodeus sericeus* (Pallas, 1776)) eggs (FSCEATB collection no. 1-2024/Sin). Abbreviation: e – A single egg exposed from opened gill tissue.

Conclusions

With little effort, an existing sample of freshwater mussels or a sample intended for inclusion into a collection can be examined to determine whether it contains gravid animals and the developmental stage of the larvae. The detection and presentation of mature larvae increases the value of such a sample and at the same time the importance to the receiving depository. The presentation of the larval type and various morphological features of the larval shell provide information about the systematic position of the animals. Accompanying information on the time and location of sampling provides important data for reproductive biology. In view of the dramatic decline in freshwater mussel populations in many areas, this information is of increasingly great importance.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

The animals were taken from the wild in accordance with the country-specific regulations applicable on the date of collection.

Use of AI

No use of AI was reported.

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Author contributions

Both authors contributed equally to conceptualization, data curation, investigation, resources, validation, visualisation, writing of the original draft and review.

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Data availability

All of the data that support the findings of this study are available in the main text.

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