A PHOTO-ILLUSTRATED DISSECTION GUIDE

FOR BOBTAIL SQUIDS

VIDAR ØRESLAND & GERT OXBY

The authors, Gert Oxby and Vidar Øresland, 50 m from the Sepietta oweniana dive area in Lysekil, west coast of Sweden. Photo: Anette Bargel

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Øresland, V., Oxby, G., Oxby, F. (2018). A comparison of catches of European lobster (Homarus gammarus) in a lobster reserve using traditional pots and scuba diving technique. Crustaceana 91: 1425-1432.

Øresland, V. (2019). The polychaete Histriobdella homari and major groups of epibionts on the European lobster and other decapods. Crustaceana 92: 189-203.

Øresland, V., Oxby, G., Oxby, F. (2020). Abundance and size of European lobsters (Homarus gammarus) and brown crabs (Cancer pagurus) inside and outside the Kåvra lobster reserve Kåvra (west coast of Sweden). Crustaceana 93 :157-169

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VIDAR ØRESLAND
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Divers and Scientists
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PREFACE

During a pilot study of the bobtail squid *Sepietta oweniana* (Cephalopoda: family Sepiolidae) Leach, 1817) off the Swedish west coast, we found no suitable dissection guide for bobtail squids that met our needs. We therefore decided to prepare one ourselves using *S. oweniana* as the dissection object. There is a huge variability among cephalopods regarding morphology which is of both evolutionary and ecological interest. The morphology of bobtail squids is generally less known, compared to many commercial cephalopods, and would therefore be worth studying in detail. However, the internal organs of bobtails are smaller and much more compressed in comparison to larger cephalopods and consequently more difficult to dissect.

The purpose of this guide is to help biology students and naturalists to obtain an understanding of the equipment and skills needed in order to carry out safe and quick dissections of bobtail squids, and other small cephalopods, including juveniles of larger cephalopods. In addition, dissecting bobtail squids is an excellent training for those who want to improve their microdissection skills for whatever purpose. The text and photo illustrations show the microdissection tools and equipment needed and provide step-by-step advice on how to remove organs from the head and the body, and how to dissect and analyze them.

The text describes some basic biology of bobtail squids together with some exercises and research areas suitable for dissection-dependent studies. Also mentioned are some important points to consider when obtaining samples for ecological studies, plus some data handling and analysis strategies that were found useful. The guide emphasizes the statoliths and the reproductive and digestive organs which are of interest to ecologists. The muscular, respiratory, excretory, circulatory, nervous and sensory systems are not, or only briefly, dealt with. A short reference list of research papers and books is included, most of which are available on the internet, to give an idea of ongoing research on bobtail squids, especially in European waters. Future challenges in cephalopod research are discussed in Zavier et al. (2015). We explain in the appendices the most frequently used anatomical terminologies, how to prepare statoliths, the equipment needed, and provide advice on how to make dissection tools and take microphotographs.

Microdissection is very much about personal experiences, choices and preferences that develop over time. It does require a great deal of time and patience, so one should be prepared for dissections that could take from a few hours to several days to complete. It is hoped that this guide will inspire students to undertake their first cephalopod dissections and to help develop personal skill, dissection strategies and photo techniques, as well as to promote further interest in cephalopod biology. One difficulty faced when writing this photo-illustrated guide was how to decide when to stop since one can always take more and better photos, given time! Comments and suggestions for improvement are welcome.

Vidar Øresland & Gert Oxby

Divers and Scientists West Coast Sweden

2021

INTRODUCTION

Bobtail squids are normally between one and eight cm long and have eight arms and two tentacles. Appealing to the eye, they have become popular objects among UW-photographers and are found in tropical, boreal, and polar regions from one meter to over thousand meters in depth. Some bobtail species may be more common in shallow waters than is generally known since much research on bobtails have been based on trawl-caught specimens. They are benthic and nektonic and some may be rather tolerant to low salinity. We have found *Sepietta oweniana* at the sea bottom three metres down in the Gullmar Fjord on the Swedish west coast where surface salinity can be as low as 18 ‰. Their life span is believed to be around one year. Females can produce multiple batches of eggs and males and females may mate several times. Females can store sperm for some length of time before they are used for fertilization. Death may occur for females soon after the final egg production and for males soon after the final copulation, but detailed information for different species is often lacking.

The biology, ecology and evolution of bobtails have intrigued scientists for over a century, and they are still of great interest. However, in order to obtain a more comprehensive understanding of bobtail squids further studies are needed on morphological development, age, growth, diet, feeding rates, maturation, reproduction, diseases and parasite infestations, etc. which requires knowledge on how to carry out dissections. Though the bobtail squid *S. oweniana* is the dissection object here, the different techniques presented can be used for any small cephalopod species or for small juveniles of larger species.

Before commencing any dissection, one should first identify the species and sex based on external characteristics, if possible. Identification of sepiolids can be difficult especially when the specimens are in bad condition or young or when species are closely related. Adults can be identified by using, e.g., Naef (1923); Bello (1995); Reid & Jereb (2005); Laptikhovsky & Ourens (2017); Bello (2019a); Bello (2020); the Marine Species Identification Portal; the World Register of Marine Species (WoRMS); the Tree of Life Web Project and the MolluscaBase. An identification guide for young cephalopods is provided by Zaragoza et al. (2015). DNA analyses may in some cases be the best alternative for sex and species identification. Some important external and internal bobtail characteristics are: overall size and shape; presence and shape of light organs; the morphology of the hectocotylus (the modified left, sometimes also the right, dorsal arm I of males, used for spermatophore transfer during copulation); number, shape and position of suckers on the arms and the tentacular clubs; the shape and position of the fins; and the morphology of the beak, the radula, the funnel organ and the presence and shape of bursa copulatrix. One should keep in mind that there is often some individual variability in morphology within most species, see e.g., Cuccu, et al. (2009) and Bello (2019b).

Identification of the internal organs of bobtail squids can be difficult for the beginner. Drawings of internal organs of squids, cuttlefish and octopus are shown in Reid & Jereb (2015). In Gestal, et al. (2019) one can find good photos and text on the internal morphology, pathogens and diseases of cephalopods. However, these show no illustrations of internal organs of bobtail squids which are very compressed and differ from the other groups of cephalopods. There is limited photographic information available on the internal morphology of adult and juvenile *S. oweniana*

but some drawings are shown in e.g., Naef (1923) and Bello (1995). One should be aware of national animal welfare laws and regulations when using cephalopods for scientific and teaching purposes and Directive 2010/63/EU (see also e.g., Fiorito et al., 2014; Fiorito et al. 2015).

SOME ADVICE

First test and then adopt a simple to use and consistent labelling system for specimen jars, organ vials, glass slides and digital photo files, as well as for specimen field collection and analyses data. Use ID codes with lower case, numbers and no spacing.

Master the dissection and photo techniques and the analyses as well as the data management and labelling procedures before starting to dissect the research specimens.

Become familiar with the selected photo software programme. Nikon NIS-Elements is an appropriate software (with several advanced research versions) that is easy to use.

Check the microscope/software scale calibrations weekly against a glass slide micrometer.

Label the vials needed for organs and fill them with 4% formaldehyde and 96% ethanol (DNA sample) before starting the dissection in order to avoid interruptions.

Start first dissection training on large specimens.

Always double check the microscope magnification against the chosen photo magnification in the software and then confirm, by writing “ok” in the photo software field for image properties. Do not show a scale or make measurements on a photo if confirmation is lacking. Here we have chosen not to show the scale in our photos since confirmation often lacked!

Take many photos and save photos in separate specimen folders which are kept in a master folder of which frequent backups should be made.

Photos for potential publications and posters, etc., should be copied immediately into special folders.

Use adequate dissection tools for each cut and have sharp back-up tools available at all times.

Dissect the objects in a scratch-free Petri dish and turn it around for optimal cut positions.

Removing all thin membranes is the key to a successful dissection!

Do not cut anything if it cannot be seen properly and make short cuts .

Save all remains from the dissections in the original specimen jar or organ vials.

Adopt a proper sitting position (do not bend your neck and keep your shoulders down).

Take notes during the dissection, drink water and take breaks!

Continue on another day if concentration waives.

TOOLS AND EQUIPMENT

The dissection tools we used are shown in **fig. 1**. The tweezers are used for all delicate dissections including the removal of thin membranes. The large forceps are useful when taking a specimen from the specimen jar and when holding the body to cut off skins and muscles using the scissors or the scalpels. The tungsten needles are used mainly for statoliths and as support for micro scalpels. The long vitrectomy scissors are expensive but extremely useful e.g., when cutting off membranes between organs and other areas that are difficult to reach. The large curved scissors cut well and is easy to handle if one has thick fingers. Tools should be sharpened frequently under the stereomicroscope, using an oilstone and finish sharpening on a piece of dry kitchen wettex (on which tools are also cleaned) and hard-rolled newspaper (**fig. 101,** Appendix IV). Always keep the tip protectors on when not using the fine tipped tweezers. Alternatively, one can keep them in a Plexiglas® holder (**fig. 104**, Appendix IV. Mark the tweezers for fast recognition. Keep all micro scissors in their original casings. Always wash the tools used in warm water directly after a dissection, and dry them and inspect them under the stereomicroscope. Pay special attention to scissors as dirt and oil tend to accumulate between the blades. Appendices III and IV provide additional equipment used and advice on how to make needle scalpels and tungsten needles.

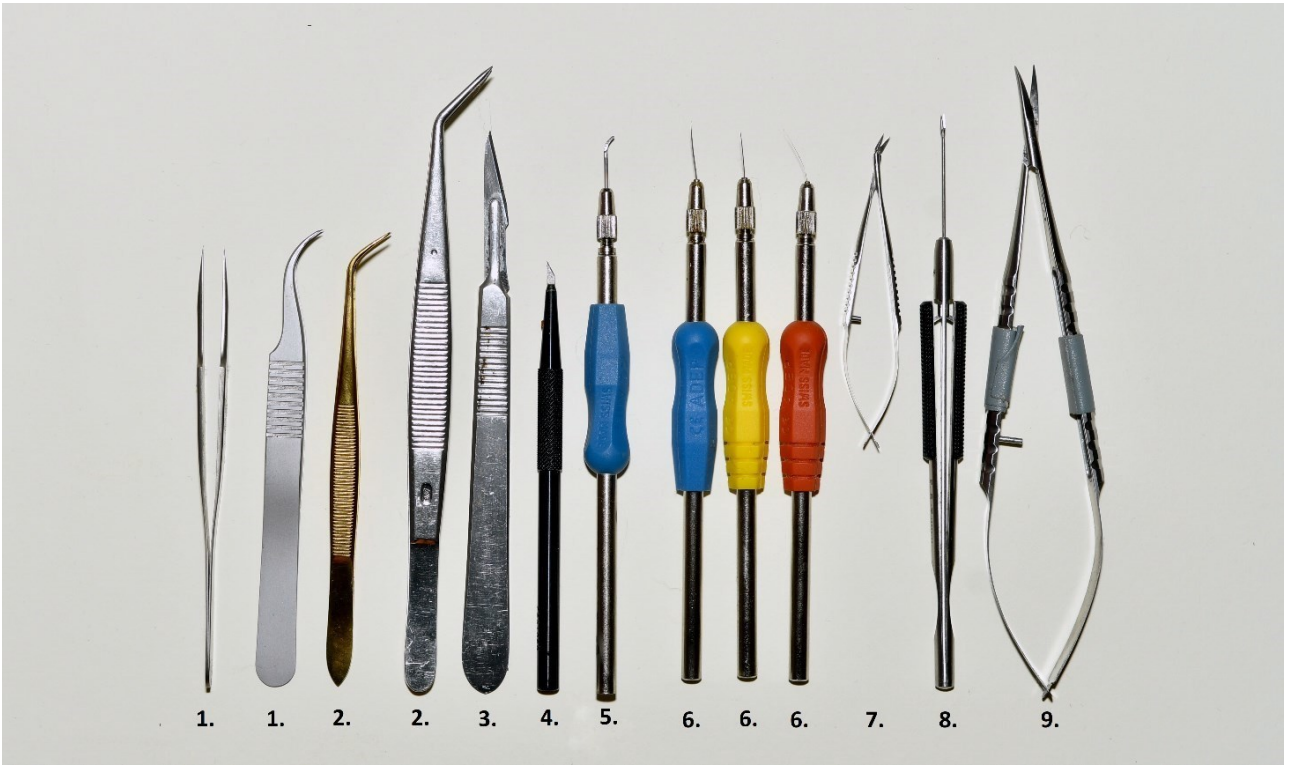

Fig. 1. Useful dissection tools. Straight and curved tweezers (1); different sized forceps (2); normal sized scalpel (3); micro scalpels (4); needle scalpels (5); three tungsten needles (0.3, 0.2 and 0.1 mm diameter sharpened tungsten wire) (6); small micro scissors (7); long vitrectomy scissors (8); and large curved scissors (9).

Equipment list

Boiled and filtered tap water in a bottle

Diamond sharpener, whetstone, Black Arkansas oil stone and surgical instrument oil

Dyes (e.g., azure B, oxytetracycline)

Ethanol 96% (molecular biology grade for DNA samples)

Forceps

Formaldehyde 4% (buffered to pH 7 using borax, $Na_2B_4O_7 \cdot 10H_2O$)

Glass slide boxes

Glass slide micrometers

Glass vials (20 ml) and labels

Household chlorine

Kitchen paper, lens paper and labels

Microscope glass slides and cover glasses

Microscopes* with cameras and software (e.g., Nis-Elements)

$MgCl_2$

Pasteur pipettes

Petri dishes of different sizes

Polyvinyl-lactophenol (PVL) or any other mounting fluid of preference

Portable digital weight scale (0.001 g)

PVC jars (500 -2000 ml) and waterproof labels

Ruler or calliper for dorsal mantle length (DML) field measurement and a cutting board

Scalpels

Scissors

Thermoplastic cement (Buehler, USA)

Tungsten needles (tungsten wire 0.1, 0.2- and 0.3-mm diameter) with coloured plastic handgrips

Tweezers (curved and straight)

Wettex for cleaning and sharpening dissection tools

*We used the Nikon SMZ18, the Wild M3Z and the inverted Olympus CK2 microscope with adjustable objectives and DFK33UX264 and DFK33UX250 5 MB colour cameras.

SAMPLING AND DISSECTION STRATEGY

In the field

When conducting ecological research, one should consider how well the collected specimens represent the subpopulation to be studied. If the data are based on just a part of a subpopulation, or a mixture of subpopulations, the conclusions can be misleading. The collected specimens are only a reflexion of the collection method used and the area, depth, and time of collection. This is even further complicated by the possibility that individuals of different sex, size, age, and stages of maturity may have different preferences regarding different habitats at different times. To shed some light on this one can; try to obtain data from deep and shallow depths at different times (day, night, monthly), using different methods, and see if any important differences can be detected in the obtained data. Nevertheless, data can easily be biased, and one should always discuss the representativeness of the samples being reported.

Bobtail squids can be obtained from fishermen (common bycatch in trawls), by using beach seines in shallow areas or during scuba diving. This guide is based on *Sepietta oweniana* specimens caught between 3 and 26 meter depths during scuba dives in the Gullmar fjord and from trawling for shrimps (>60 m depth) in the Gullmar fjord and the Koster fjord on the Swedish west coast. Small bobtail squids can be difficult to spot during diving and thereby underrepresented. Note that beach seine catches and especially trawl catches are highly size selected, and that juveniles can be absent or underrepresented. When using specimens from trawling in a population study, it is important to know the mesh sizes of the trawl used and to ensure that all bobtails are saved (not only the large and easy to spot ones). *S. oweniana* can be found on or above gravel/sand/mud bottoms. During both day and night some may be buried in the bottom substrate, but monthly 24-h dive data from different depths and habitats are lacking. In comparison with trawling, diving for bobtails will provide more detailed data (depth, habitat, light condition, time, microhabitat, temperature, etc.) as well as fresh specimens in perfect condition suitable for diet/feeding, morphological studies and reference collections. The specimens should be kept separated in numbered plastic bottles with wide openings during the dives and the individual catch data noted on an underwater writing pad. Immediately after capture one should take notes if the specimen is damaged, if gut contents have been vomited, or if there is anything else of possible importance.

The anaesthetics used for experimental purposes and prior to the killing of cephalopods have given rise to different opinions regarding their execution (see e.g., Lincoln & Sheals, 1985; Fiorito et al., 2014; Fiorito, et al. 2015; Polese et al. (2014); Pugliese et al. (2016); Butler-Struben, et al., 2018; Winlow, et al. (2018) and references therein). New knowledge and guidance can be expected in the near future, but meanwhile, we are here only concerned with the killing of the specimens in a practical and humane way. We have, for the time being, adopted the following: the muscles of a live specimen in a jar with seawater can be relaxed by adding, gradually, an equal amount of a nearly isotonic solution of magnesium chloride (7.5% $MgCl_2$ *$6H_2O$ dissolved in tap or distilled water) for approximately 15 minutes. Alternatively, one can follow the protocol suggested by Polese et al. (2014) using a true anaesthetic that will work perfectly in relaxing the animal before manipulating it.

As soon as the specimen is fully relaxed, put the specimen on a cutting board and kill it quickly by cutting the brain using a scalpel. Dry the specimen on kitchen paper and measure the total wet mass (TWMfresh) when stabilized to the nearest 0.01 gram. Measure the dorsal mantle length (DML fresh) to the nearest mm (**fig. 2**). The standard measurements of the cephalopod body are described in Roper and Voss (1983). DML measurements on live non-relaxed cephalopods can be highly unreliable due to contraction and extraction, so one needs to explain the conditions during measurements. It is also important to note and report whether the mass and length estimates are based on fresh, defrosted, formaldehyde or ethanol preserved material, etc. If the brain is needed one can do the measurements first and then quickly cut the head off (severing the dorsal aorta) of the relaxed specimen.

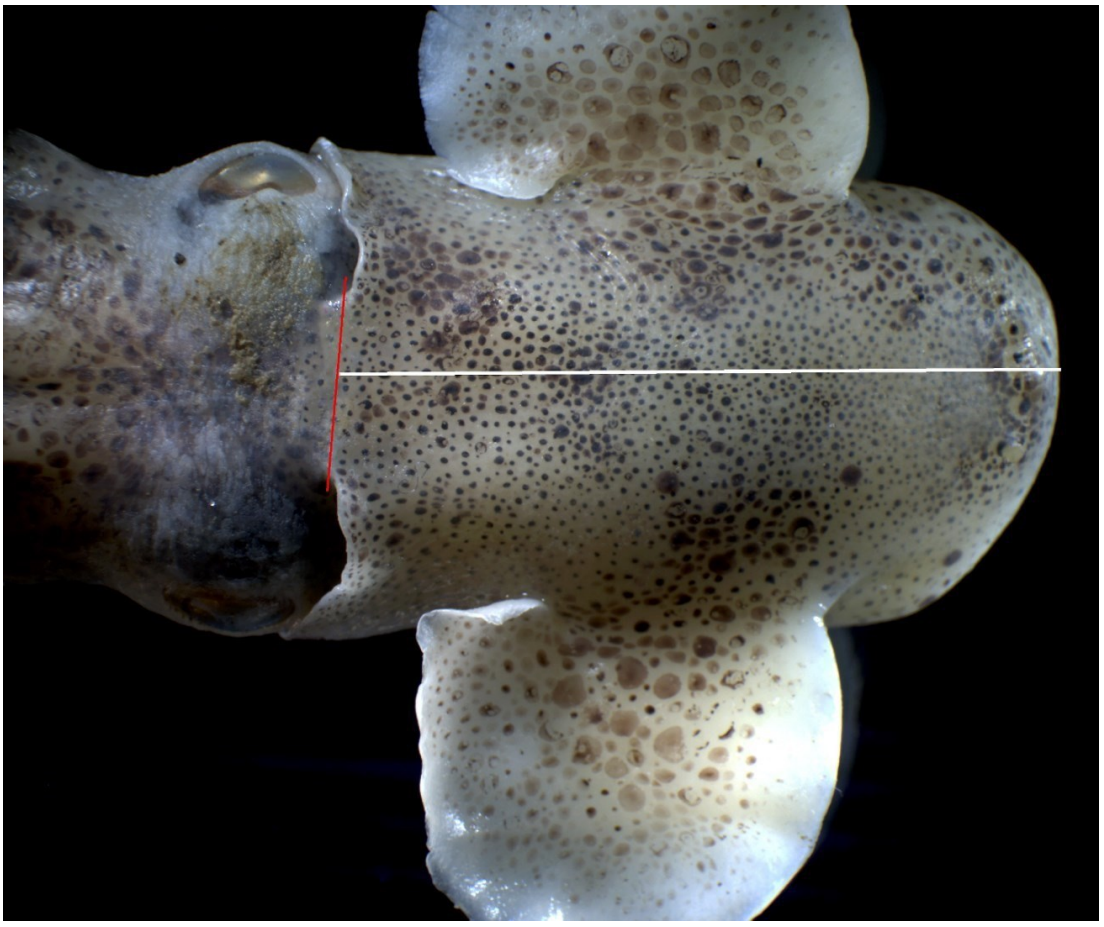

Fig 2. The dorsal head-cut (red line) close to the mantle and the dorsal mantle length (white line).

Use a large scalpel to cut the head off from the dorsal side so that the dorsal part of the mantle remains intact (**fig. 2)**. The cut should be made at a slight angle away from the head (without cutting into the brown digestive gland). Place the head in a labelled jar of seawater. It is important that the jar has a wide opening so the specimen can be taken in and out easily. The head should be held in the head skin using forceps in order not to damage the suckers which can fall off easily. When returning from field collection, the jar with the fresh head can be stored in a refrigerator for a maximum of three days before dissection.

Preserve the body in 4% formaldehyde in tap water in a labelled jar (one part concentrated formaldehyde and nine parts water). It is important, if diet analyses are to be done, that the specimens are is

killed as quickly as possible. Otherwise, quickly digested prey items might be lost or more difficult to identify. This is especially important when analysing trawl-caught specimens and the estimated time between catch and preservation should always be reported. When epibionts of organs such as the gills are of interest one should, remove the organs whilst fresh (or defrosted) and preserve them in a vial. It is important that the preservative (in tap water) in the vial is filtered for epibionts since they tend to fall off their host organs when in contact with formaldehyde (Øresland, 2019). Tap water is used since epibionts might occur naturally in seawater.

Exercise: Test whether using $MgCl_2$ and ethanol makes the epibionts fall off their host.

Reference collection

When saving an intact specimen for a reference collection the specimens should be relaxed /anaesthetized in seawater and the arms preserved in a straight position. Cut the brain in order to kill the specimen. Needles can then be used to pin the arms (across, not through, the arms) on a rubber or cork plate glued to a flat container. Remove the seawater and add 4% formaldehyde or ethanol. When using ethanol, one could start with 20% (one day) and change to 40% (one day) before adding 70% (1 week). Use a spray bottle with preservatives to clean the specimens between changes. An alternative is to start with the formaldehyde (three days) and then change to ethanol in order to avoid handling formaldehyde-preserved specimens when removed from their jars. However, ethanol makes the specimens much harder and thus less suitable for dissection. With large specimens, a low volume of the preservative can be injected into various, deeper parts of the head and body. Remove the needles and the preservative and photograph the specimen together with a ruler ensuring that the photos are consistent so that different specimens can be compared.
Mount the specimen, using white or black polyester thread, onto a PVC plate with small holes and put the plate into a container with a wide opening. Add the final preservative (a volume ten times that of the specimen, if possible). Mounting the specimen on a plate protects it during handling, especially as the suckers can fall off. Label the inside of the container with all relevant data and the outside with a reference code. The final preservative should be changed again after a few months. Remember to save a sample for DNA analyses before fixation and keep it in a refrigerator. Check every six months that there is enough preservative in the container.

Dissection strategy

The strategy chosen for dissection and analysis of specimens will obviously depend on the research goals. The three-step-dissection strategy suggested here is a general one enabling dissections and analyses of organs to be spread over time. This is practical when: there are many fresh specimens at a time; when some dissections need to be done on fresh material and some on preserved; and when some material is sent in bulk to someone else.

The work schedule in the laboratory can then look like this:

1. Species, and sex determination and primary dissection of the fresh head. Remove organs from the head and preserve them individually in glass vials and make glass slide preparations of the statoliths.
2. Primary dissection of the preserved body. Measure DML (formaldehyde). If the whole specimen is intact, one should measure the TWM (formaldehyde). Remove organs from the body and preserve them individually in glass vials.
3. Secondary dissection and analyses of preserved organs; make glass slide preparations and take photos.

The reason why we dissect the head fresh (or defrosted) is because formaldehyde will quickly dissolve the statoliths and possibly affect the thin walls of the beak. All other organs should be well preserved in 4% buffered formaldehyde. Never let the thin lateral walls dry. A solution of 4% formaldehyde is preferable to 70 or 95% ethanol since it does not harden the organs as much and is cheaper. We use 95% ethanol for DNA samples (which are stored in a deep freezer). This guide does not, or only briefly, deal with the muscular, respiratory, excretory, circulatory, nervous and sensory systems. However, Appendix IV provides some tips regarding tools to be used for the microdissections of such systems. Note that it may be necessary to use specialized techniques for the fixation and preservation of specimens to be used for histological studies and certain staining methods a well as for SEM and TEM.

PRIMARY DISSECTIONS

General guidelines

Any items for dissection that have been preserved in formaldehyde should first be rinsed in tap water to eliminate the smell. A Plexiglas® cover with air ventilation can be used over the microscope during dissection to further eliminate the risk of toxic and vaporized formaldehyde. The stereomicroscope dissections should be done in a Petri dish, with or without tap water, using a black background. It is advisable to start the first training on large mature specimens for easier dissection and identification of organs, species and sex. The appendices provide a glossary and advice regarding grinding statoliths, equipment, how to make dissection tools and microphotography. Save all parts of the specimens in individual organ vials (digestive organs, reproductive organs, gills etc.), in the original specimen jar (arms, mantle, head remains etc.), or on glass slides (statoliths, stomach content etc.). There may be a need to analyse the material later on in the light of new findings as the study progresses. Multiple photographs of the dissected organs and glass slide preparations should be taken for the purposes of documentation and analysis. It is important to keep dissection and analysis spreadsheets for all work done (and what remains to be

done). The data columns in the table should go from left to right as the different data are obtained during the dissection to make the notations faster and safer. Use separate spreadsheets (in the same file and master folder for ease of backup) for the analyses of the digestive organs, statoliths, reproductive organs, etc. As mentioned above, a simple to use and consistent labelling system is crucial.

Polyvinyl-lactophenol (PVL) can be used as a mounting fluid for slide preparations but there are many others available. It is, however, important that the fluid does not solidify too fast or too slowly. The use of PVL provides a permanent preparation and time to arrange, e.g., food items (approximately 20 minutes depending on the amount/thickness on the slide and the condition of the PVL). The consistency and clarity of PVL makes it easy to work with but one must be aware that it is toxic. PVL renders some objects more transparent over time which can reveal new structures, especially in high magnification. The slides should therefore be re-checked after a month or two. Sealing the cover glass (using e.g., nail polish) is not always necessary for PVL slides but is nevertheless recommended. For thick preparations, however, where two or more cover glasses are used on both sides of the object (sandwich style) and another one covering it, sealing after a few days is always needed.

Removing organs from the head

Dry the fresh head gently on a kitchen paper so that the suckers do not loosen and put it into a Petri dish without water. Put some drops of ethanol or water under the Petri dish to prevent it from moving during the dissection, which also makes the background appear darker. Place a piece of wood or Plexiglas® between the Petri dish and the microscope stand so the Petri dish cannot move forward (see **fig. 106,** Appendix V). Change to clean and scratch-free Petri dishes during the dissection in order to obtain a better background for taking photographs.

DNA

DNA and morphological analyses are important research tools, especially when used in combination (Groenenberg, et al., 2009; Sanchez, et al., 2019). Take a sample of the muscles from the fresh head for species and sex identification and store it in a vial containing 95% ethanol, in a deep freezer. It may be necessary to take DNA samples from other parts (when fresh), e.g., from spermatangia (see below, “Reproductive organs”), stomach content, and egg clutches), depending on research interests. When taking samples for DNA analysis: tools should be cleaned with household bleach (10%) and distilled water in order to prevent cross contamination.

Statoliths

Before dissection can commence, one end of a thermoplastic cement (TC) rod should be hold over a laboratory heater(100-150 ℃, see **fig. 96**, Appendix II) and formed into a point. Put a

clean glass slide on the heater and hold the tip of the TC rod one mm above it to create a 5-10 mm diameter droplet. Usea tungsten needle to remove any air bubbles. Use a glove when handling the hot glass slide. Let the slide with the TC cool down. It will save time if several slides are prepared in advance and kept in a dust free-slide box.

The goal is to mount the two statoliths in TC on separateglass slides and, later on, to grind them on each side (see below) so the rings used for age analy- sis can be seen. There are two calcareous statoliths behind the brain that can be used to estimate age and growth by counting and measuring the width of the assumed daily rings (see below, "Secondary dissection and analyses"), in the same way as can be done for fish larvae otoliths.
Each statoliths is found inside a statocyst which is a sense organ for gravity, orientation, etc. The statocyst itself is found inside the cephalic cartilage, that envelops the posterior part of the brain.

Place the head with the arms downward into a Petri dish. Begin the dissection by identifying the two statoliths. If the head-cut was successful one should be able to see them in the head as in **fig. 3**: if not, carefully remove the white tissue in the head until the statoliths become visible. If they cannot be found, they are most likely destroyed in the body by the formaldehyde. It is for this reason that the head-cut should be made at a slight angle away from the head. We have found no data as to whether the statoliths are destroyed within seconds or minutes by formaldehyde (which should be investigated for bobtails). Do not try to remove the statoliths at this point since they could easily get lost inside the head (though this may work with larger squids). Make a second head-cut just behind the eyes as shown in **fig. 4** and turn the posterior part over so the statoliths can be seen (as in **figs 3 & 5**).

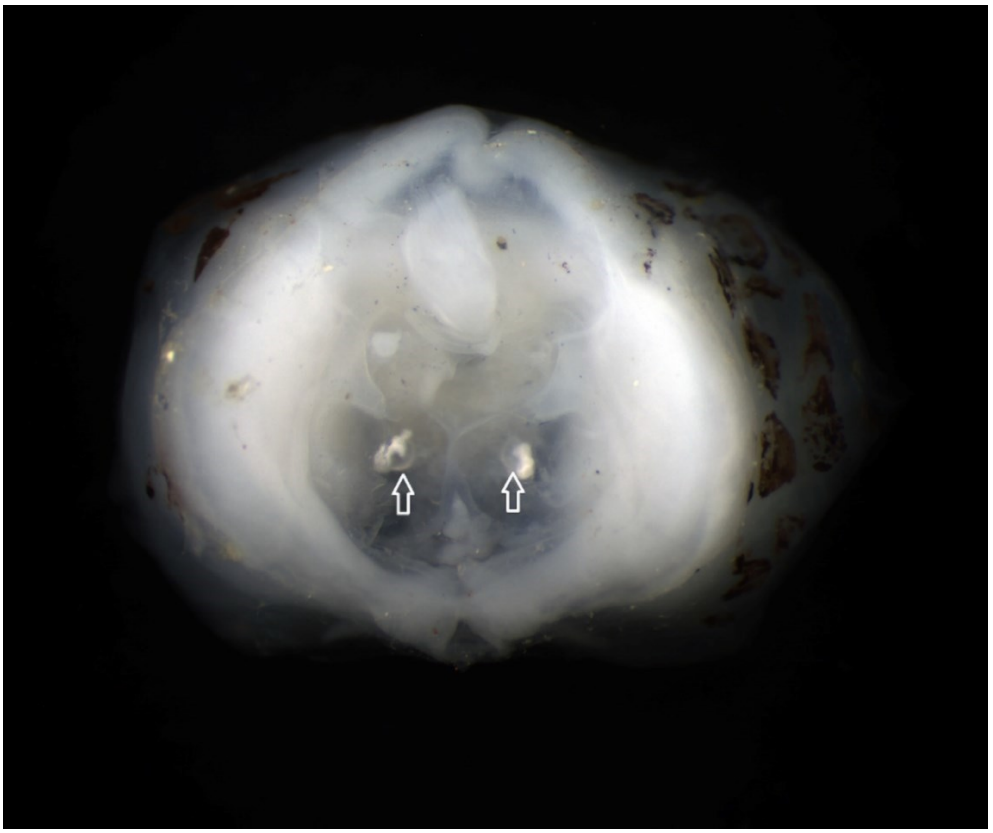

Fig. 3. The two statoliths inside the statocysts.

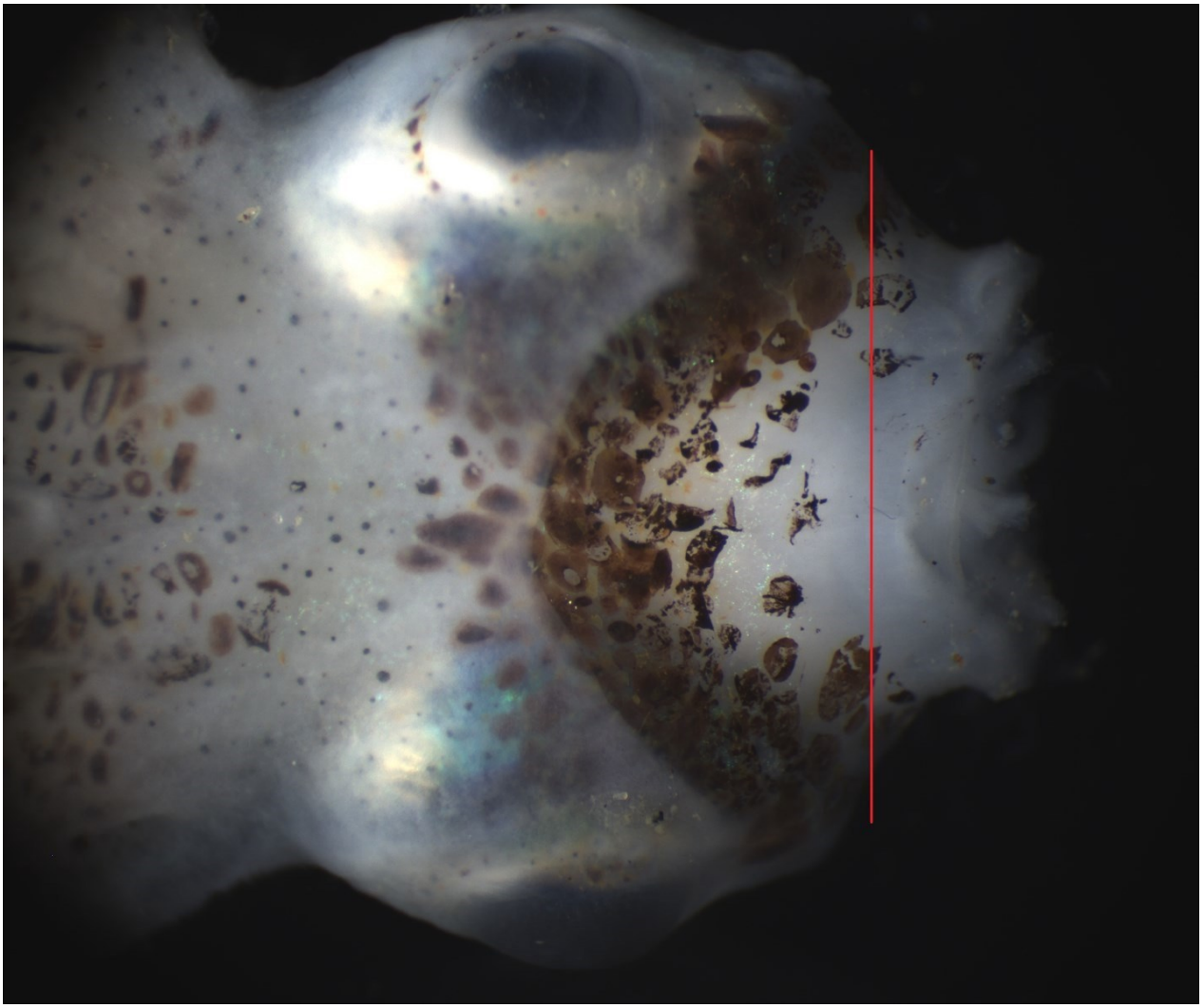

Fig. 4. The second head-cut behind the eyes.

Add some water into the Petri dish then hold the tissue using a tweezer and cut out the statoliths as shown in **fig. 5** and move them into two separate drops of water. Keep track of the right and left statoliths at all times.

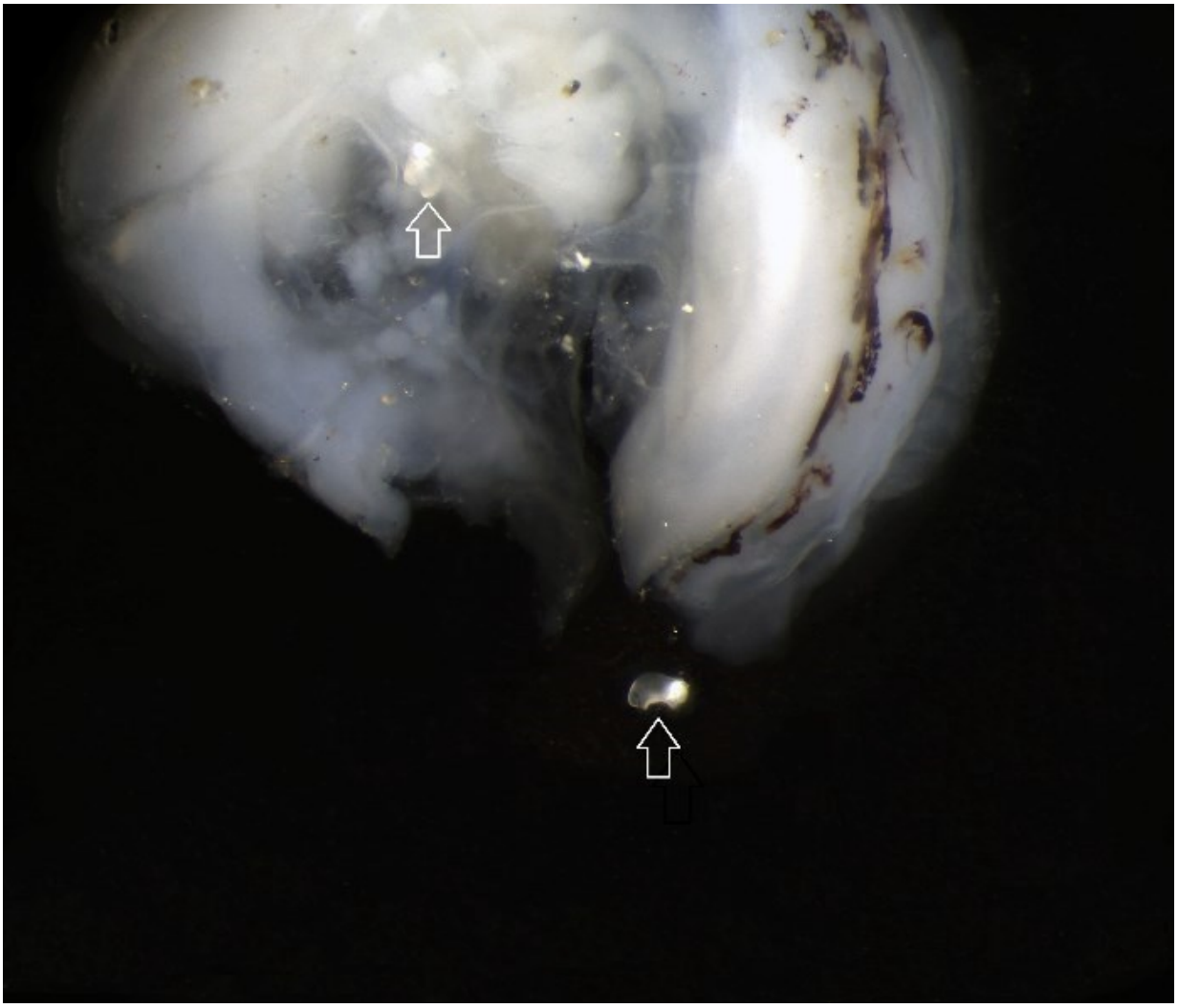

Fig. 5. After the second head-cut the right statolith has been cut out and placed in a drop of water (arrows = statoliths).

If a statolith has enough surrounding tissue, seize it using fine tweezers, and move the statolith to a drop of water close to the TC on the glass slide. If the statolith has too little surrounding tissue one can push it (in water), using tungsten needles, to a microscalpel blad and move it over to the glass slide water drop. Cut away the remaining tissue and clean the statolith carefully on both sides in the water droplet using tungsten needles. Then slide the statolith in the water (using a curved tungsten needle) over to the top of the TC (**fig. 6**). Statoliths of bobtail squids are convex on one side and rather flat on the other. Make sure that the statolith does not become dry. Repeat the procedure for the other statolith.

Fig. 6. Cleaned, but not ground, statolith on top of the TC. Note that the rings cannot be seen at this point.

Put the heater under a stereomicroscope and heat the first slide so the TC melts while the statolith is observed through the stereomicroscope. Use a tungsten needle to cover the statolith with the melted TC and drag redundant TC and any air bubbles away from the statolith in different directions. When finished, the convex side of the statolith should be resting on the glass slide in a perfectly horizontal position with only a thin layer of cement covering its flat side. Some may prefer to place the flat side down first in order to get a better horizontal position. The reason why we prefer to mount statoliths with the convex side downwards is that we want to grind the flat side first since it makes it easier to turn the statolith over without breaking it. It may also be easier to evaluate how close to the centre one is when grinding the thicker convex side later on. If a scanning electron microscopy (SEM) photo of the statolith´s convex surface structure is needed, the TC should be heated just enough for the statoliths flat side to become glued to the glass slide (or glued to a SEM specimen stub). **Fig. 7** shows a light microscopy photo of an *S. oweniana* statolith with a roof tile structure on the convex side (which would have looked better as an SEM photo). This structure is not always evident. Write ID codes on the opposite side of the glass

slide (due to later grinding in water). Put the slide in a slide box until grinding. The grinding procedure and analyses are dealt with below in “Secondary dissections and analyses” and in Appendix II.

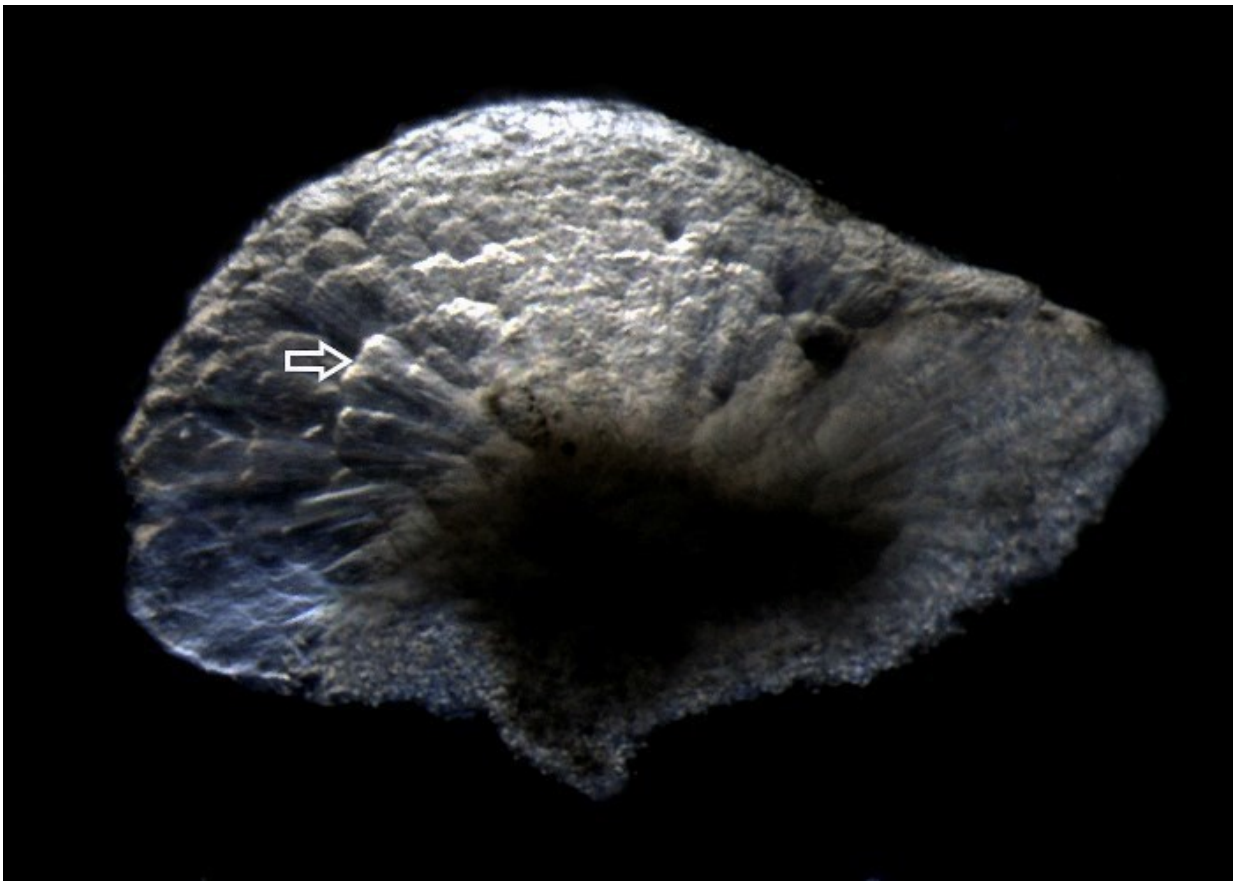

Fig. 7. Light microscopy photo of a roof tile structure on the convex side of a *Sepietta oweniana* statolith**.**

Eye lenses

The diameter of the lenses can be correlated to age, mass and dorsal mantle length (DML). If the brain is not needed, the lens is easily dissected by a third head-cut just in front of the eyes (**fig. 8**). Cut out the lens but save some of the brownish structure that surrounds it. Photograph the lens and measure its diameter at a 90-degree angle to the brown ring left as shown in **fig. 9**. It is important for all measurements to be taken exactly in the same way since the lens is not perfectly spherical.

Fig. 8. A third head-cut just in front of the eyes has been done.

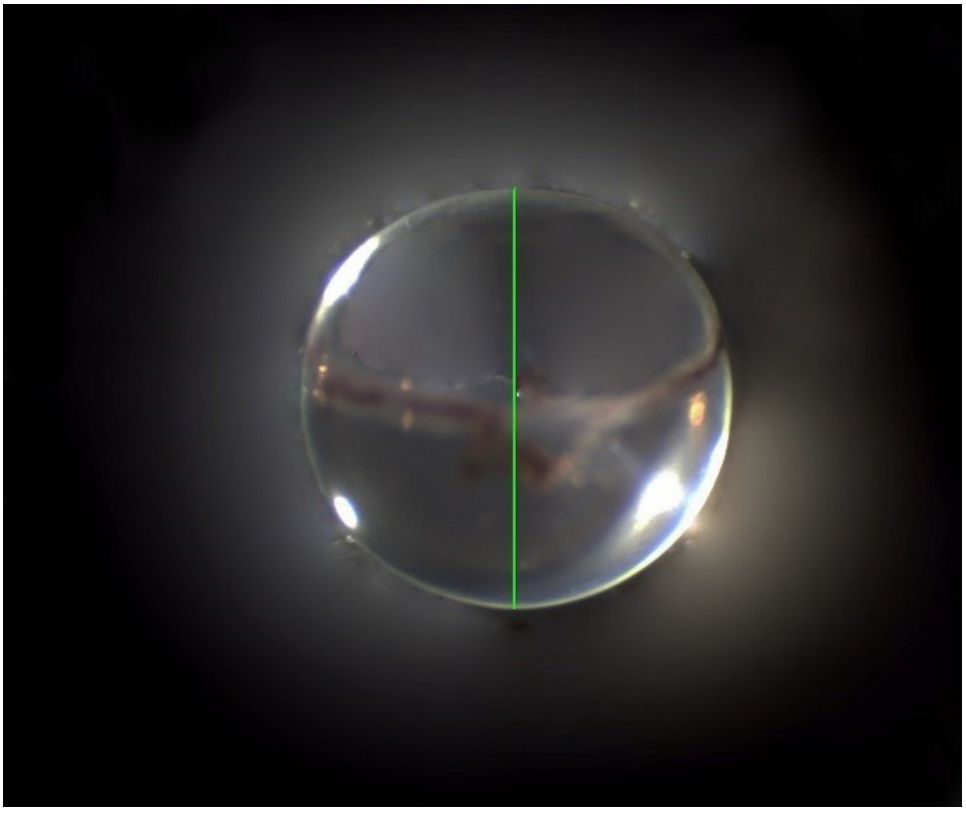

Fig. 9. Diameter measurement of an eye lens.

Buccal mass

The buccal mass is a muscular bulb between the bases of the arms which consists mainly of the beak, the tongue with the radula, the primary salivary glands and the buccal muscles. The beak comprises an upper and a lower part that are used for determining the age of certain cephalopod species. The beak and the radula can also be used for species identification (useful also when studying the diet of predators feeding on cephalopods). The buccal mass is easily removed by making a cut between the two ventral arms and another cut around the beak as shown in **fig. 10**. Save the buccal mass in a vial in a deep freezer since preservatives might affect the thin lateral wall of the beak.

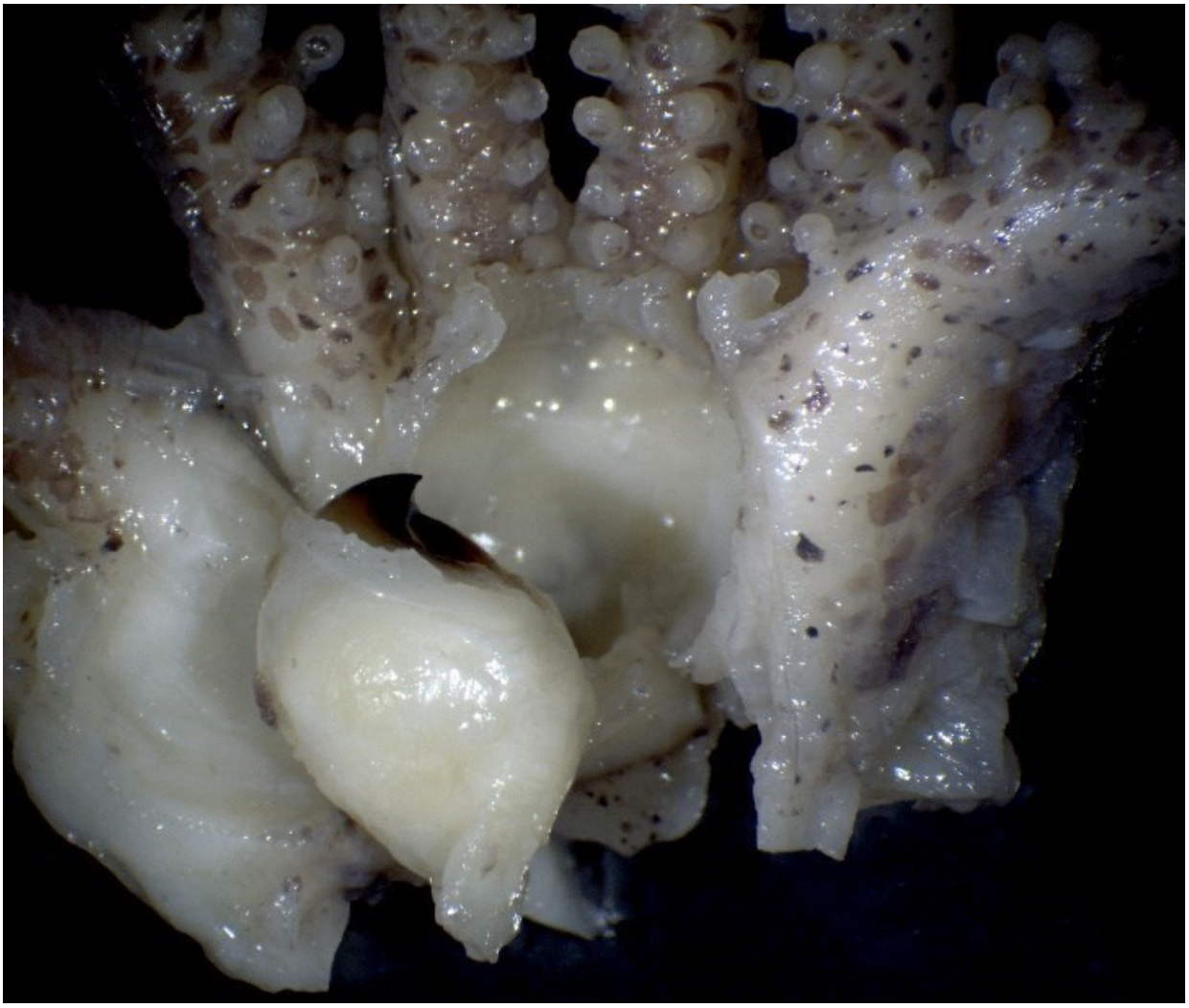

Fig. 10. Buccal mass cut free.

Sex and species determination based on arms

Handle the arms gently so that the suckers important for sex and species identification, are not lost. The four pairs of arms (I to IV) and the pair of tentacles with the buccal mass and the beak in the centre, and the white funnel below them, are shown in **fig. 11**. Once the buccal mass has been removed, cut out the RAI and LAI together from the inside (taking care not to destroy the suckers at the base of the arms). Photograph them either individually or together if analyses are to be done later. Save the arms in a vial.

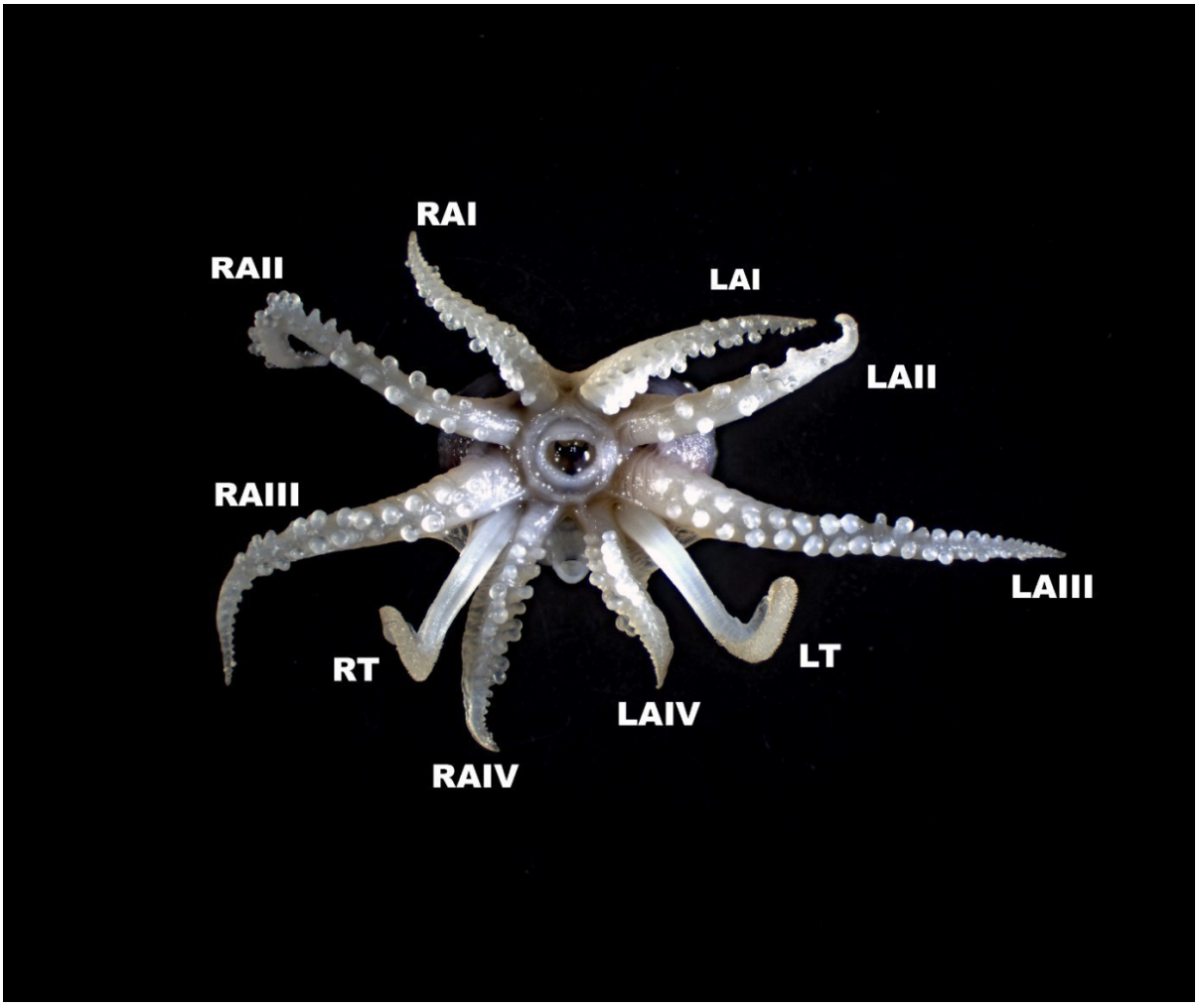

Fig. 11. The four pairs of arms and the pair of tentacles of a female *Sepietta oweniana*. A is arm, T is tentacle, R is right, and L is left.

The sex and species (males) can be determined according to the shape of the first left arm (LAI). If hectocotylized it´s a male (**fig. 12**), otherwise it´s a female (**fig. 13**). Note that early juveniles do not have a hectocotylus. Hectocotylization is the modification of an arm in order to transfer spermatophores from the male to the female`s bursa copulatrix (BC) (in *Sepietta*) during copulation (Bello 2020). The hectocotylus can be subdivided into three parts (**fig. 12)** according to Naef (1923): the basal part; the copulatory apparatus (with modification of from one to several

sucker/stalk structures); and the distal part (where suckers often become smaller and smaller towards the tip of the arm). Note the different sizes of suckers, typical for *S. oweniana* male*s,* as shown in **fig. 12**.

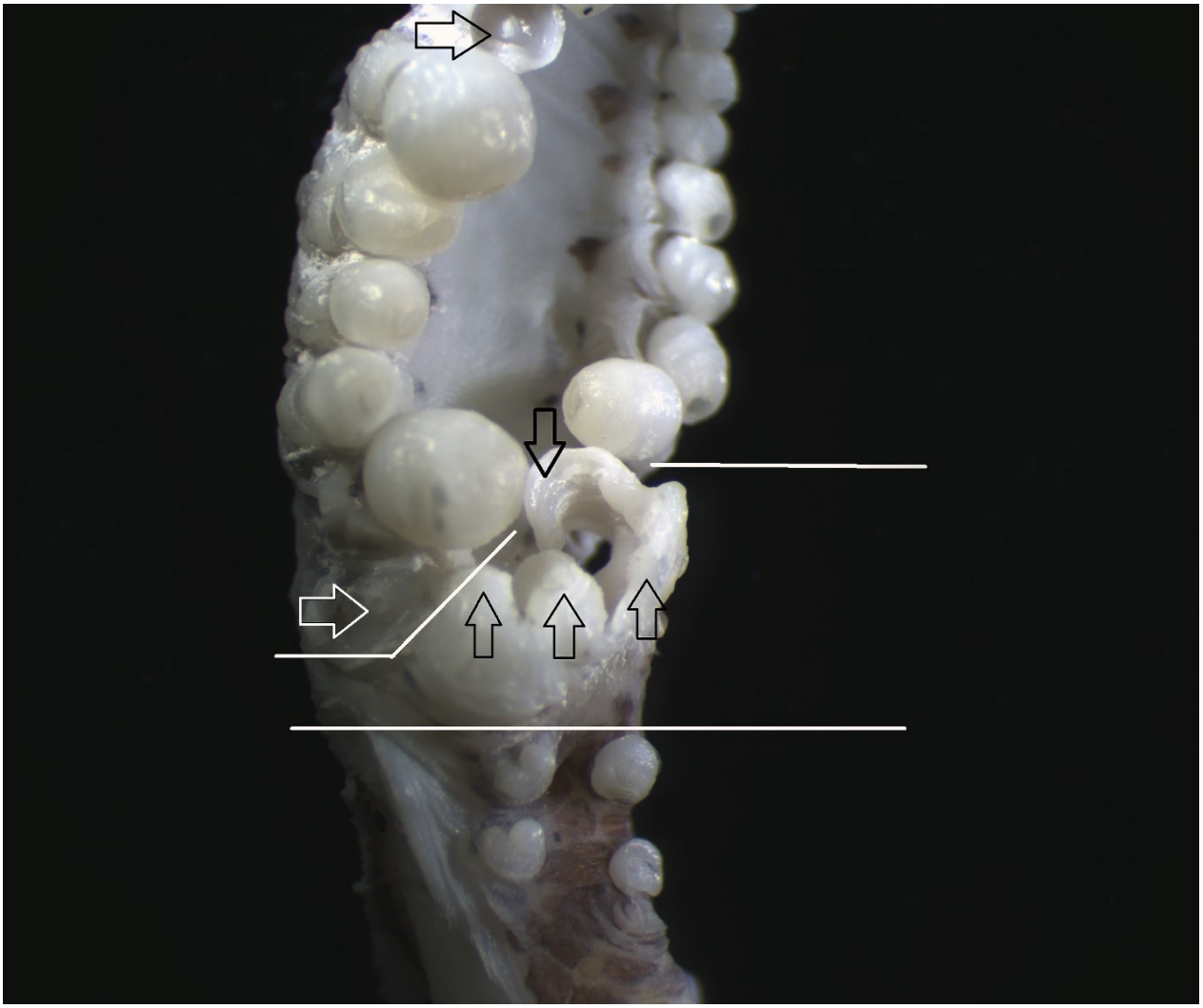

Fig. 12. Hectocotylized left dorsal arm (LAI) of a normal male *Sepietta oweniana.* The upper right arrow shows the pronounced hook-like modification and the lower arrows show three other modified sucker/stalk structures. The lines separate the three different parts of the hectocotylus. The left row of suckers is on the dorsal side of the arm (closest to the dorsal midline of the body). Two large suckers are missing (far left arrows).

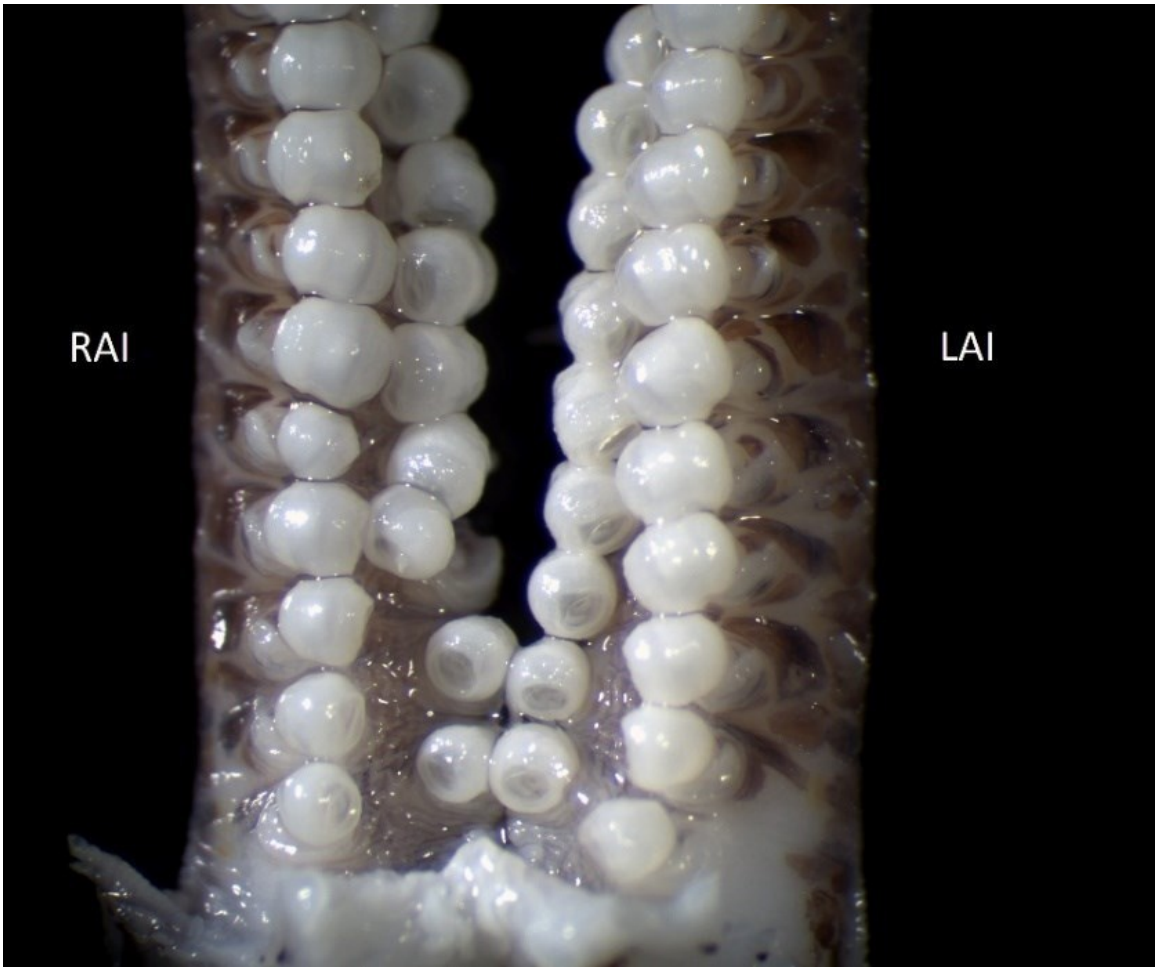

Fig. 13. Right (RAI) and left (LAI) dorsal arms of a female *Sepietta oweniana.*

The morphology of a normal hectocotylus of *S. oweniana* is shown and discussed in Bello (2019b). As a rule, for this species, there are four suckers in the basal part, plus, on the dorsal side of the arm, two enlarged suckers followed by two to four small suckers and two more enlarged suckers. **Fig. 12** shows the modified suckers. Note that the morphology of the hectocotylus changes during development and can be difficult to observe in small males (stain the small hook-like structure and use a curved tungsten needle to lift it up). Consequently, for the sake of comparison, specimens should preferably be of the same size and at the same stage of maturity. Cuccu, et al. (2009) and Bello (2019b) reported variations among the hectocotylus of *S. oweniana* specimens from Sardinian and Spanish waters, respectively. Subpopulations that manifest differences among morphological sex characteristics (and other morphological features) are of interest, especially in species like *S. oweniana* with a large geographic distribution covering very different habitats. Collaborative anatomical/morphological studies, comparing newly caught and well-preserved material throughout different species' distribution areas, might therefore provide important new insights as regards the biology and speciation processes of bobtails. Further sex determination will be carried out during the dissection of the reproductive organs.

Removing organs from the body

Removing several complete organ systems from the same individual can be challenging, but for just one organ system it suffices to merely cut off everything else. Ecologists would normally need the statoliths, the beak, the radula, the spermatophoric sac with spermatophores, the oocytes, the bursa copulatrix and the oesophagus/stomach/caecum/intestine.

Opening the body

Dry the body on a kitchen paper and put it into a Petri dish without water. Measure the DML (formaldehyde) to the nearest 0.01 mm from a photo of the preserved body. Since the mantle is connected to the head on the dorsal side it is important to take the DML in a uniform way (see **fig. 2**). The body should have been in formaldehyde for at least three days so its effect on length has been more or less stabilized. However, we have found no data describing the effect of formaldehyde on length and mass of bobtails over time.

Before the organs can be removed, one needs to cut away the mantle and its muscular layer. We prefer to take the ventral part of the mantle first, so that all the organs are attached only to the muscular layer of the dorsal mantle. The dorsal part of the mantle has to be removed later in order to free the visceral mass (see below), a necessity when one has to dissect the individual organs from all possible angles since bobtail organs are compact and closely attached to each other.

Hold the body with large forceps and use a micro scalpel to make a u-shaped cut just through the dorsal mantle skin and its muscular layer close to the fins, as shown in **fig. 14.** Check that the cuts go through all the muscles. If they are made close to the fins there is little risk of cutting into any organs.

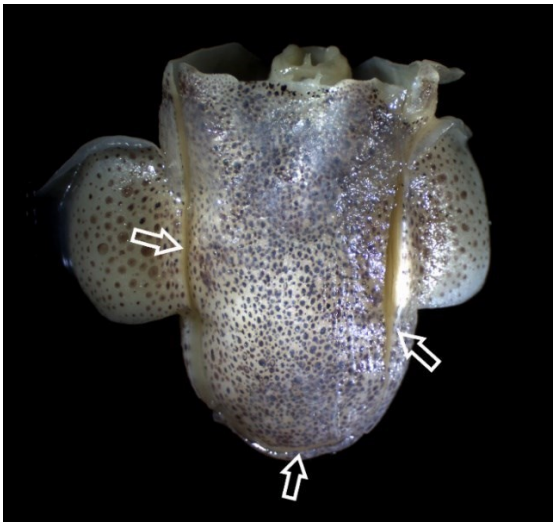

Fig. 14. The u-shaped cut (arrows) through the dorsal side of the mantle and the mantle muscular layer.

Keep track of the left, right, dorsal and ventral sides of the body during all remaining dissections. Place the body against the Petri dish wall (**fig 15**) with the ventral side downwards. Use two forceps to hold the dorsal side of the mantle upwards and to press the ventral side of the mantle downwards so that the gill attached to the ventral skin muscle close to the fin can be seen. Cut the left gill free close to the mantle muscle **(fig 16**) so that it´s connected to the branchial heart only (via the large gill/heart blood vessel at the base of the gill). The mantle sides should then be separated further so the large central/ventral muscle connected to the ventral mantle side can be cut off (**fig 15**).

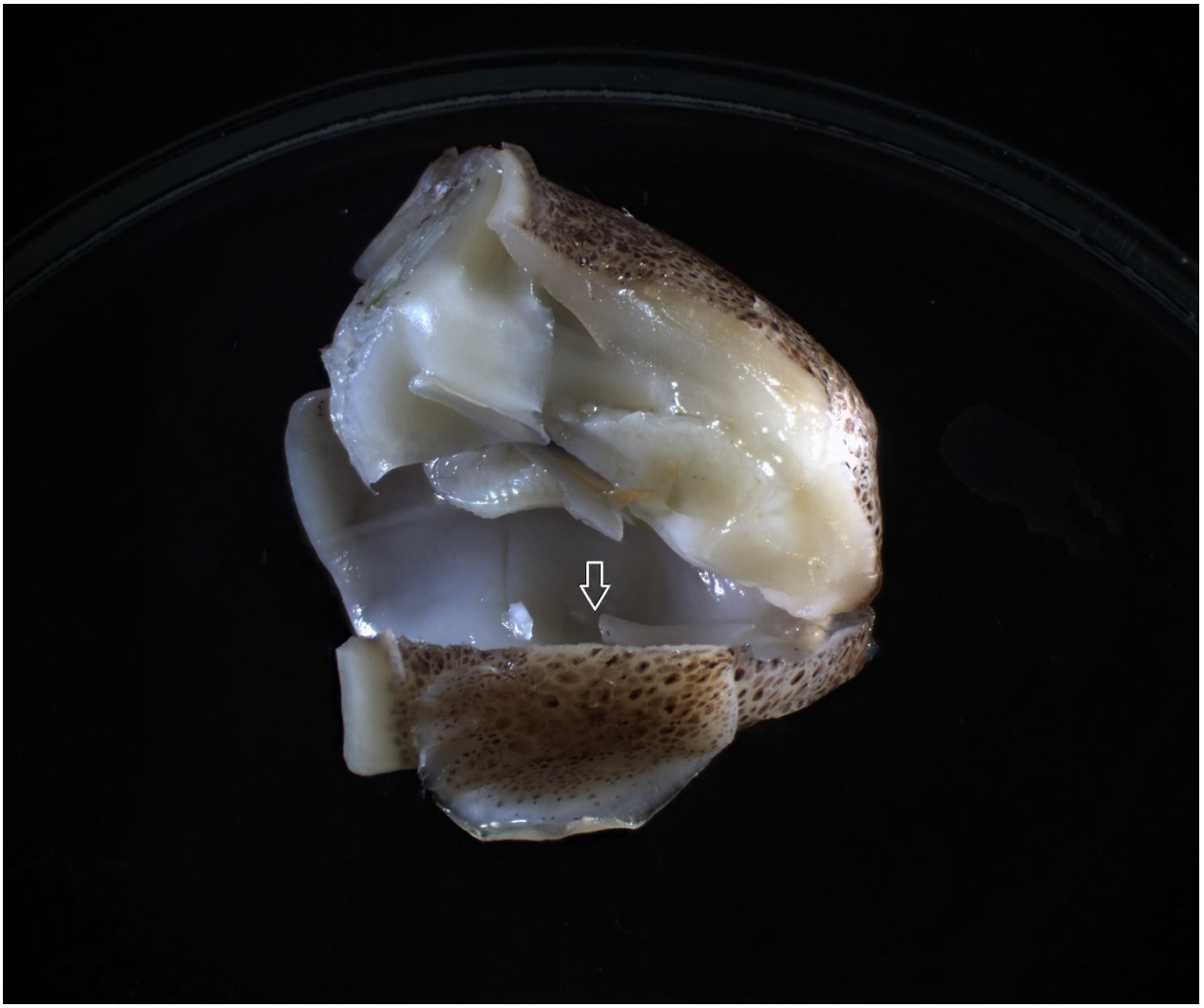

Fig. 15. The body with the ventral side downwards and lying against the petri dish wall. The left gill (removed) and the large central/ventral muscle (arrow) has been cut off close to the ventral mantle.

Fig.16. The gill is being removed.

Turn the body and cut off the other gill. Finally, cut off, close to the mantle muscular layer, any other obstacles in the posterior and anterior parts of the body so that both sides of the mantle can be separated (**fig. 17**). Save the ventral part of the mantle in the specimen jar.

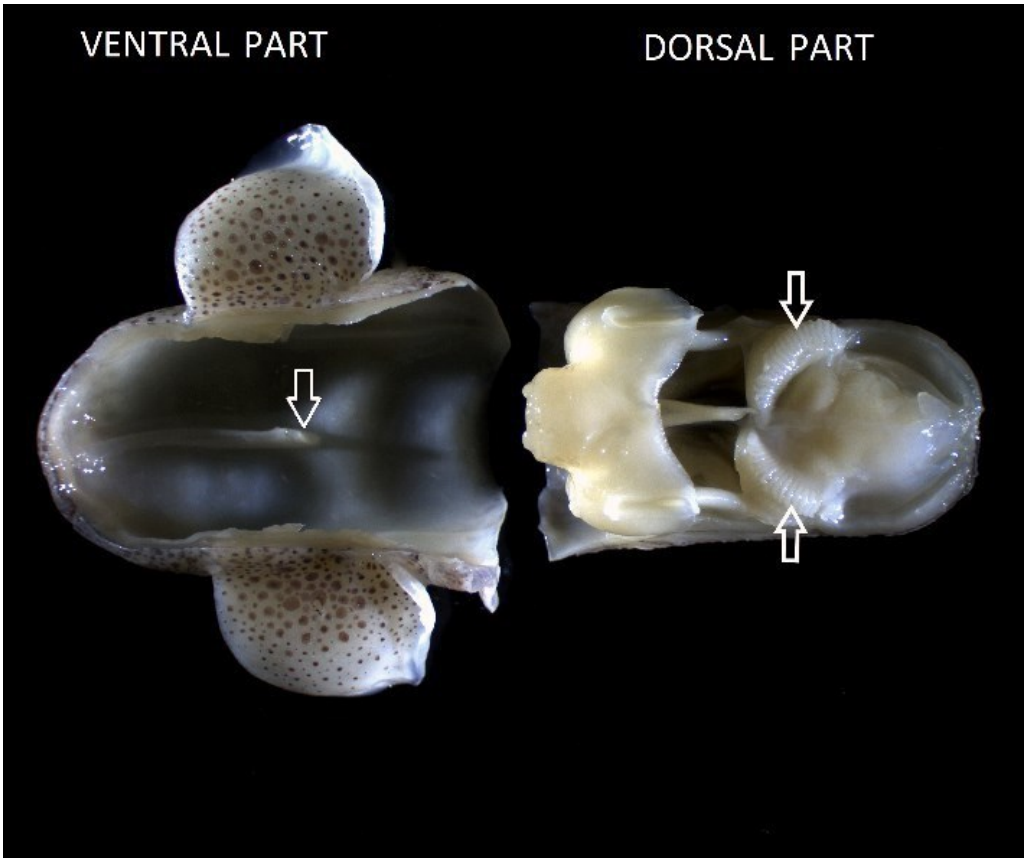

Fig 17. The empty ventral part of the mantle (the arrow shows where the ventral muscle was cut off) and the dorsal part with all organs including the gills (arrows)

Removing or not removing the gills

If one is interested in dissecting the gills, the three hearts and the main blood vessels all together, this should be done at a later stage (see below, "Separating the gills and the hearts from the digestive organs". Otherwise, remove the gills and preserve them in formaldehyde in a vial (**fig. 18**). One reason for saving the gills could be to look for the presence of epibionts/parasites about which little is known (see short review by Roumbedakis, et al., 2015).

Fig. 18. A gill connected to the branchial or gill heart.

Removing the digestive gland

The dorsal mantle should not be removed until the large brown digestive gland (DG) has been removed in one piece. To get access to the DG, the large muscle complex in the anterior part of the body (**fig. 19**) covering the DG needs first to be removed. There are two pairs of large ventral and dorsal muscles attached anteriorly to the muscle complex and posteriorly to the left and right mantle musculature**.** These four muscles need to be cut free at one end.

Start by cutting off the two ventral muscles close to the mantle (as shown in **fig. 19**) still leaving them connected to the muscle complex. Carefully remove the thin (but strong) membranes that cover the muscle complex. Removing membranes is the most time-consuming part of bobtail dissections. Use two tweezers (one fine-pointed and straight and the other curved) to remove the membranes. Grasp the membrane with the straight tweezers (by holding it against the curved one

to get optimal precision) and then use the curved one to hold the organ while removing the membranes with the straight tweezers. Never use fine-pointed tweezers to hold any organs as they can easily be damaged.

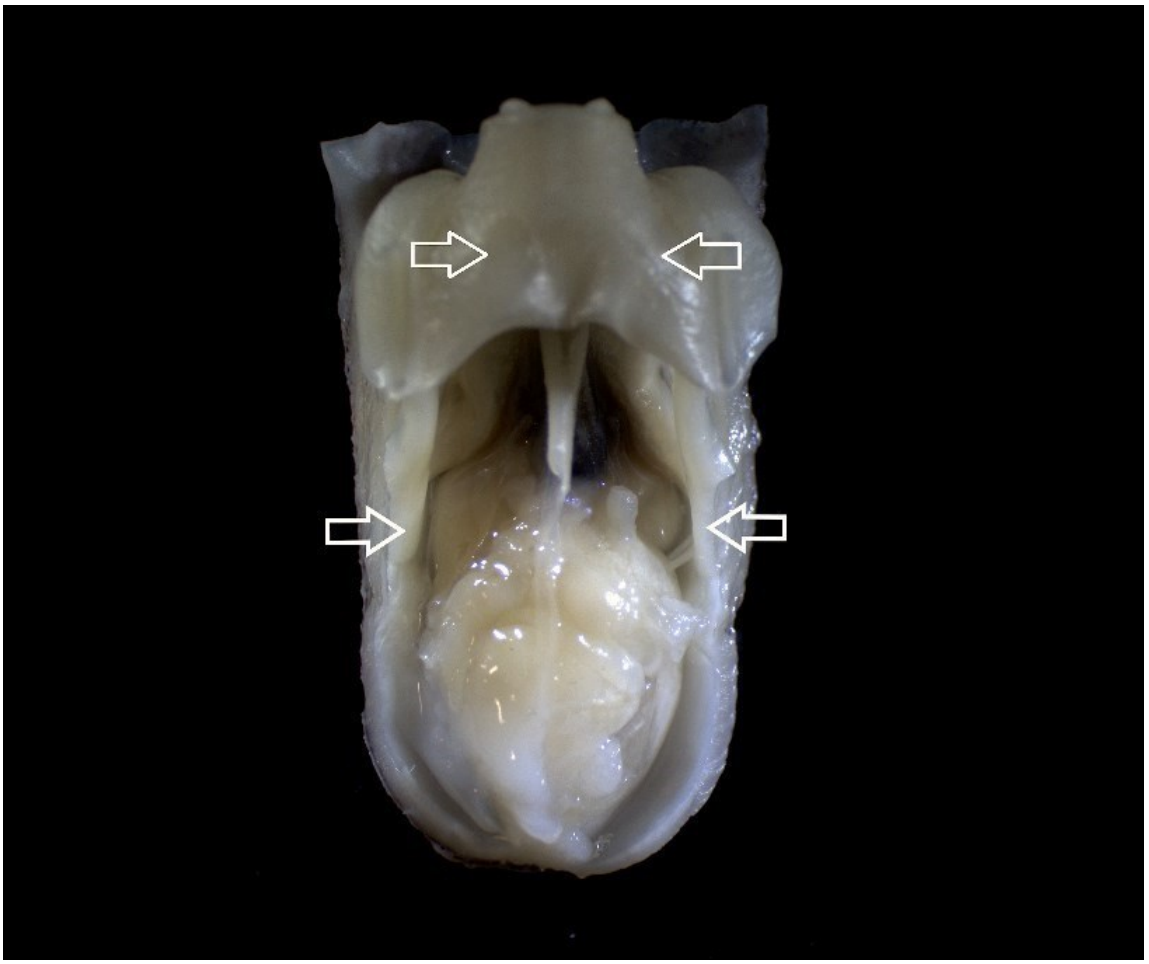

Fig. 19. The gills have been removed and the upper arrows show the large muscle complex. The lower arrows show the cut positions for the two large ventral muscles, close to the mantle on each side of the body.

If it was decided to keep the gills together with the hearts, they should be moved to the side so the pair of dorsal muscles can be cut off close to the large muscle complex, as shown in **fig. 20**. Be careful not to damage the brown digestive gland underneath these muscles when making the cuts. The large ventral muscles remain connected to the large muscle complex and the dorsal muscles are still connected to the mantle as seen in **figs 20 & 21**. Cut off any remaining minor obstacles and the anterior muscle package can be carefully removed (**fig. 20**).

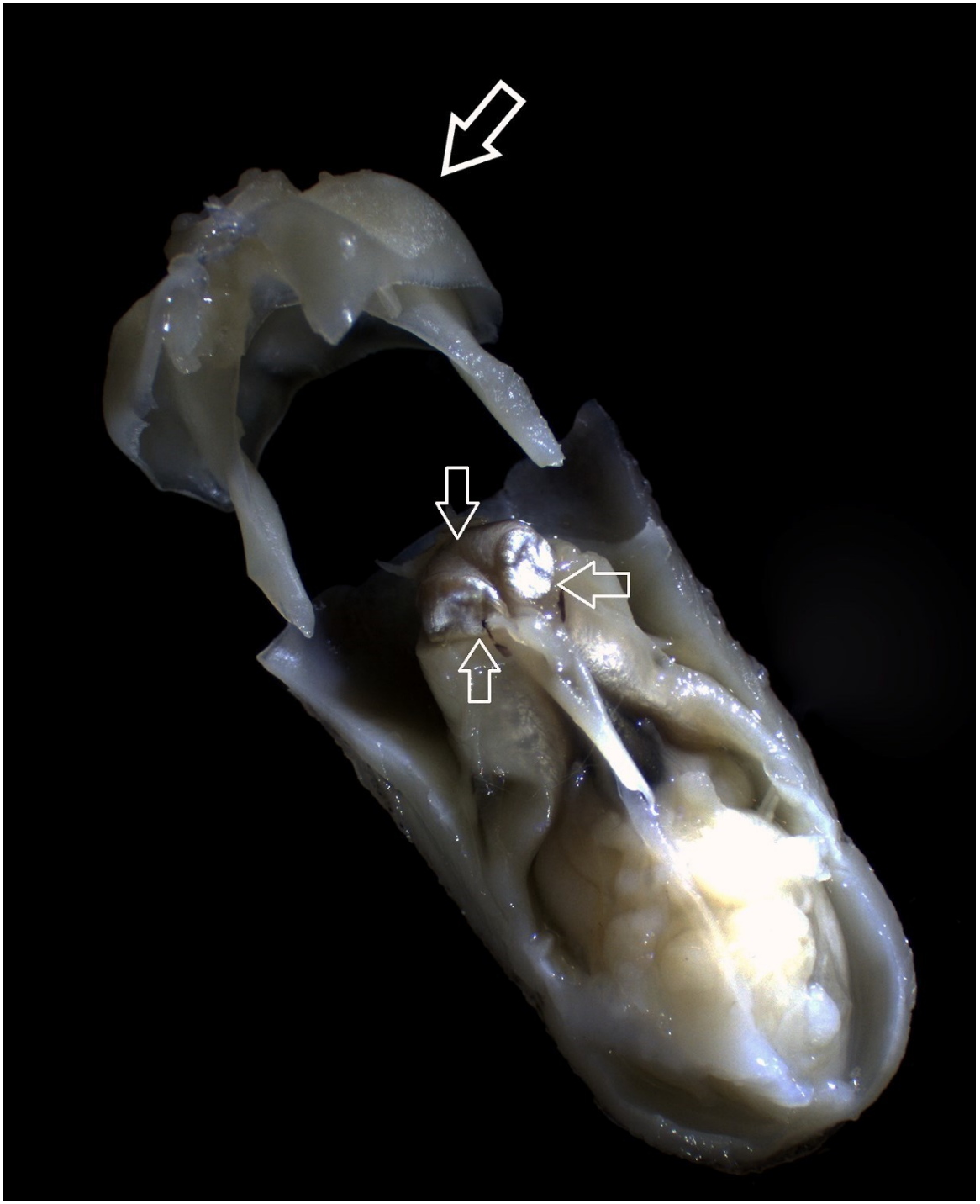

Fig. 20. The large muscle complex (upper arrow) is removed together with the ventral pair of large muscles. The brown digestive gland can be seen (arrow) where the large dorsal muscles were cut off close to it (lower arrows).

The next task is to cut off the large central/ventral muscle (which posterior mantle connection was cut off when removing the ventral mantle, **fig. 15**). As can be seen in **fig. 21**, the central/ventral muscle divides around the end of the intestine. Remove all the membranes between the central/ventral muscle and the intestine and cut off the muscle around the intestine as indicated in **fig. 21.**

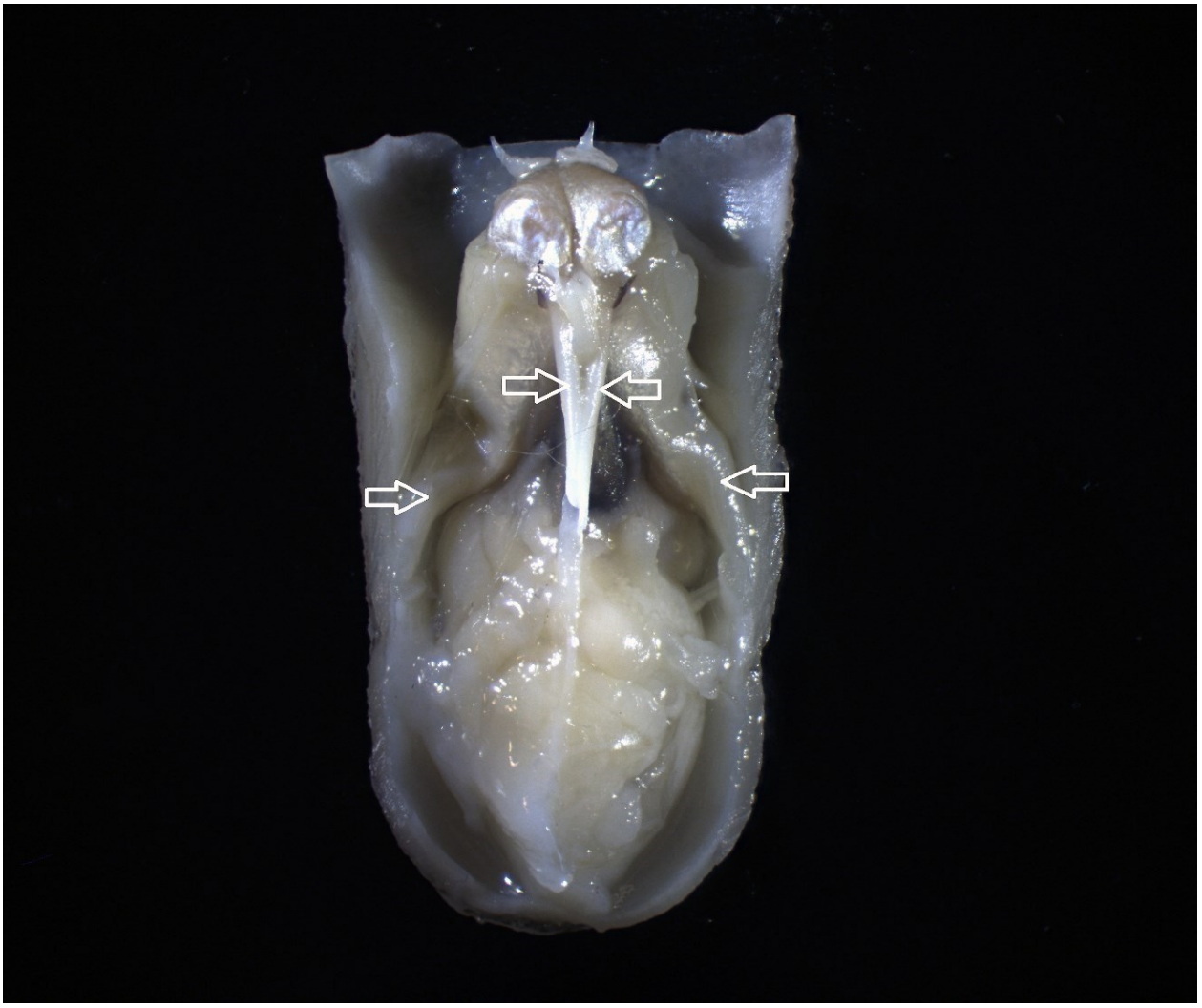

Fig. 21. The two central/ventral muscle cuts close to the intestine (upper arrows) and the two dorsal muscle cuts close to the mantle (lower arrows).

Once the central/ventral muscle has been removed, one can cut off the two large dorsal muscles where they connect to the mantle musculature (**fig. 21**)**.** Carefully remove the remaining thin membranes and the muscle package covering the DG, as seen in **fig. 22.**

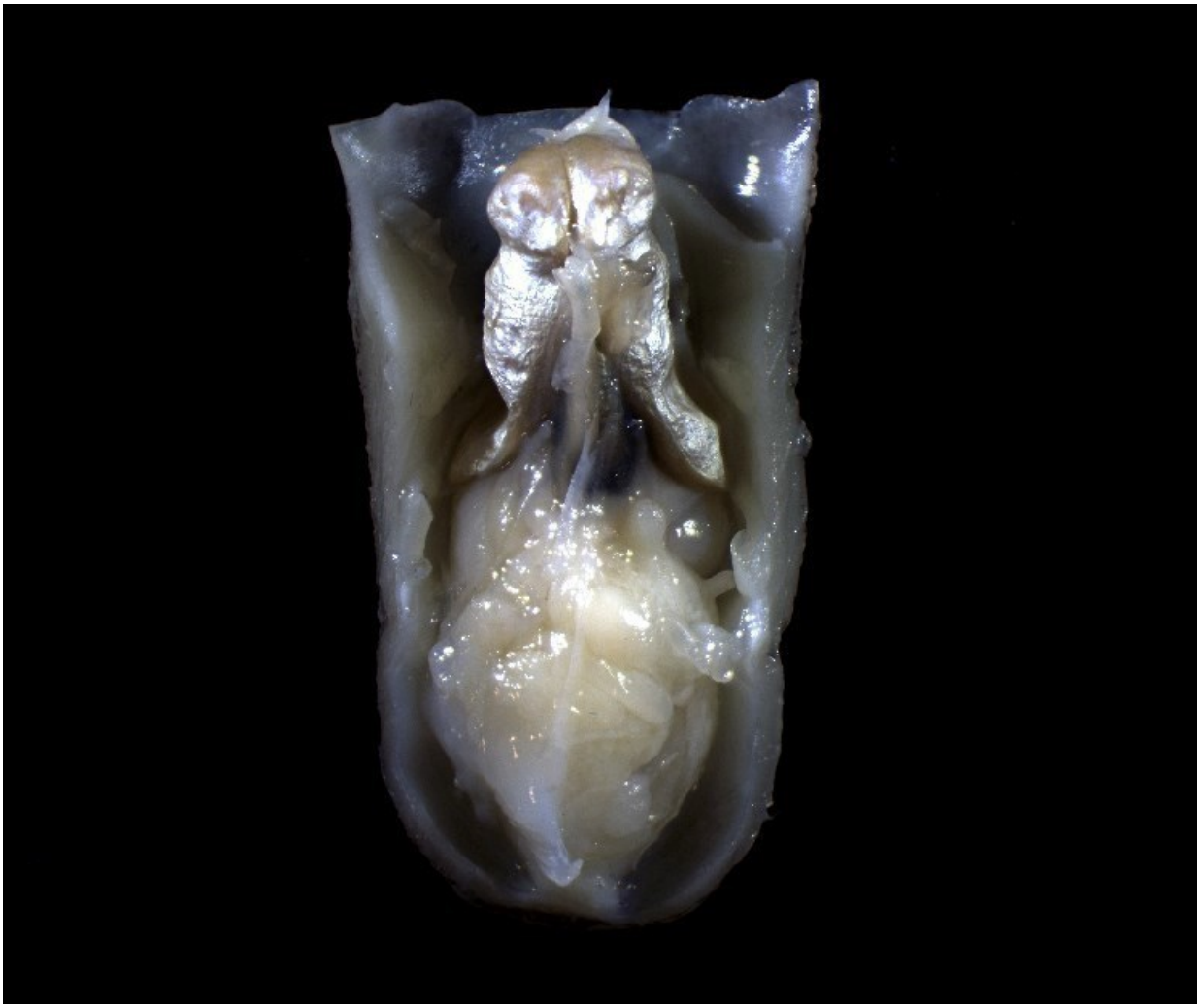

Fig. 22. The muscle package covering the digestive gland has been removed so it can be seen in full.

Before taking away the DG, one must slightly separate its two halves to free the oesophagus and the dorsal aorta that go through the DG. Insert the curved side of the curved tweezers between the two halves and release the pressure slightly. The oesophagus and the dorsal aorta are now free but connected to each other by thin membranes and the DG (**fig. 23**) can be removed. Photograph and determine the wet mass (formaldehyde) of the DG and preserve it in vial. Later, one can use the same vial for the remaining digestive organs. Note that the shape and the size of the DG differs considerably between individuals and especially between those of different sizes.

Fig. 23. The brown digestive gland has been lifted out. The oesophagus (upper arrow), with the closely connected aorta, and the intestine (lower arrow) that is stained dark here due to leakage from the ink sac are now free.

Removing the visceral mass

To dissect the individual organs, the visceral mass has to be cut free from the dorsal mantle and its muscular layer. However, for females, it is preferable to keep the dorsal mantle when removing hearts and reproductive organs (see below). Place the anterior part of the mantle against the Petri dish wall (**fig. 24**). At the anterior part there are many minor muscles and membranes to be removed before the organ package can be freed (**fig. 25**). One can also dissect the organ package from the side (as was done when removing the ventral side of the mantle). For mature females, one can use tweezers to remove the membranes between the oocytes and the dorsal mantle muscles while holding the body with large forceps. Save the dorsal mantle in the specimen jar.

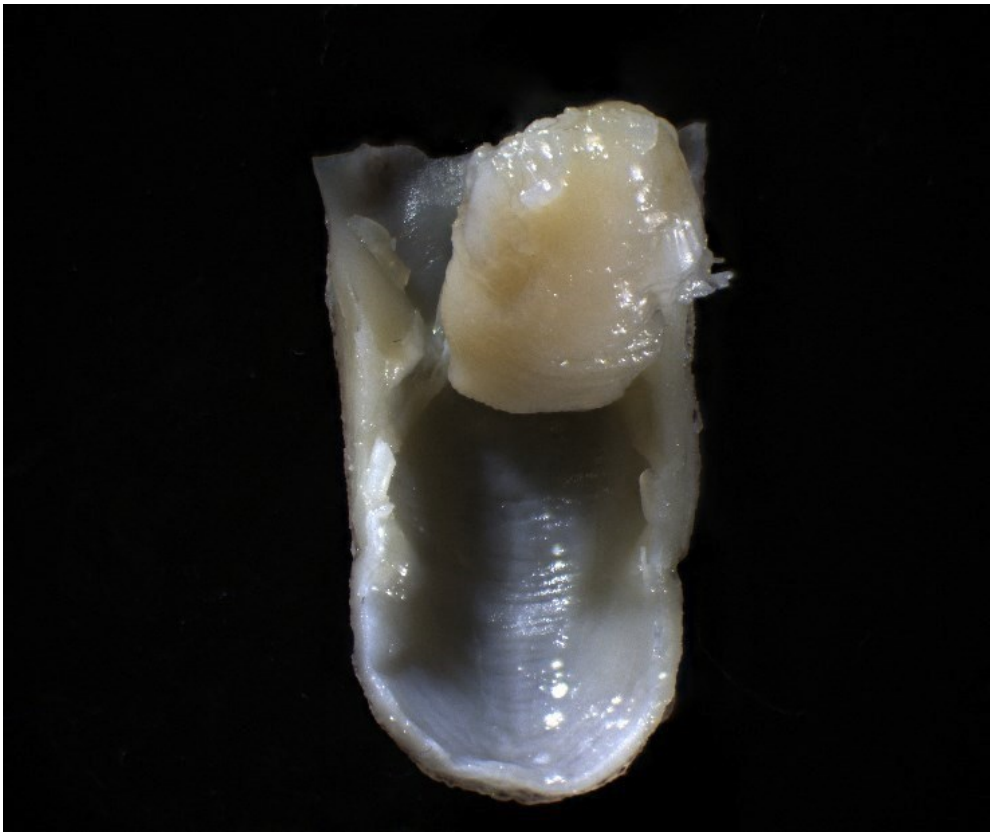

Fig. 24. Removing the organ package from the dorsal mantle.

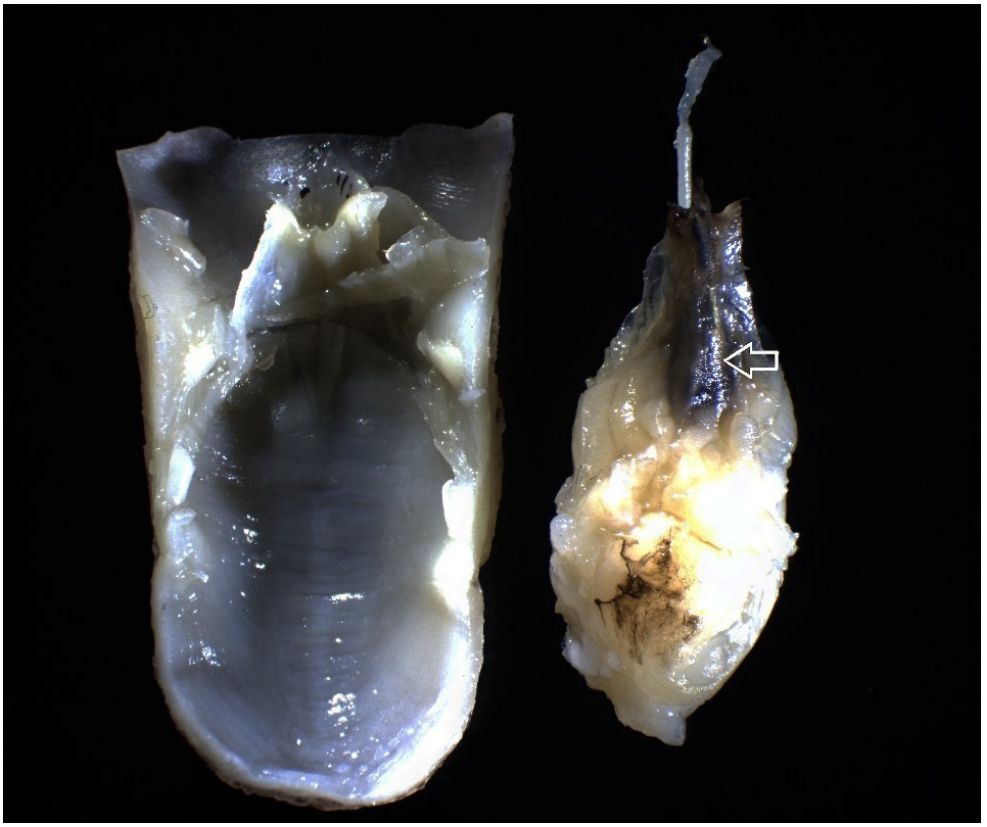

Fig. 25. The organ package is free from the dorsal mantle. Note the muscles that are cut off in the anterior part of the ventral mantle. Arrow = ink sac.

Removing the ink sac

It is important that the ink sac is removed before dissection continues (**fig. 25**). The ink sac and the intestine are very close to each other and connected by membranes. The ink sac goes into the end of the intestine close to the two anal flaps as shown in **fig. 26**. Peel off the membranes, piece by piece, starting below the anal flaps, using fine-pointed tweezers. Be extremely careful not to cut into the ink sac as the ink would stain the organs. Have a Pasteur pipette ready to suck up any ink. Once the ink sac is completely free from all membranes (**fig. 27**), save it in a vial of its own.

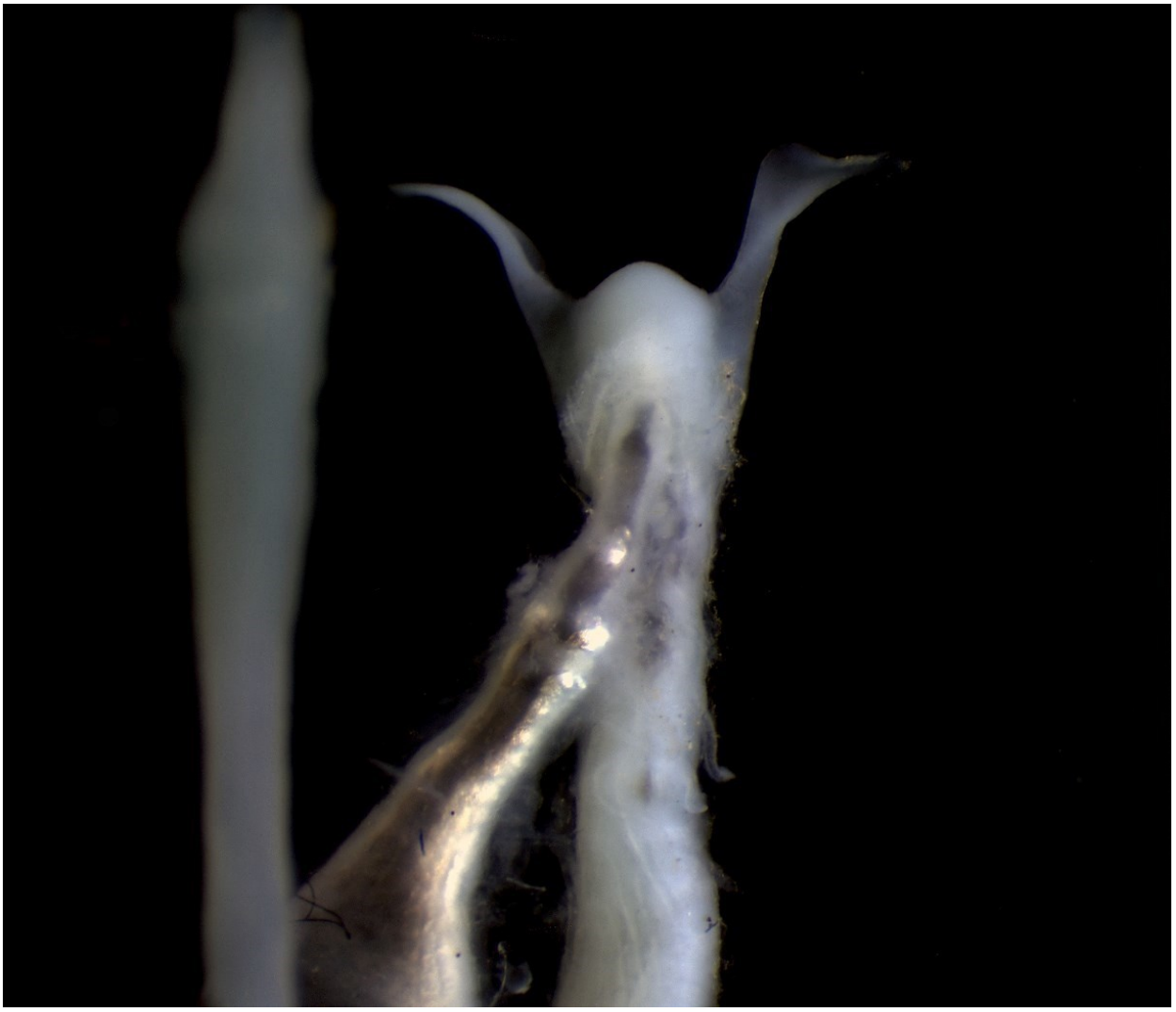

Fig. 26. The ink sac goes into the end of the intestine close to its anal flaps. The membranes connecting the ink sac and the intestine have been removed (the oesophagus in the foreground is out of focus).

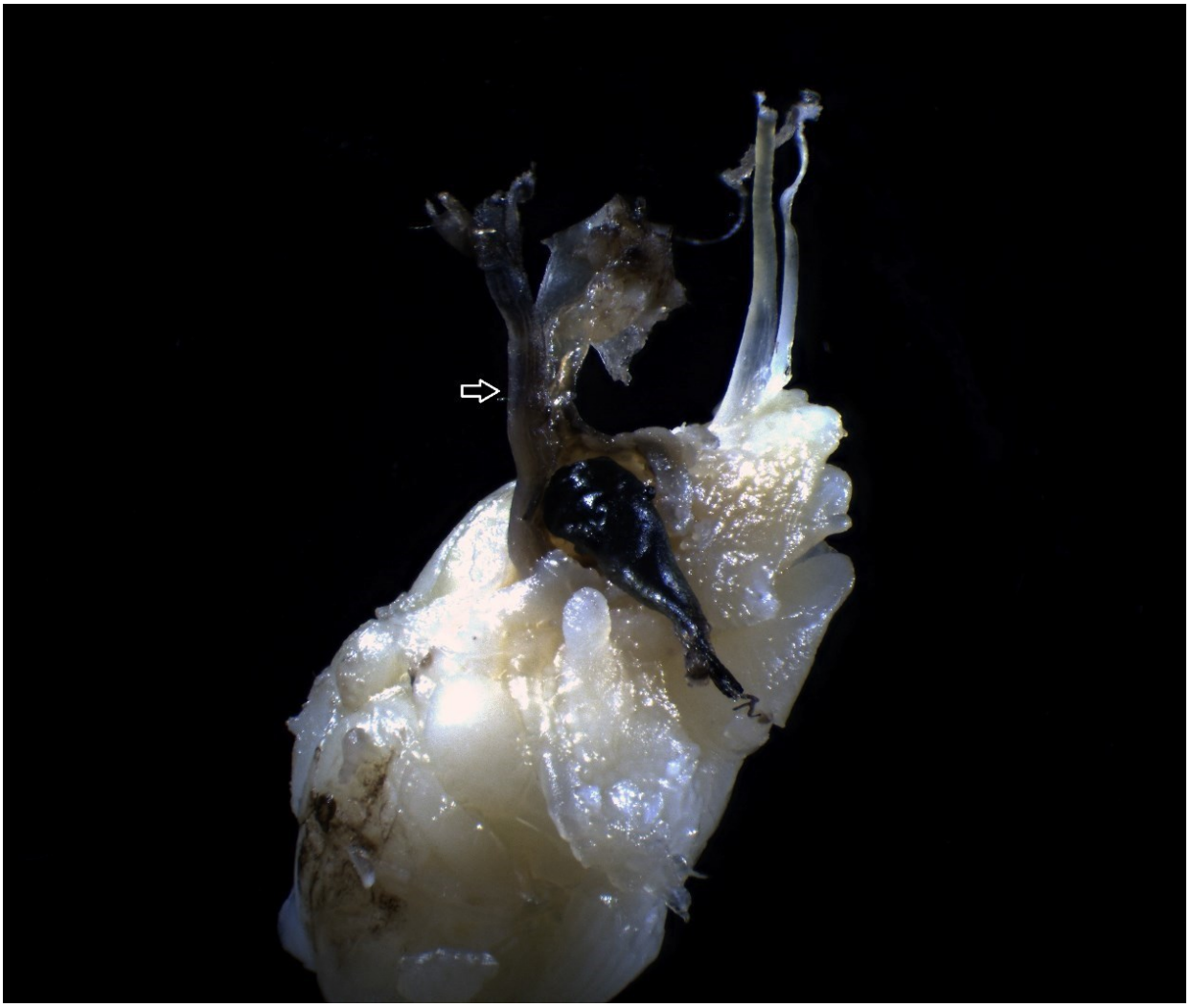

Fig. 27. The thin membranes connecting the ink sac and the dark intestine (arrow) have been removed. The ink sac is laying down and ready to be carefully removed. In the background is the oesophagus with the thinner dorsal aorta to the right (the membranes between them have been removed).

The main organs in the visceral mass

Three groups of organs found in the visceral mass can now be removed (keeping together the organs within each group) in the following order:

1. The reproductive organs consisting mainly of: the male`s testis; the spermatophoric organ; and the spermatophoric sac with its terminal organ: the female`s two nidamental glands; the two accessory nidamental glands; the ovary (with oocytes); the oviduct; the oviductal gland; and the bursa copulatrix.
2. Parts of the respiratory and circulatory organs consisting of: the two gills; the three hearts (the two branchial or gill hearts and the systemic heart); and some major blood vessels.

3. The digestive organs consisting mainly of: the buccal mass; the digestive gland (both already removed and saved); the digestive gland duct appendages; the oesophagus; the stomach; the caecum; and the intestine.

Removing the reproductive organs

The goal is to remove all the reproductive organs together as a group. See below, "Secondary dissections and analyses" how the organs are connected. One should begin by removing the outer membrane that covers the entire visceral mass (**fig. 28**).

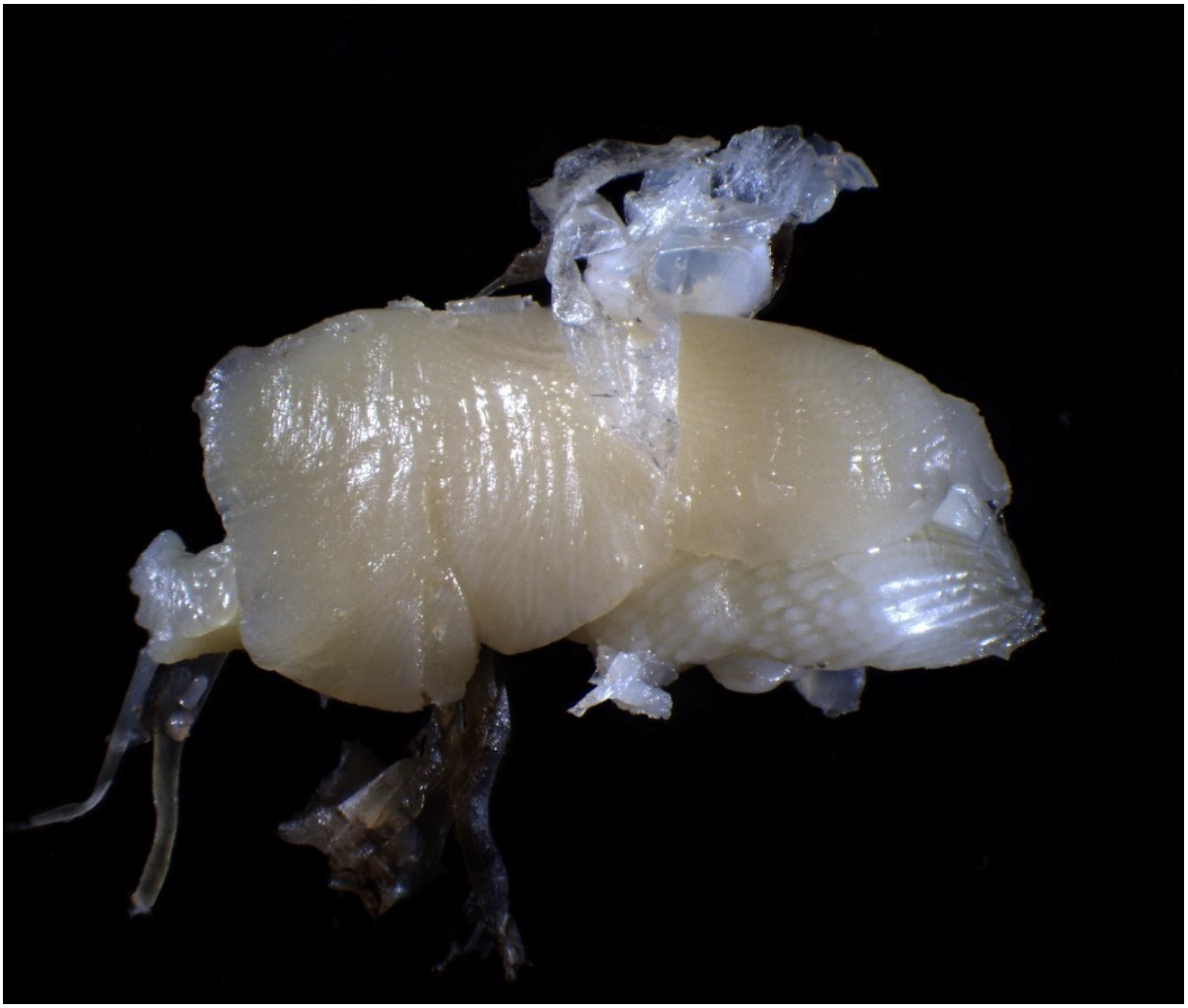

Fig. 28. The outer membranes have been partly removed from the visceral mass without staining.

Staining the membranes with a weak water solution of the thiazine dye azure B (II) makes them blue and much easier to see and remove (**fig. 29**). Azure B is produced by oxidation of methylene blue (Horobin & Kiernan, 2002). Observe the staining process in the stereomicroscope for a few seconds and stop the staining by moving the object to a new Petri dish with water. Repeat the staining when removing the inner membranes later on. Note that this stain will fade

away in ethanol and polyvinyl-lactophenol but remains well in formaldehyde. When the outer membranes are removed, the organs should appear as in **fig. 30**.

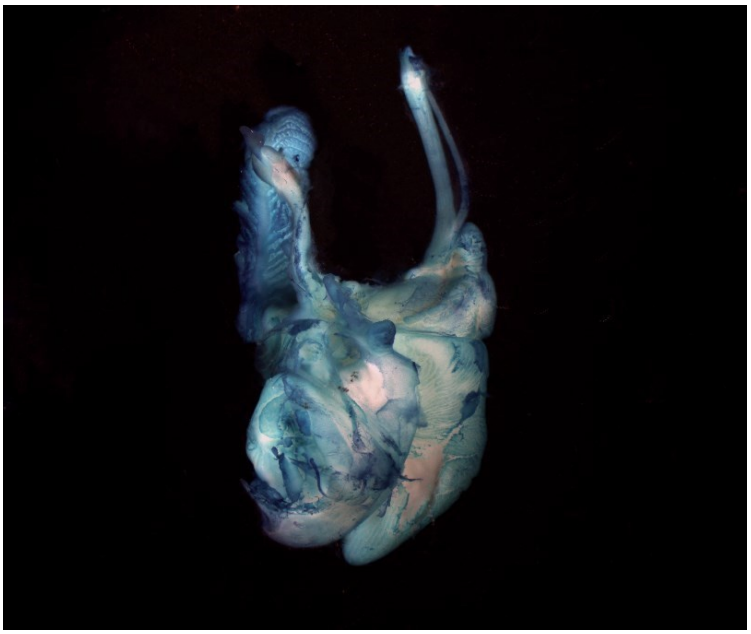

Fig. 29. The outer membranes stained with azure B**.**

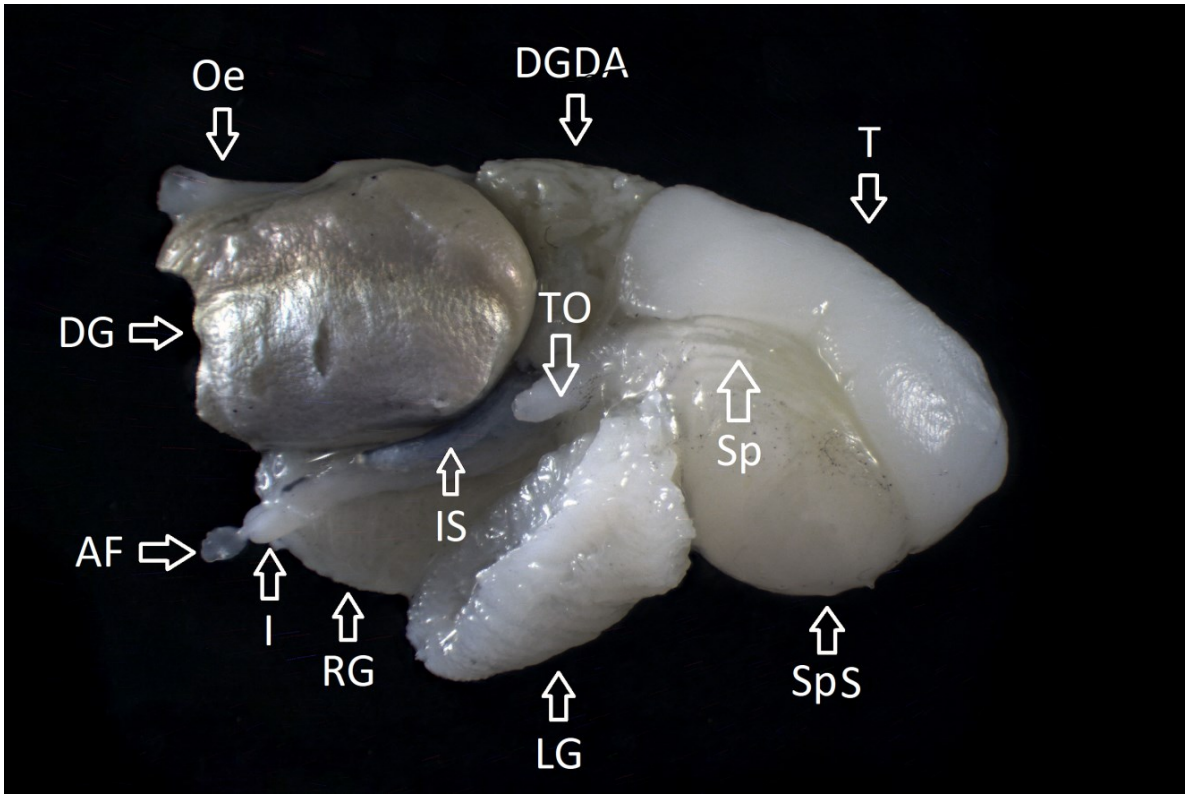

Fig. 30. A sideview of a male *Sepietta oweniana,* without outer membranes, showing: oesophagus (Oe); digestive gland (DG); digestive gland duct appendages (DGDA); testis (T); spermatophoric sac (SpS); spermatophores (Sp); terminal organ (TO); left gill (LG); right gill (RG); ink sac (IS); intestine(I); anal flaps (AF).

Exercise: Remove the outer membranes of a female in order to compare with the male as in **fig. 30**.

The different organ groups can now be separated by inserting the curved side of the curved tweezers between the organ groups and then releasing the pressure gently. Meanwhile, any membranes in between should be cut off. The needle scalpels and the long vitrectomy scissors shown in **fig. 1** are very useful for this and other delicate dissections. This method can be used to separate all individual organs in the following dissections. Make sure that the connections (including the membranes) between the different reproductive organs are intact when separating them from the other organs.

Males. Start the dissection by identifying the major male organs as shown in **fig. 30**. Remove all membranes from the other nearby organs so that the male organs can be removed together and put with the testis (which may be separated from the other male organs) in a male organ vial (**fig. 31**). The thin sperm duct between the testis and the first gland in the spermatophoric organ can easily break. However, in most studies this would probably not matter.

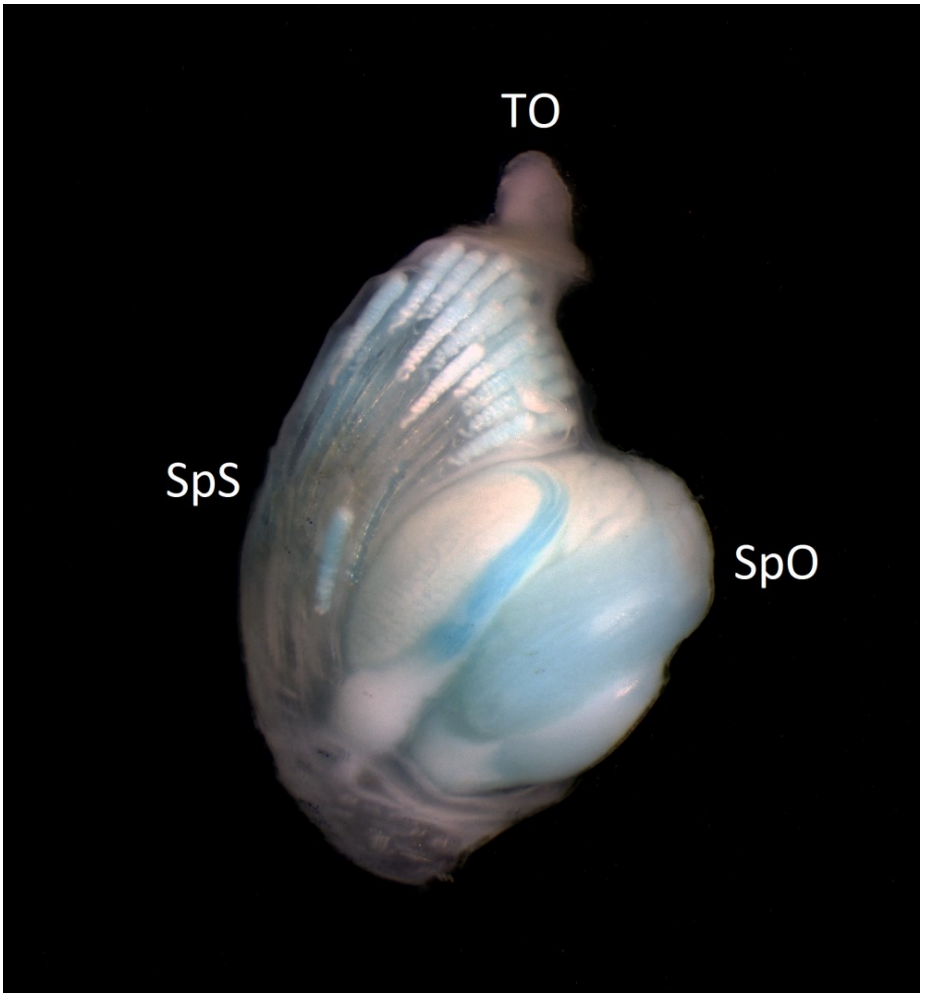

Fig. 31. The spermatophoric organ complex: the spermatophoric organ with its different glands (SpO); the spermatophoric sac (SpS) with spermatophores; the terminal organ (TO), held together by an outer membrane stained blue with azure B.

Females. Commence the dissection by identifying the major female organs as shown in **fig. 32**. Note that the dorsal mantle is not yet removed. Cut off the outer membrane that connects the nidamental glands to the oocytes (**fig. 32**) just enough so the nidamental glands (that are closely connected) can be moved forward and pin them to the body mantle (**fig. 33)**. Remove all the membranes that connect to the other nearby organs including digestive organs, gills, hearts and the major blood vessels (**fig. 33**). When all membranes and other obstacles are removed (including the pins), the dorsal mantle can be cut free. Remove the female organs in one piece if possible (**figs 34 & 35**) and put them in a female organ vial.

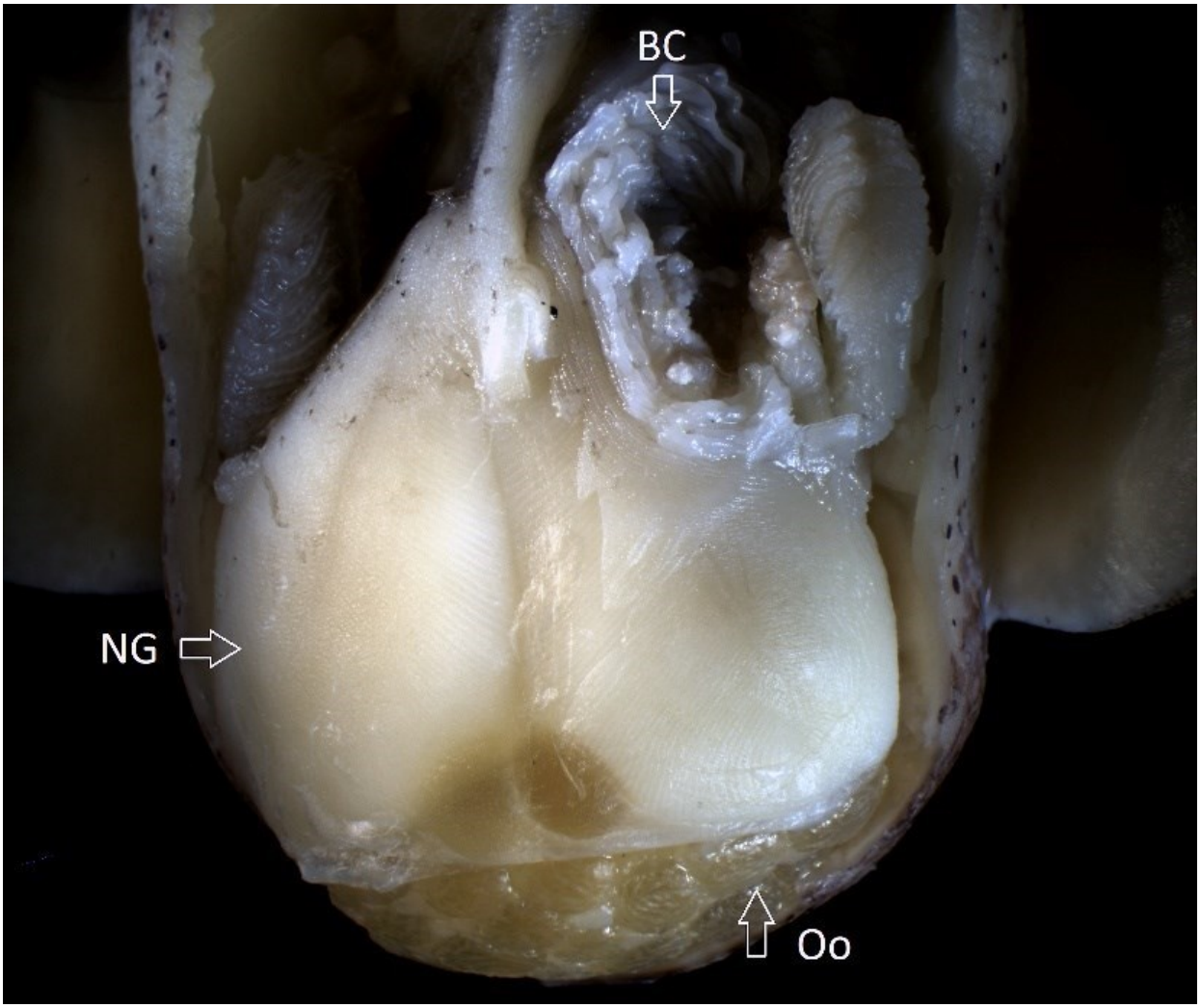

Fig. 32. Ventral view showing the major female organs with the outer membrane connections between the whitish nidamental glands and the oocytes intact: bursa copulatrix (BC); Oocytes (Oo); nidamental glands (NG)**.**

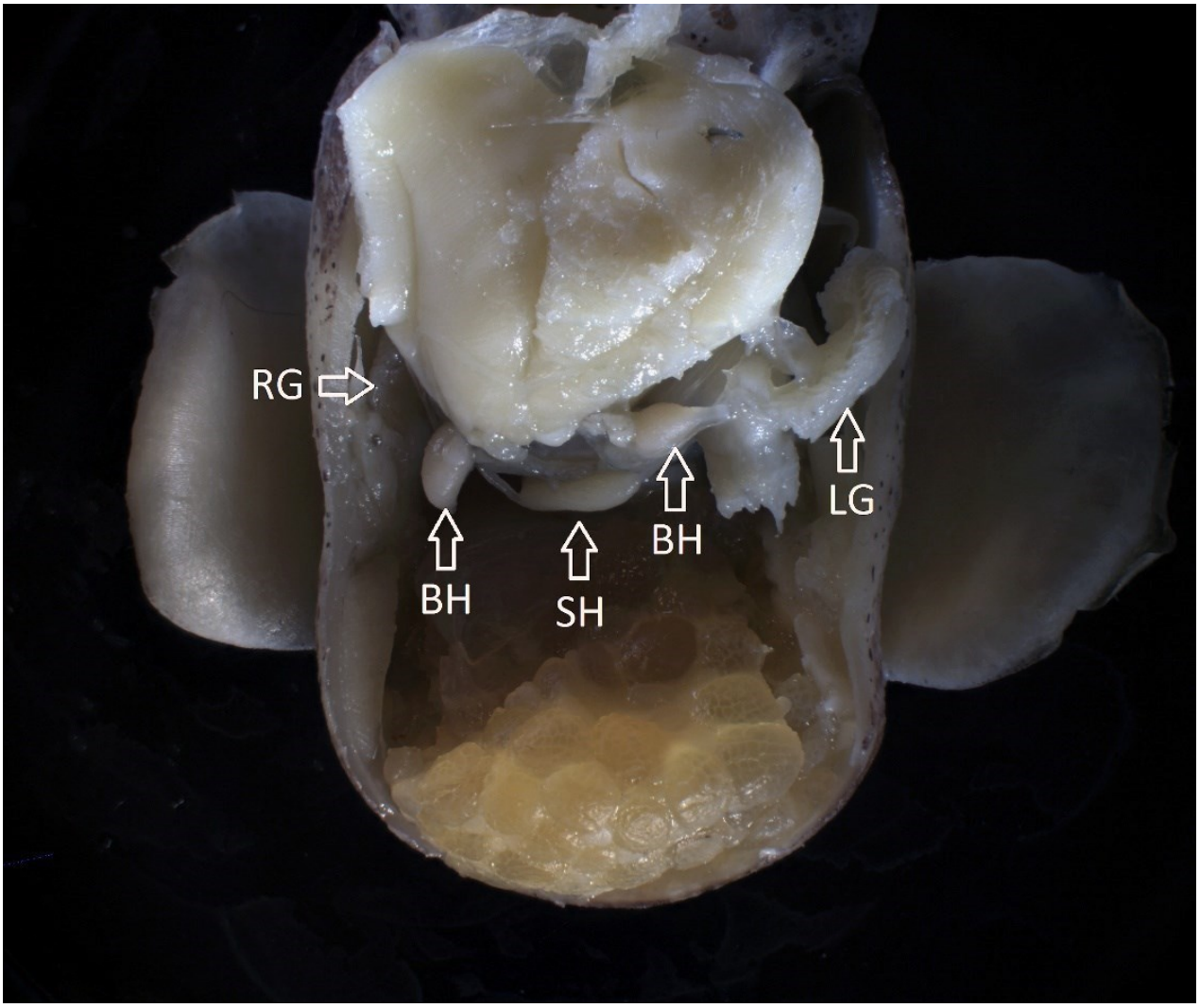

Fig. 33. Ventral view showing that the outer membrane connections between the nidamental glands (NG) and oocytes have been removed. The NG have then been moved forwards and pinned to the dorsal mantle. The organs underneath the NG are: right gill (RG); left gill (LG); branchial heart (BH) or gill heart and; systemic heart (SH).

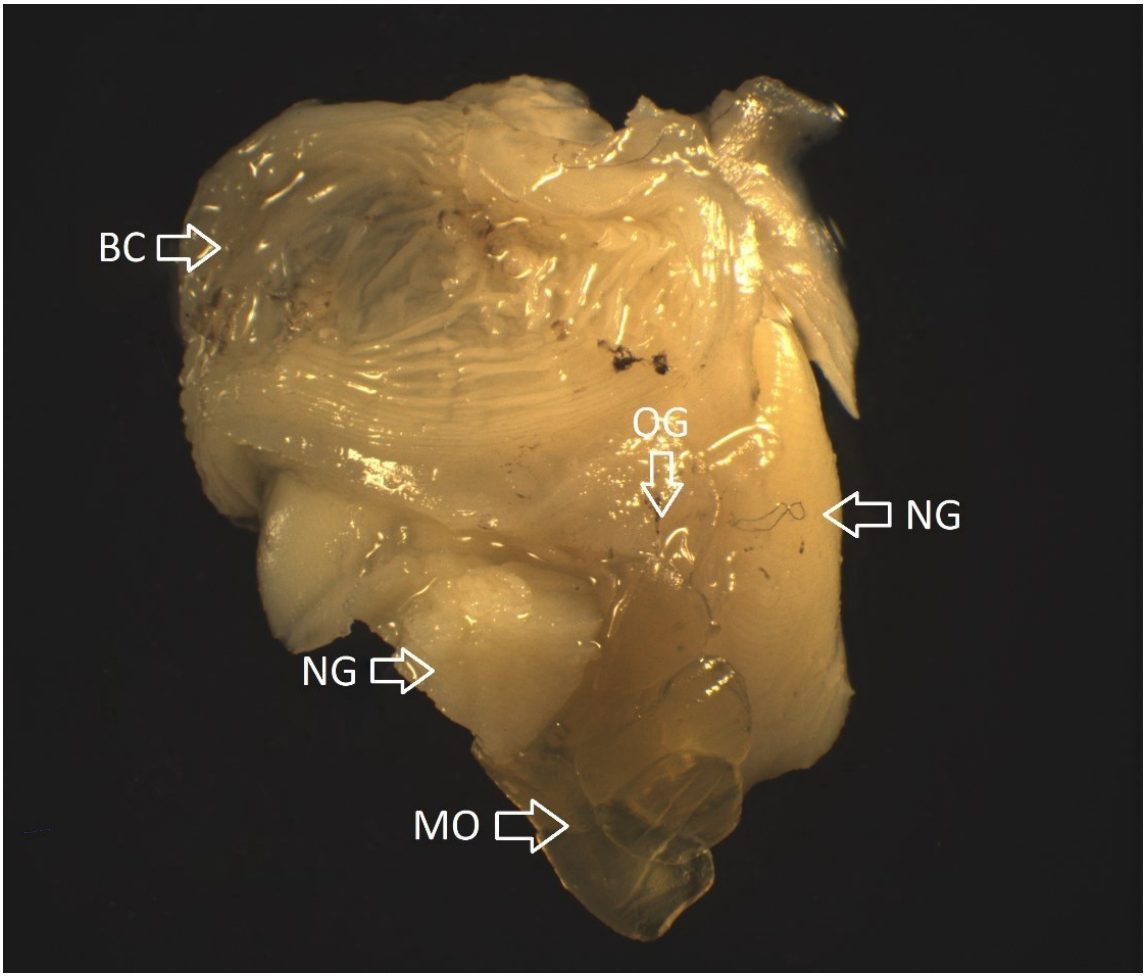

Fig. 34. Side view of the removed bursa copulatrix (BC), the oviducal gland (OG), the whitish nidamental glands (NG) and the mature oocytes (MO).

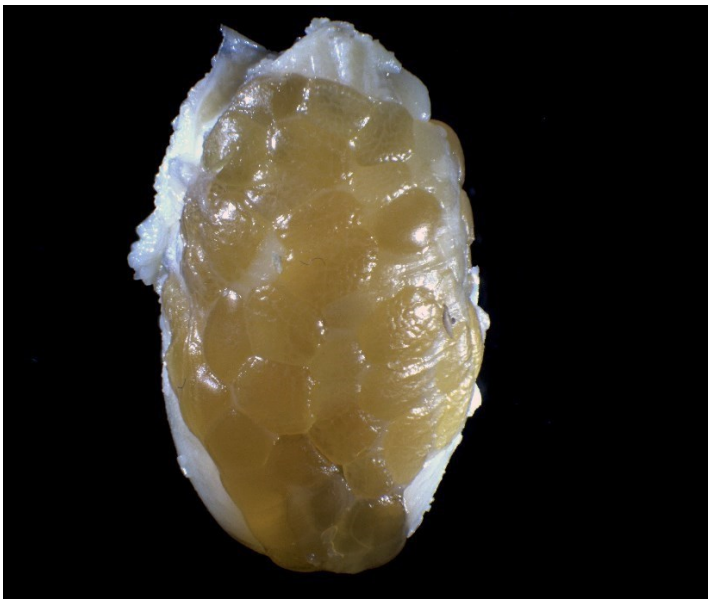

Fig. 35. Dorsal view of separated female organs showing reticulated oocytes.

Removing the gills and the hearts from the digestive organs

Remove the membranes, starting with those from the gills to the gill hearts and then the ones to the systemic heart. Cut the gills and hearts free and save them in an organ vial (**fig. 36**).

Fig. 36. The two pairs of gills and branchial hearts, and the systemic heart.

Dissecting blood vessels requires staining and some special techniques that are not dealt with here. If blood vessels are of interest, one has to remove the dorsal mantle since blood vessels are connected to the digestive and other organs. If the digestive gland duct appendages (**fig. 30**) are not required they can be removed, little by little, in order to simplify the later dissection.

Exercise: Try to trace the blood vessels from the systemic heart (test injecting a dye solution into the heart).

The remaining organs are the main digestive organs (the oesophagus, the stomach, the caecum and the intestine, which are connected in a U-shape). All these organs should be cleared from membranes and blood vessels, with the use of curved tweezers, fine tipped tweezers, the needle scalpels and the long vitrectomy scissors. First work along the oesophagus and the dorsal aorta

close by, carefully removing all the membranes in between (**fig. 27**). Continue removing membranes connected to the stomach (found at the end of the oesophagus) and the nearby caecum, followed by the intestine with its anal flaps. Note that the shape and volume of the stomach and the intestine can vary considerably depending on the amount of food present (**fig. 37**). Save the digestive organs in a vial.

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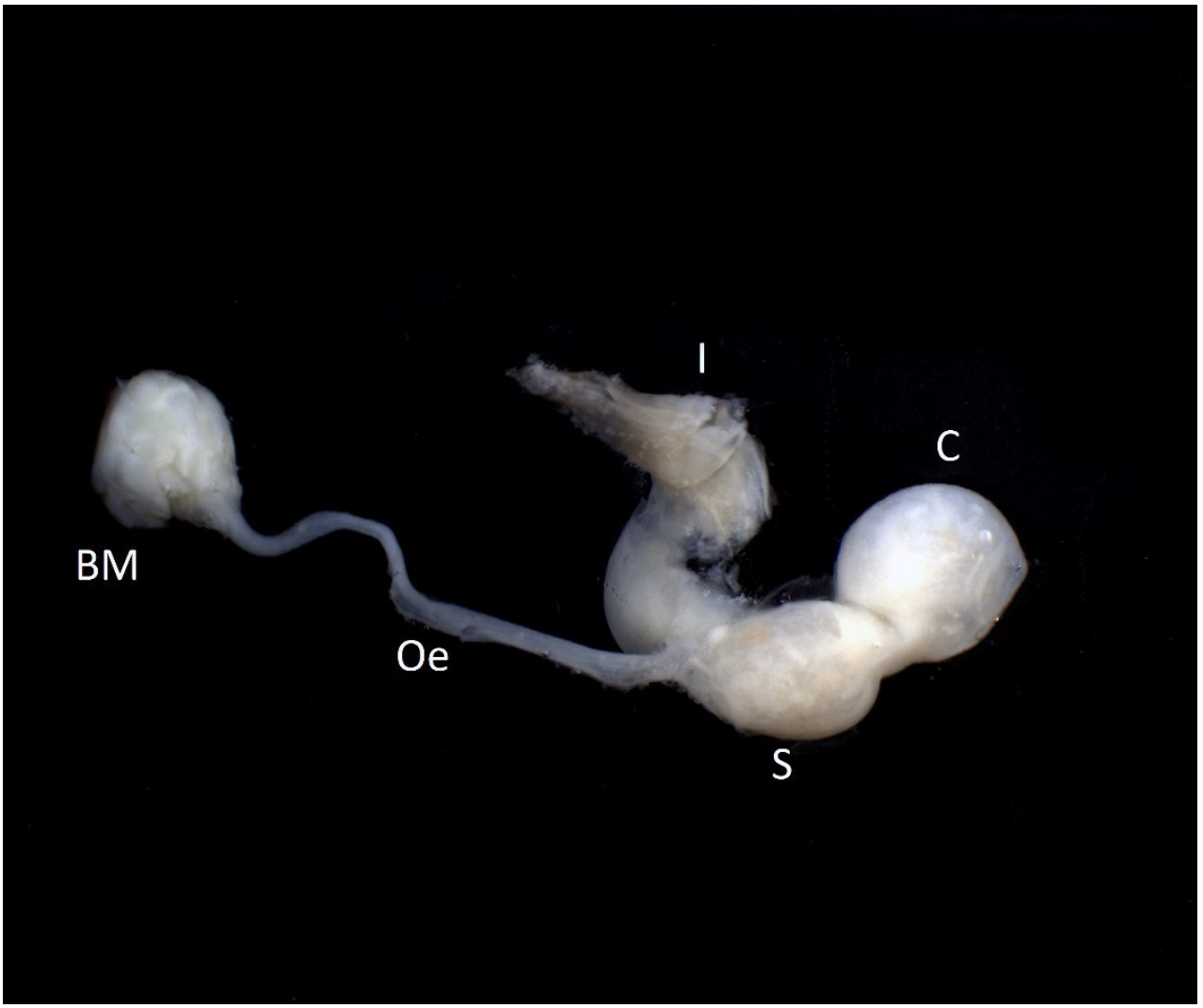


Fig. 37. The digestive organs: buccal mass (BM); oesophagus (Oe); stomach (S); caecum (C); intestine (I). The digestive gland and digestive gland duct appendages have already been removed.

SECONDARY DISSECTIONS AND ANALYSES

Statoliths

The one pair of statoliths of cephalopods and the three pairs of otoliths of fish larvae have similar functions and are essentially composed of calcium carbonate. The rings that appear during their growth are used for estimating age and growth since they have often been proved to be formed daily (or assumed to be so) and the width of the rings are used as a proxy for growth. The analysis techniques used for statoliths and otoliths are almost identical, though the latter are generally far easier to analyse and much can be learned from otolith research. However, the statolith rings of some species are much easier to count compared to others. Statolith morphology varies between species and the shapes, structures and terms used to describe statoliths are provided by Clarke (1978). Different other techniques for estimating the age and growth are discussed by Arkhipkin, et al. (2018).

A day (24 hour) ring consists of one dark and one light ring. The ring formations, from the centre of the statolith to its edge, are not uniform, and groups of nearby rings can show common characteristics although some individual ones can stand out. Periodical growth rings in statoliths have been discussed since the beginning of statolith research (Spratt, 1978; Lipinski, 1978; Kristensen, 1980) but, although the basics are well researched, a comprehensive understanding of ring formation is still lacking. Therefore, it is important that the interpretation of the different kinds of ring structures found in both statoliths and otoliths is made with caution until they have been evaluated for the species and area in question, (see reviews by Stevenson & Campana, 1992; Campana, 2001; Arkhipkin & Shcherbich, 2012).

The aim is to grind the statoliths so that all dark and light rings can be seen clearly and then to count the day rings and measure their width (**fig. 38**). The grinding process is shown in Appendix II and is the same for both statoliths and fish larvae otoliths. In order to obtain estimates of the age and growth of statoliths/otoliths one needs to know which ring represents the hatch day, that all the following rings can be counted and measured correctly, and that each pair of dark and light rings represents one day.

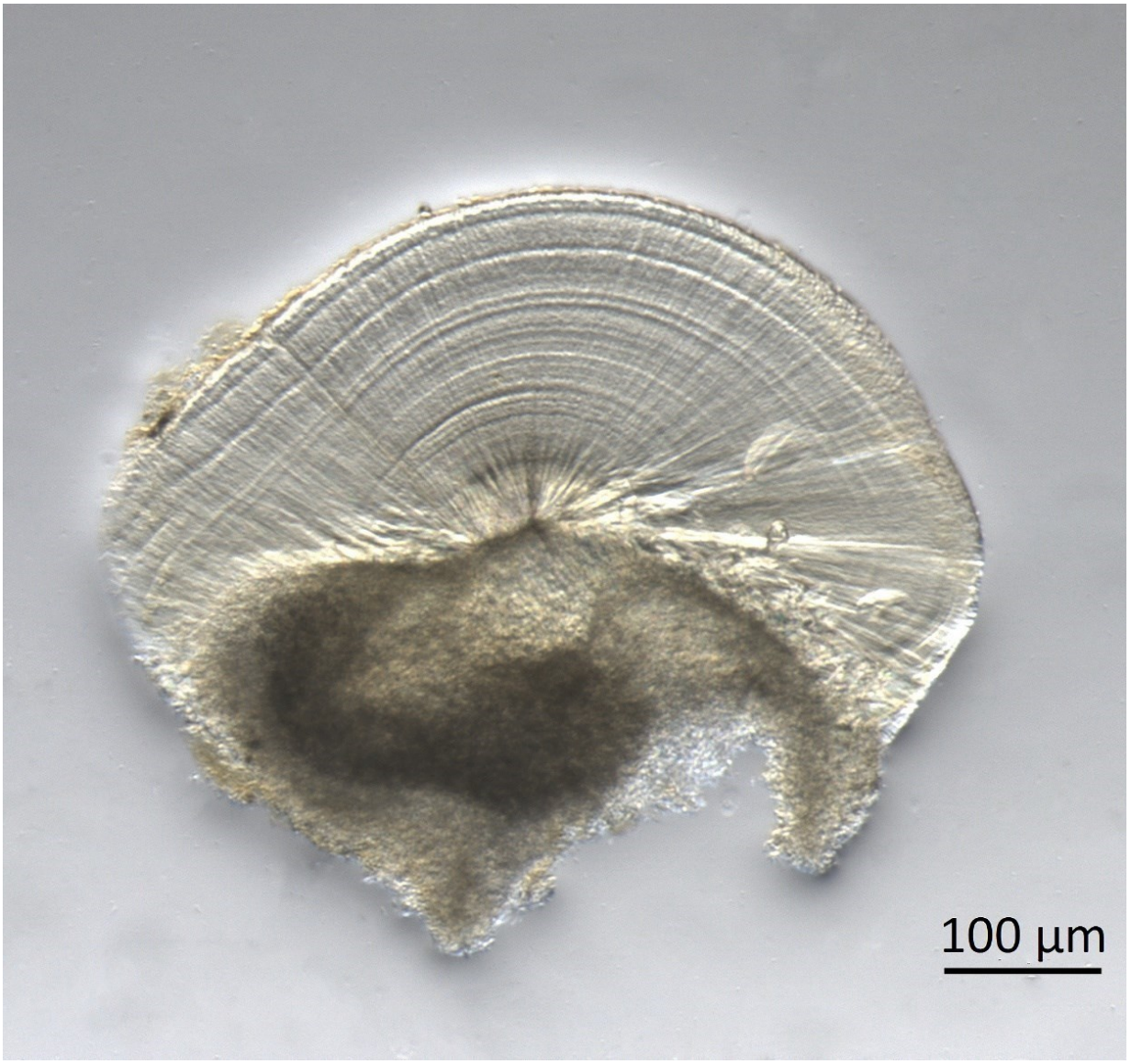


Fig. 38. A grinded statolith of a *Sepietta oweniana* showing the opaque and cracked area around its centre, making it difficult or impossible to identify the hatch ring even in high magnification. *S. oweniana* statolith rings are not that easy to count.

Hatch rings

Finding hatch rings in bobtails can be difficult, or even impossible, since the area near the centre of the statolith is often opaque and cracked (**fig. 38**). Therefore, ascertaining the maximum expected radius from the statolith centre to the hatch ring of newly hatched individuals at different temperatures (Sakai, et al., 2004) can be useful when counting rings. There is still a lack of documentation on hatch rings for many cephalopod and fish species. The otoliths of newly hatched cod larvae have a distinct, dark hatch ring which is not found in the otolith of a cod embryo just prior to hatching (Øresland & Andre, 2008).

Counting and measuring rings

When studying cephalopods, it is not uncommon to find that only a minor portion of all statoliths can be counted with confidence, and this is a problem. In comparison, Øresland & Andre (2008) were able to count the otolith rings of all cod larvae *(Gadus morhua*) in their study and thereby estimate their hatch period by back-calculating the otolith ages. One should be aware of possible optical effects when counting and measuring the width of rings (Stevenson & Campana, 1992; Campana, 2001; Arkhipkin, et al., 2012). Two nearby small rings can, for example, merge into one wider and more distinct looking one. However, the use of adjustable objectives (often with inverted microscopes) can separate the rings and thus avoid underestimating their number and overestimating their width. **Fig. 39** shows what double rings look like in a cod otolith when the adjustable objective was not focused on the rings close to the centre. After focusing, the double rings were all clearly separated. Ordinary objectives are made for cover glass use and adjustable ones for transparent objects of different thicknesses, such as statoliths, otoliths and cell culture containers. The use of adjustable objectives is therefore recommended. With modern cameras there is really no need for a 100x oil objective since further magnification can be done on the computer screen. In addition, the oil, if not removed, will affect the statoliths/otoliths over time making later independent age reading unreliable.

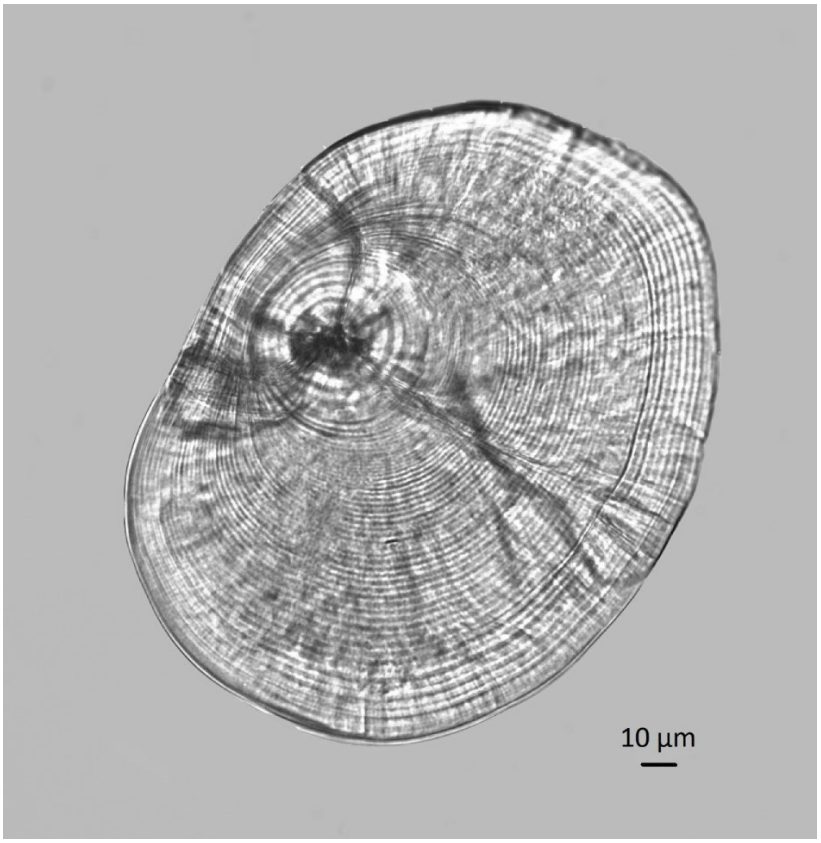


Fig. 39. A grinded lapillus otolith of a 62 days old cod larva*, Gadus morhua*. The rings in the centre are double rings since the adjustable objective used was not focused in this photo.

Before starting the ring counts, one should estimate the ring countability in the photos that were taken after the grinding. Record in the data worksheet these estimates as: 1 = perfect; 2 = good

and 3 = uncountable. Obviously, statoliths in group 3 do not need to be counted although their radius could still be estimated if the inner starting point and the direction of the radius are defined. When possible, the rings of both statoliths of a specimen should be counted and compared. If significant differences are found between the ring counts of groups 1 and 2 from the same specimen, one might consider discarding group 2 counts altogether. One can, of course, decide from the start that only group 1 statoliths should be counted but, in any case, the counting strategy should be made clear.

Mark all the rings on the photo(s) and they will be automatically counted by the software. It is seldom possible just to follow a straight track from the hatch ring to the last ring. It often becomes necessary to stop at some point and move over to a new track. Sharp photographs of the markings should always be saved for later checking. There is always a risk that some rings are missed or counted twice when changing between marking tracks or photos. An image compare module (e.g., used with Nikon NIS-element) would help to reveal such mistakes.

The width of individual rings is most often so small that exact measurements are impossible. Groups of close pairs of dark/light rings of similar widths should therefore be measured together in order to obtain a mean estimate. Correlations between ring widths and, e.g., age, mass, DML, food abundance and temperature are usually made when investigating growth.

Verification of daily rings

Verification that rings are formed daily can be achieved through various experiments (Campana, 2001). One common method is to keep cephalopods of different sizes in aquariums, stain their outer statolith ring(s) with a vital dye such as, e.g., oxytetracycline, and then kill them after a specified period of time and then count the rings. Usually, the dye will stain more than one ring. The stained ring(s) can be identified using fluorescence imaging, which is constantly becoming more advanced, and for the purpose of research one should always try to obtain the optimal microscopes, cameras, fluorescence lightning and software, despite the expense. However, for students and the amateur naturalist cheaper alternatives are available, see Appendix III.

The dye can be injected, given in food, or dissolved in the aquarium water (the specimens are then moved to an aquarium with clean water). The number of rings counted from the first stained ring to the last ring on the edge of the statolith should be equal to the number of experimental days. However, if the statolith is not stained within 24 h the number of experimental days will be higher than the number of rings (assuming they are daily). In addition, the last ring on the edge of the statolith can be difficult to count correctly. A second staining a few days before the experiment is finished can solve both these problems, assuming that the time taken to achieve staining is the same on both occasions. Take a photo and mark the first stained ring on both occasions, then take a second photo without fluorescence light and duplicate the markings onto the second photo. Count the marked rings and the rings in between them.

It is more difficult to count rings in fluorescent light (affecting contrast and resolution); many of the dyes used are fluorescent and can cause blurring, making the stained rings less distinct (**fig.**

40). It is therefore important to use dyes that act quickly and stain as few rings as possible with little or no blur effect. Statoliths of different species may be stained differently, and a testing period to ascertain the optimal dye concentration, pH level, and application time for a certain dye should be foreseen. It appears to us that a dye dissolved in the aquarium water might be the method of choice to best control dye concentration and application time, and also be less stressful to the specimens. Note that ring deposition and growth rates of fish larvae have been shown to be significantly affected by rearing conditions, especially during long experiments (see e.g., Geffen, 1982). Therefore, many short experiments, including specimens of different sizes, should be considered rather than a few long ones, and experimental conditions should be carefully monitored. Due to the importance of accepting rings as being daily, any experimental claim that they are so should be supported by photographic evidence of the statoliths showing the stained rings and the marked and counted rings.

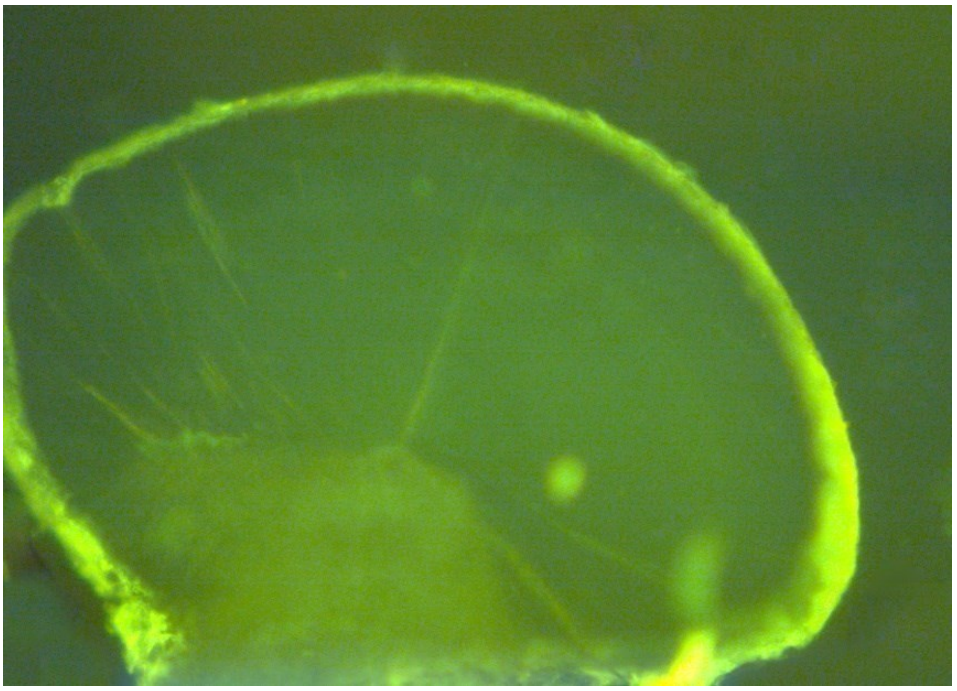


Fig. 40. An *S. oweniana* statolith stained with oxytetracycline during a test trial demonstrated some common problems. Observe the fluorescent blur effect in the outer rings and that the statolith as a whole is greenish making it impossible to count the rings (compare with **fig.38**). In addition, the test trial period was too short since no new unstained rings appeared. Here we used the NIGHTSEA® fluorescence viewing systems, see Appendix III.

Exercise: Compare ordinary objectives with adjustable ones when counting rings.

Exercise: Test different fluorescent dyes, staining methods, fluorescence cameras and software to improve contrast and visibility of rings as well as to confirm whether the rings are formed daily.

Exercise: Compare rings in an embryo close to hatching with rings in newly hatched individuals in order to find the hatch ring (and any pre-hatch rings).

Beak and Radula

The aim is to dissect the beak and the radula from the buccal mass (**figs 41 & 42**) and to make glass slide preparations for analyses of morphology and age. Although part of the digestive system, the beak and the radula are dealt with separately. Their morphology (see Boucaud-Camou & Boucher-Rodoni, 1983) is of interest for cephalopod feeding studies but, above all, they can be useful in taxonomic/systematics studies, separating even closely related species and in predation studies on cephalopods (see Boucaud-Camou & Boucher-Rodoni, 1983; Clarke, 1986; Samuel & Patterson, 2003). However, since both molecular and morphological methods may fail to reveal species specific differences, they should be combined to correctly assess cephalopod diversity (see Fernández-Álvarez, et al., 2021, and references therein). The beak is also used to determine the age of cephalopod species (see e.g., Peralez-Raya, et al., 2014) and for stable isotope analyses (see Golikov et al., 2020); Xavier et al., 2015).

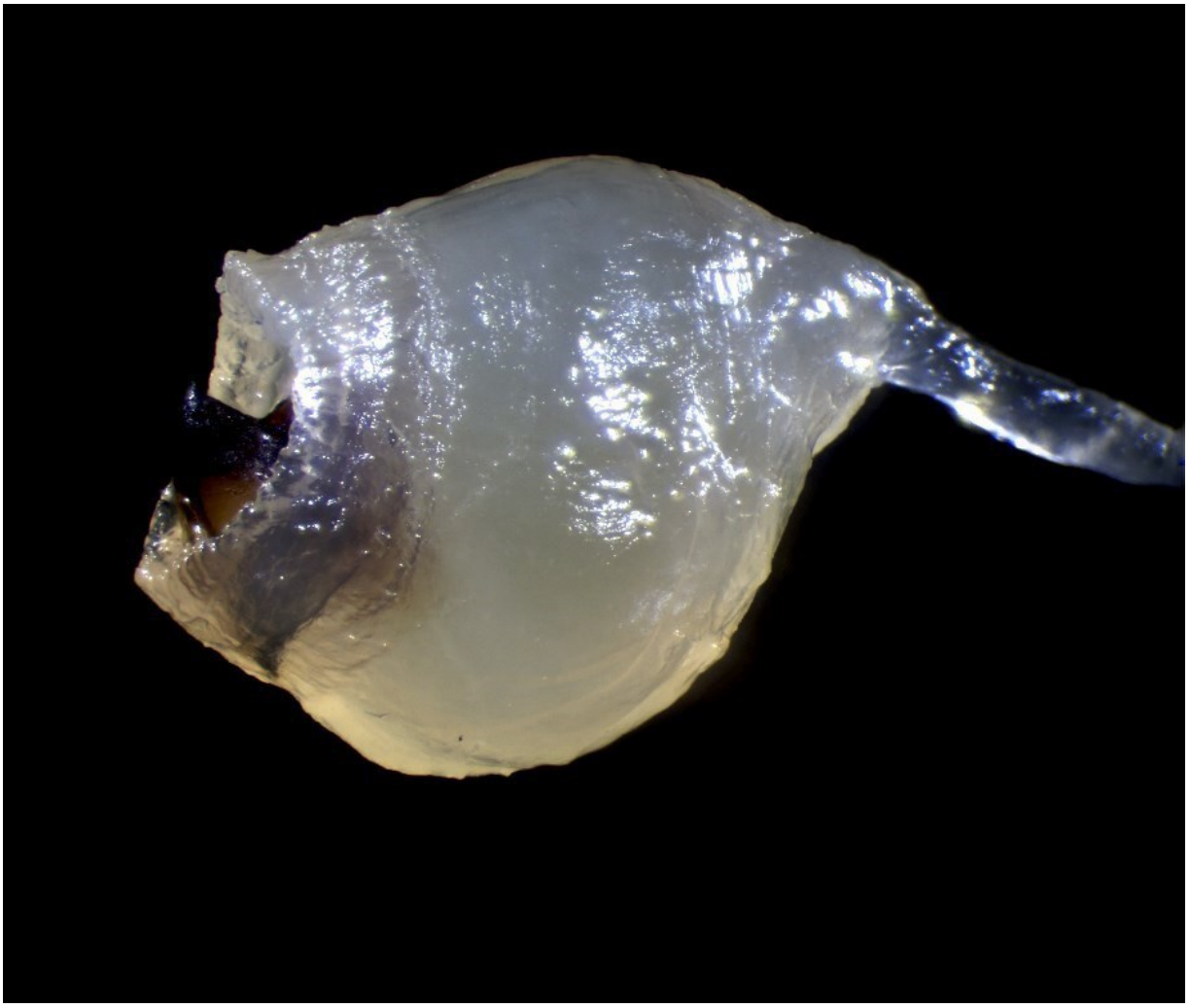


Fig. 41. The buccal mass with the lower beak visible and the oesophagus coming out the buccal mass to the right.

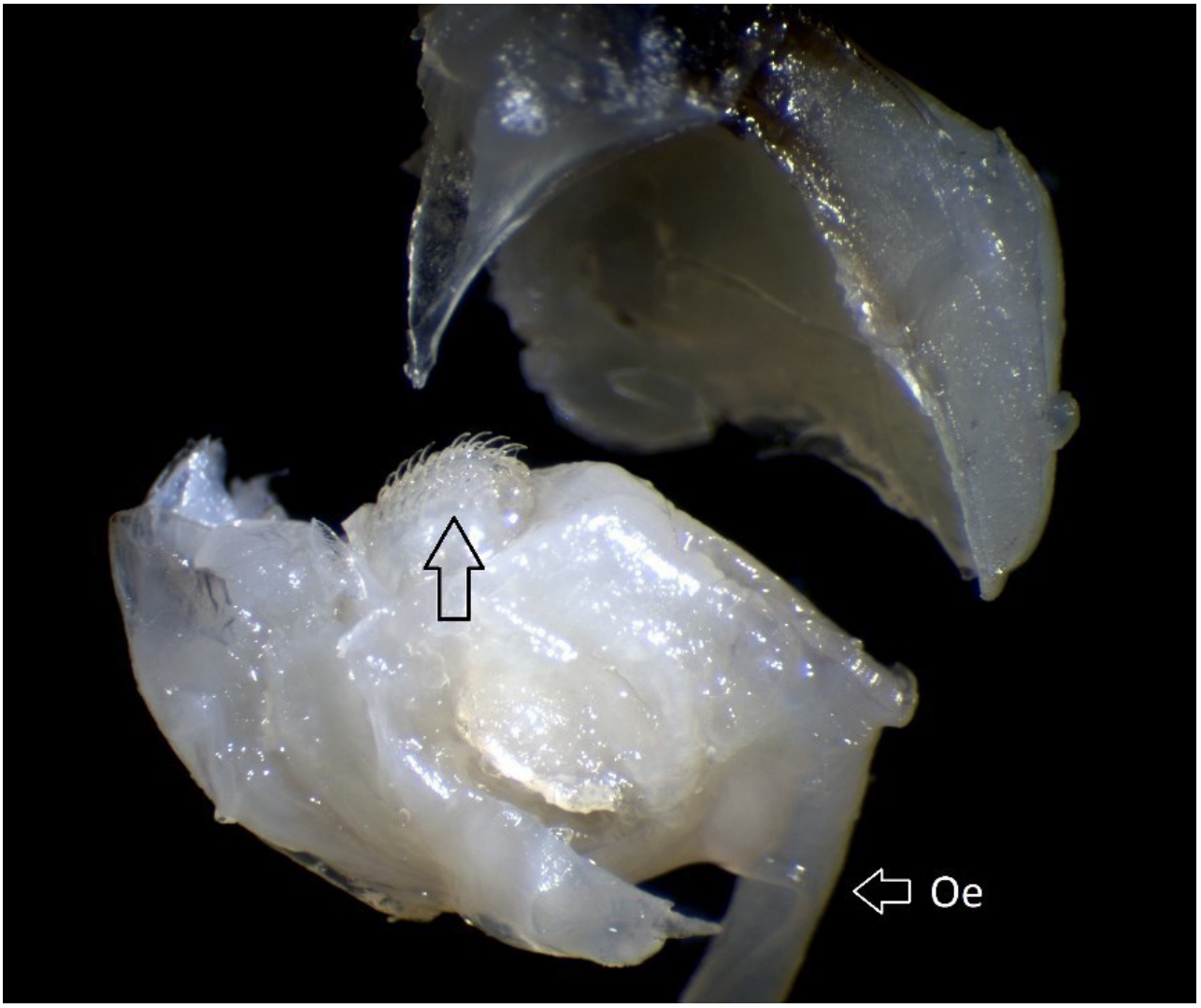


Fig. 42. The outer muscles have been removed making the upper beak, the lower beak and the radula with teeth visible (arrow). The radula is connected to the tung-like odontophore. Note that the teeth are pointing backwards towards the oesophagus (Oe).

The beak with its very thin lateral walls (**figs 43 & 44**), the radula with its teeth, and two other lateral tooth-bearing structures above the radula, are all extremely delicate and almost impossible to dissect. An easy method to obtain them is to put the buccal mass into a Petri dish of concentrated household chlorine for between one and three hours until all the muscles disappear. Observe the muscles under the stereomicroscope during the latter part of the process and, when they have disappeared, transfer the various remaining items to a Petri dish with tap water to stop the process and eliminate the chlorine. Do not put items covered with chlorine into PVL since they will react chemically together. Always put a small Petri dish with chemicals, like chlorine and glycerine, into a larger Petri dish to protect the microscopes from spillover.

Fig. 43. The upper beak with a ring pattern on its lateral wall.

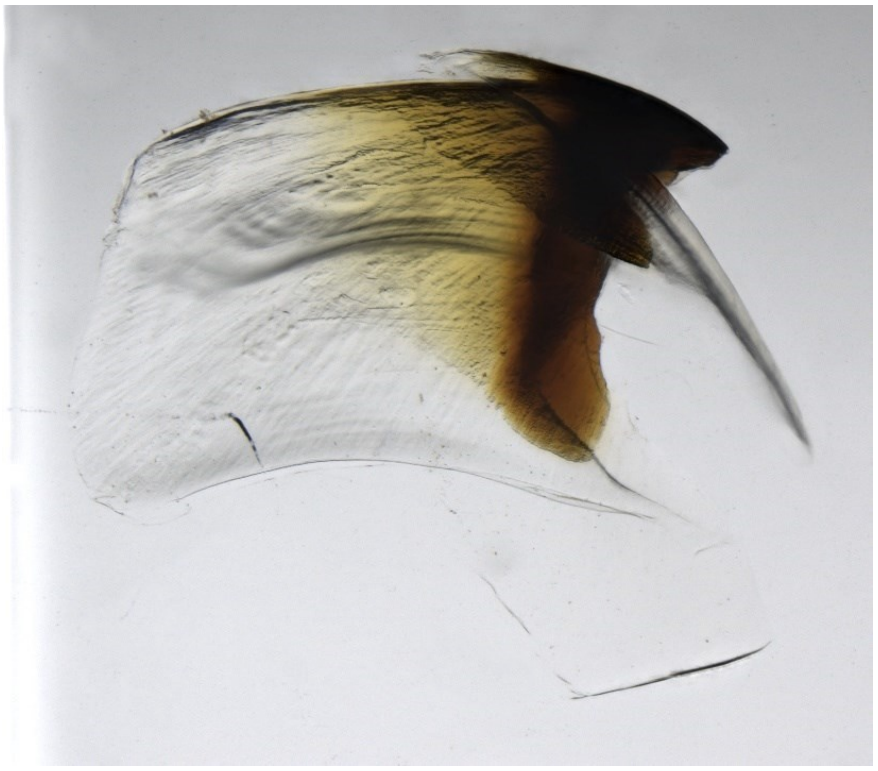


Fig. 44. The lower beak with lateral wall.

The dark/light ring pattern that is seen in the lateral wall of especially the upper beak **(fig. 45**) is interesting since daily rings were found in the lateral wall of *Octopus vulgaris* (Peralez-Raya, et al. 2014). Working from the inside and using a very sharp microscalpel, cut the beak and the lateral wall in half in order to obtain a left and a right side, and make two PVL sandwich slide preparations. This will prevent any rings in the background (as in **fig. 43)** interacting with rings in the foreground during analysis. However, it is critical to be able to clearly locate the first ring produced after hatching, as well as the last outer ones, for age estimates to be trustworthy (see also statolith hatch ring and daily ring verification above). In high magnification and optimal light and contrast, some very thin rings (inside the dark and light rings shown in **fig. 45**) become visible, as indicated in **fig. 46**. What they represent and how they are formed in *S. oweniana* remain to be investigated.

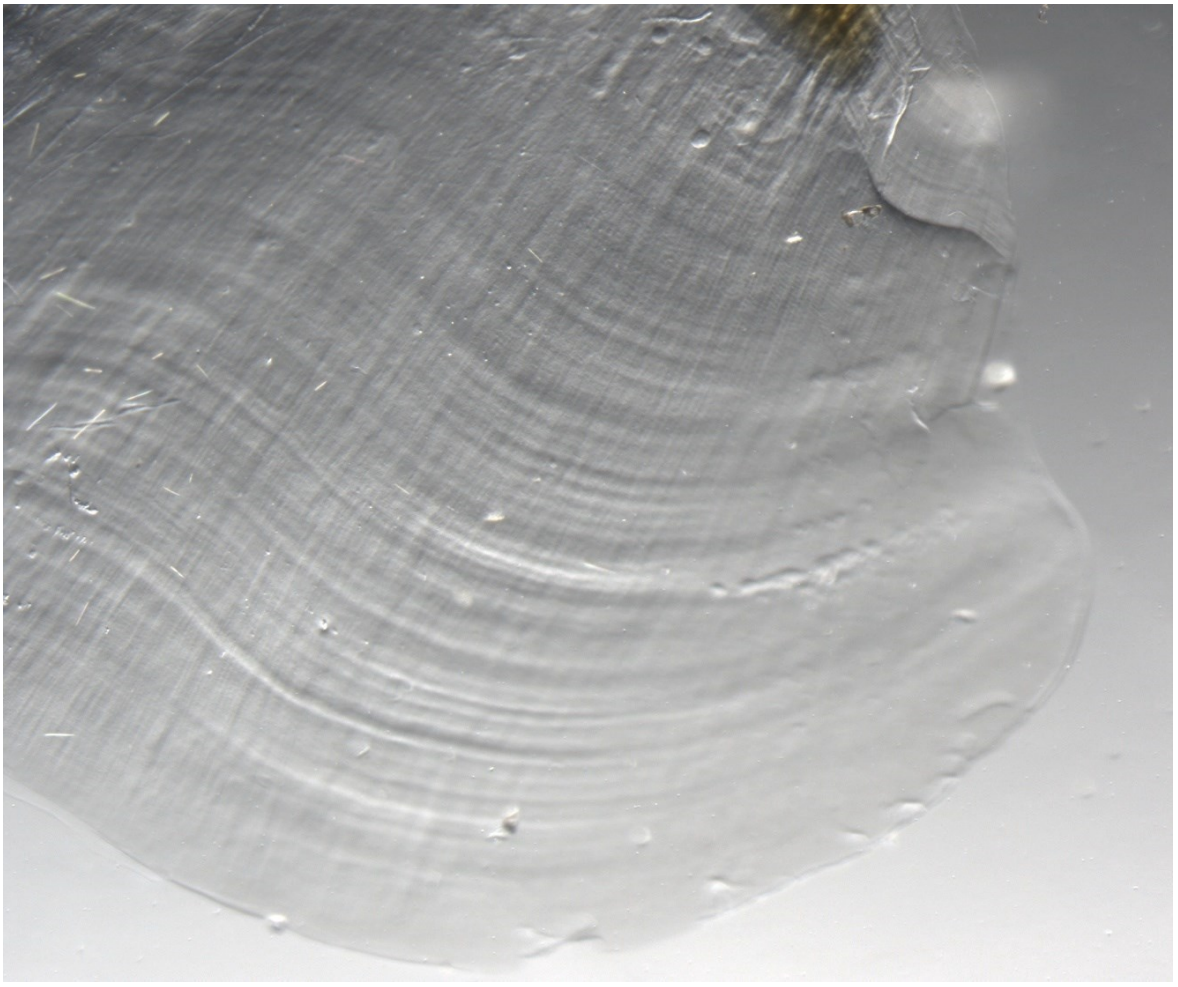


Fig. 45. The ring pattern in low magnification on the lateral wall of the upper beak.

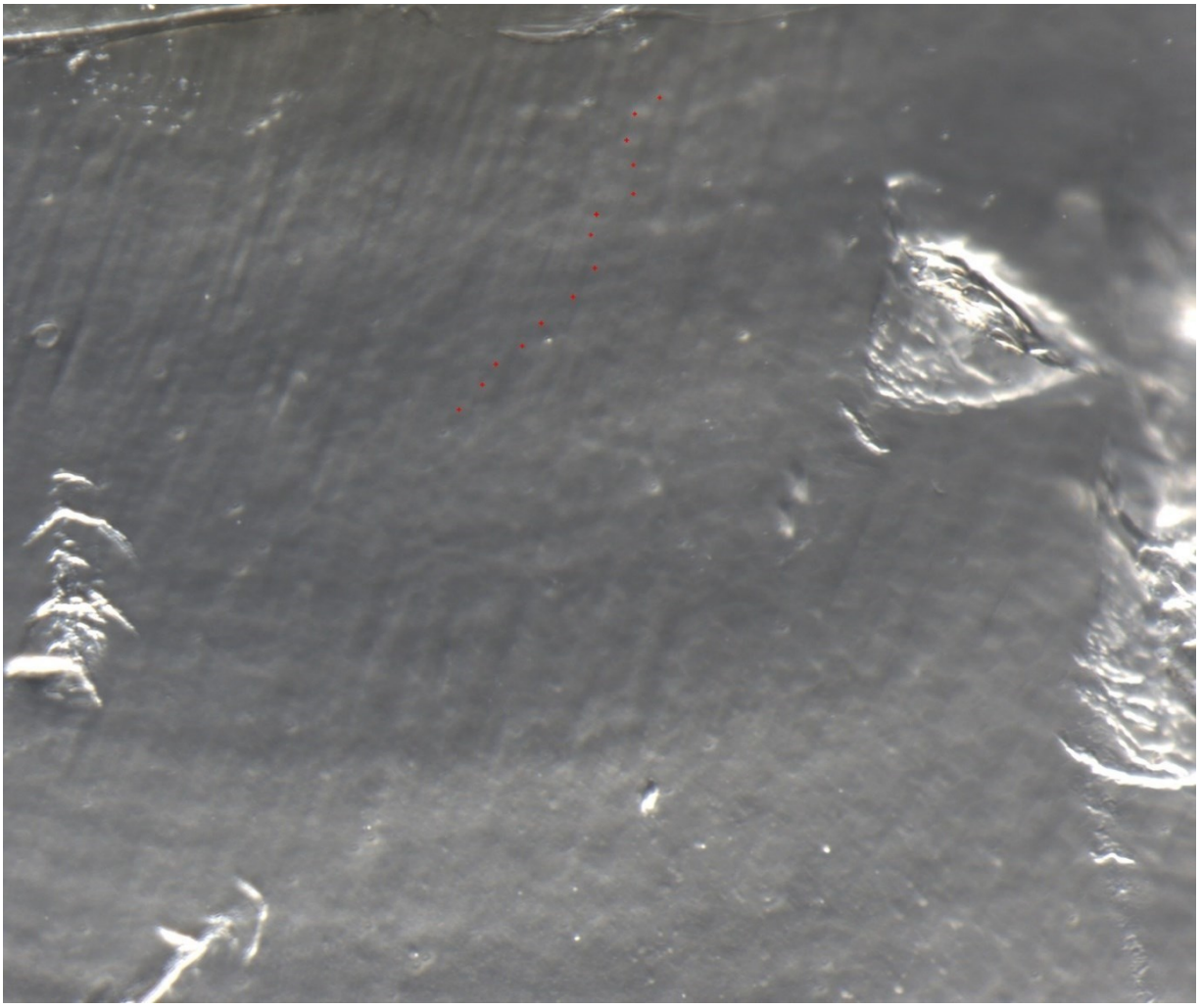


Fig 46. A small portion of the lateral wall of the upper beak with some much thinner dark and light rings, within the thicker dark and light rings as shown in **fig. 45,** can be discerned in higher magnification. Seven thin rings have been marked in red within a thicker dark and light ring, respectively.

The bent radula is lined with teeth (**figs 47 & 48**) and above it two other very thin objects with teeth can be found, as shown in **fig. 48**, for which we have found no name (lateral tooth structure?)**.** It is difficult to flatten the radula without breaking it or losing some of the teeth. Put the radula into a Petri dish with glycerine for three days to soften it, clean it and then mount it stretched in PVL on a glass slide (**fig. 49**). The teeth are replaced over time and comparisons between radulae should be made from specimens of similar size /age. The seven rows of teeth are shown in **figs 50 & 51** and the teeth of one of the two lateral tooth-bearing structures close to the radula are shown in **fig. 52**.

Fig.47. Ventral view of the bent radula and the ring pattern on the lateral wall of the upper beak. The white areas in the background are muscles not yet destroyed by the chlorine.

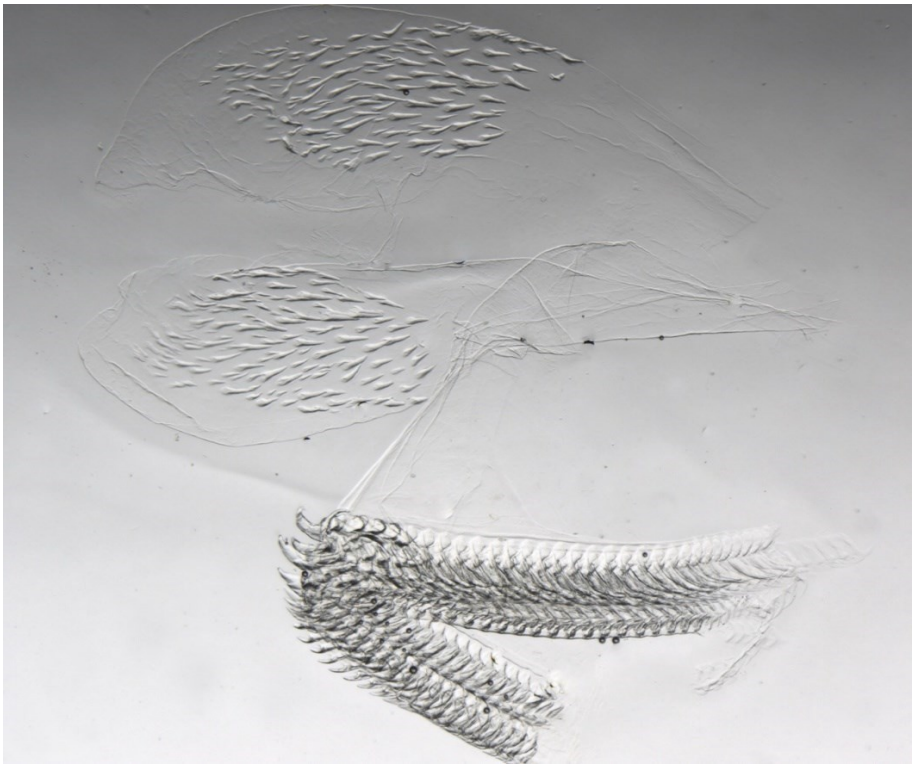


Fig. 48. The bent radula and the pair of lateral tooth-bearing structures found close to the radula.

Fig. 49. The streched radula after three days in glycerine (some glycerol is still present).

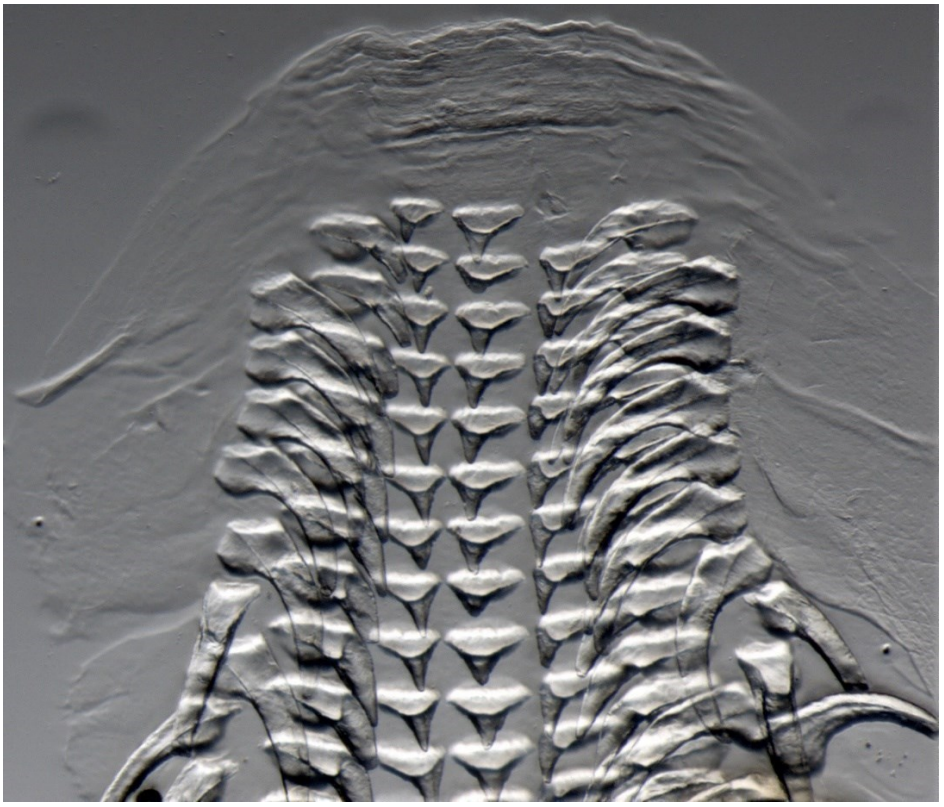


Fig. 50. Anterior part of the radula.

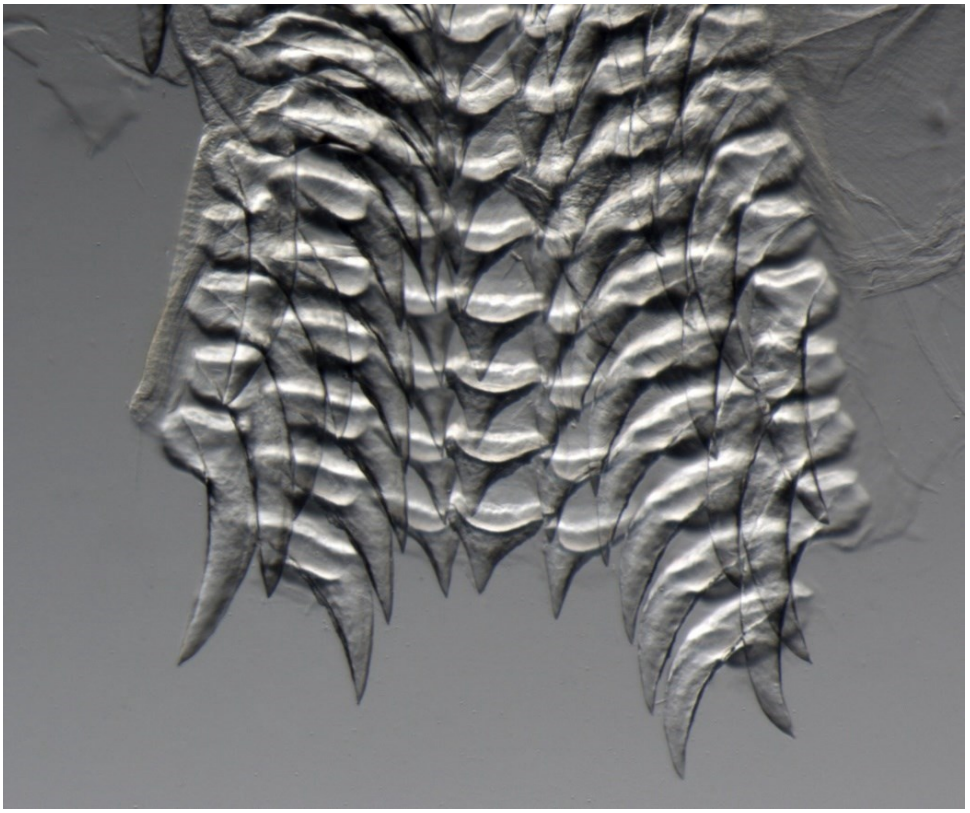


Fig. 51. Posterior part of the radula.

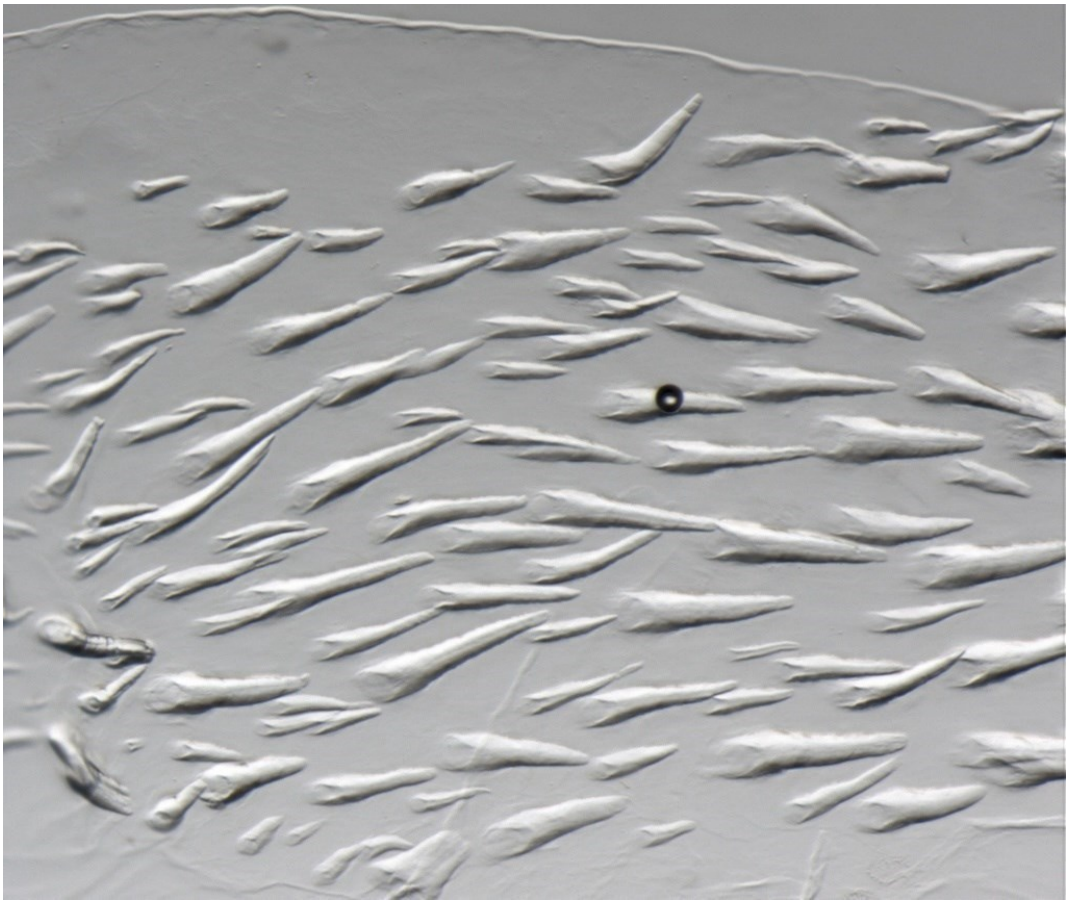


Fig. 52. The teeth of one of the two thin lateral tooth-bearing structures found close to the radula.

Exercise: Make glass slide preparations and compare the radula and tooth structures of different sizes of bobtails and closely related species. Can early teeth be found? Compare also the tooth structures shown in fig. 52.

Exercise: Compare radula dissected teeth with chlorine obtained teeth using a scanning electron microscope in order to see whether fine structures are affected by chlorine.

Exercise: Make slide preparations of the lateral wall of the upper beak and compare ring counts with those of the statoliths from the same individual.

Reproductive organs

Reproduction biology and research goals

Some basic understanding of male and female gamete production is crucial for the interpretation of maturity data. From such data, one may obtain knowledge on maturity status, fecundity, time for copulation, fertilization, egg-laying, expected appearance of juveniles, life span, energy utilisation, etc. There is great variability among cephalopod species when it comes to the temporal development and morphology of reproductive organs and the mating process. Differences regarding the morphology of sexual organs between subpopulations may therefore disclose speciation processes. Jereb, et al. (1915); Robin, et al. (2014) and Lishchenko, et al. (2021) review the life history and ecology of European cephalopods, including bobtail squids.

Photos of the organs mentioned here are shown below in the chapters on dissection of the male and female organs. The spermatozoa are produced in the testis and transported via the sperm duct to the first of several consecutive and morphologically distinct, sac-like and tubular glands (Sabirov, et al., 2012) in the spermatophoric organ where they are encapsulated into spermatophores. These then travel via the spermatophoric duct to the spermatophoric sac (= Needham´s sac) where they are stored before ending up in the terminal organ. All these organs (except the testis) together make up the spermatophoric organ complex. Comprehensive knowledge on the morphology and the temporal development of the male reproductive system is lacking for most European bobtail species.

The female gametes develop in the ovary and go through several early developmental stages, that are not dealt with here. The later stages that are visible in a stereomicroscope are: the small whitish oocytes of different sizes; the yellowish, reticulated oocytes; and finally, the mature oocytes which are somewhat transparent with a smooth surface and are slightly smaller than the reticulated oocytes (at least in *S. oweniana*). The mature oocytes have been given different names in the cephalopod literature which can be confusing (mature oocytes, ripe oocytes, eggs, ripe eggs, mature ovarian eggs, and ova). The mature oocyte goes through the oviduct and passes the oviducal gland (connected to the bursa copulatrix), from which it receives a coating, and should thereafter be named ovum (ova pl.), since it is then ready to be fertilized. The ovum goes through

a thick duct (at least it appears so in *S. oweniana*, see below) under the bursa copulatrix and reaches the outward, wrinkled side of the bursa copulatrix which is in direct contact with sea-water. We do not know the life span of an *S. oweniana* ovum but assume it is short since we have never encountered any in our bursa copulatrix samples**.**

The mating process in *Sepietta* starts with the male using its modified left dorsal arm, hectocotylus (LAI), to take the spermatophores from its own terminal organ and place them on the female`s bursa copulatrix during copulation (Akalin & Salman, 2018). Note that in other groups of cephalopods, the spermatophores can be placed elsewhere on the body. A comprehensive knowledge of the copulation behaviour of most bobtail squids is still lacking (see Rodrigues, et al., 2009 and references therein). Once on the bursa copulatrix, the spermatophore goes through a process named the spermatophoric reaction, resulting in a spermatangium (Marian, 2012; Salman, 2014; Sato, et al., 2014; Akalin & Salman, 2018) which in *S. oweniana* will be embedded into the cuticle of the bursa copulatrix. After some unknown storage time the spermatozoa are released from the spermatangia and the ova are fertilized. We use the term "egg" once an ovum has been fertilized. The egg will receive a coating, produced by the two nidamental glands and the two accessory nidamental glands, prior to spawning (Huang, et al., 2018). The symbiotic bacteria that are present in this outer coating may play a role in the egg`s defence against threats from fungi and bacteria during the embryonic development (Kerwin, et al., 2019; Suria, et al., 2020). This is a general picture of the reproductive processes of bobtail squids; further detailed studies for the different species are needed.

Maturity stages

Different maturity stage systems have been used to describe the maturity development of male and female cephalopods (see e.g., Arkhipkin,1992; Rodriguez, et al., 2011; Czudaj et al., 2012; Salman, 2014; Lipinski, 1979; Lipinski & Underhill, 1995). The most important aspects of any such system are that the maturity stages should be as clear cut as possible and serve the specific needs of the study in question. Problems with stage separation will increase by increasing the number of maturity stages and criteria. We have modified earlier stage systems and limited the criteria to the development of the hectocotylus, the oocytes and the spermatophores/spermatangia in order to simplify the maturity scale for ecological studies of *S. oweniana*. Photos are shown below.

Table 1. Maturity scale for *Sepietta oweniana*

Stages	Females	Males
I Juvenile	Gonads are not visible or identifiable to sex in a stereomicroscope	
II Maturing	a) Early oocytes only b) Early oocytes and RO	SpOC and Hc: developing SpO: contains developing Sp
III Mature	Stage II b characteristics and MO are precent	Hc: developed SpO: contains developing Sp SpS and TO; contains developed Sp
IV Mating	Stage III characteristics and Spt on the BC	Omitted for males, see comments
V Spent	Few gametes, see comments	Few gametes, see comments

Females: RO = reticulated oocytes; MO = mature oocytes; Spt = spermatangia: BC = bursa copulatrix
Males: SpOC = spermatophoric organ complex; Hc = hectocotylus; SpO = spermatophoric organ; Sp = spermatophores; SpS = spermatophoric sac (Needham`s sac); TO = terminal organ

Comments
Stage I, Juvenile. DNA testing can be used for sex determination if sexual organs cannot be detected. This stage is important for the detection of recruitment time.

Stage II, Maturing. Dividing this stage into a and b for females is easy to do and might be useful in different studies.

Stage III, Mature. Sexual organs are fully developed but no evidence of immediate mating. Developed Sp = most Sp have reached final length and final internal morphological characteristics.

Stage IV, Mating. Females with spermatangia on the BC is the only sure visual sign of mating. Difficult to determine if a male has copulated and if Sp have been used.

Stage V, Spent. This stage may be regarded as unusual due to death close to last egg laying or last mating (males). Though, observations of such individuals are important to life span studies.

Note: Maturing females of some species have been found with spermatangia.

Male reproductive organs

The aim is to locate and dissect the male organs and to photograph all spermatophores in the spermatophoric sac for analysis. The spermatangia are dealt with together with the female organs, since they are found on the female bursa copulatrix. There are often fewer known details regarding the morphology of male organs as compared to those of females. The production of spermatophores and spermatozoa can be studied, for example, to learn about the reproductive periods and to compare the fecundity of males of different species and from different sub-populations and habitats, in order to obtain a better understanding of the factors affecting fertility and its evolutionary consequences. Note that counts and measurements of spermatophores and estimates of the number of spermatozoa are only a reflection of a temporal situation which changes during stage II and III.

Separating the male organs

Place the male organ package into a cup with tap water until there is no smell of formaldehyde and then put it into a Petri dish with tap water and stain its membranes with azure B. Start the dissection, in a new Petri dish with water, by removing the thin membranes surrounding the white testis. The hollows on the testis that are shown in **fig. 53** illustrate how compressed the organ systems are in bobtail squids. The white testis has no pronounced inner structure. Cut off the sperm duct connecting the testis to the first gland of the spermatophoric organ and save the testis in a male organ vial.

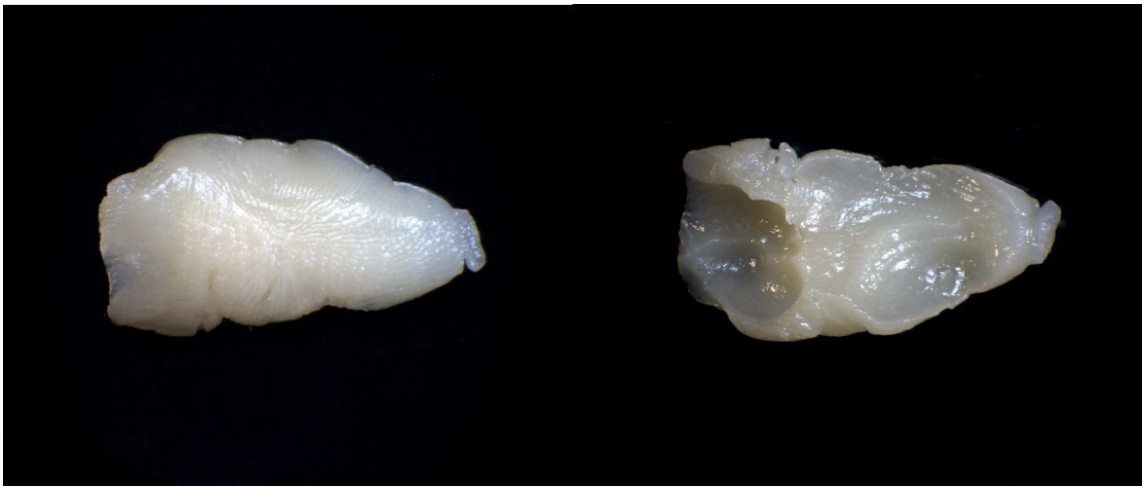


Fig. 53. The testis viewed from both sides with hollows on the inner side where other organs were found.

When all the connective membranes have been removed from the remaining male organs (**fig. 54**) one can, using curved scissors, separate the spermatophoric sac from the spermatophoric organ, but not yet the spermatophoric duct (**fig. 55**).

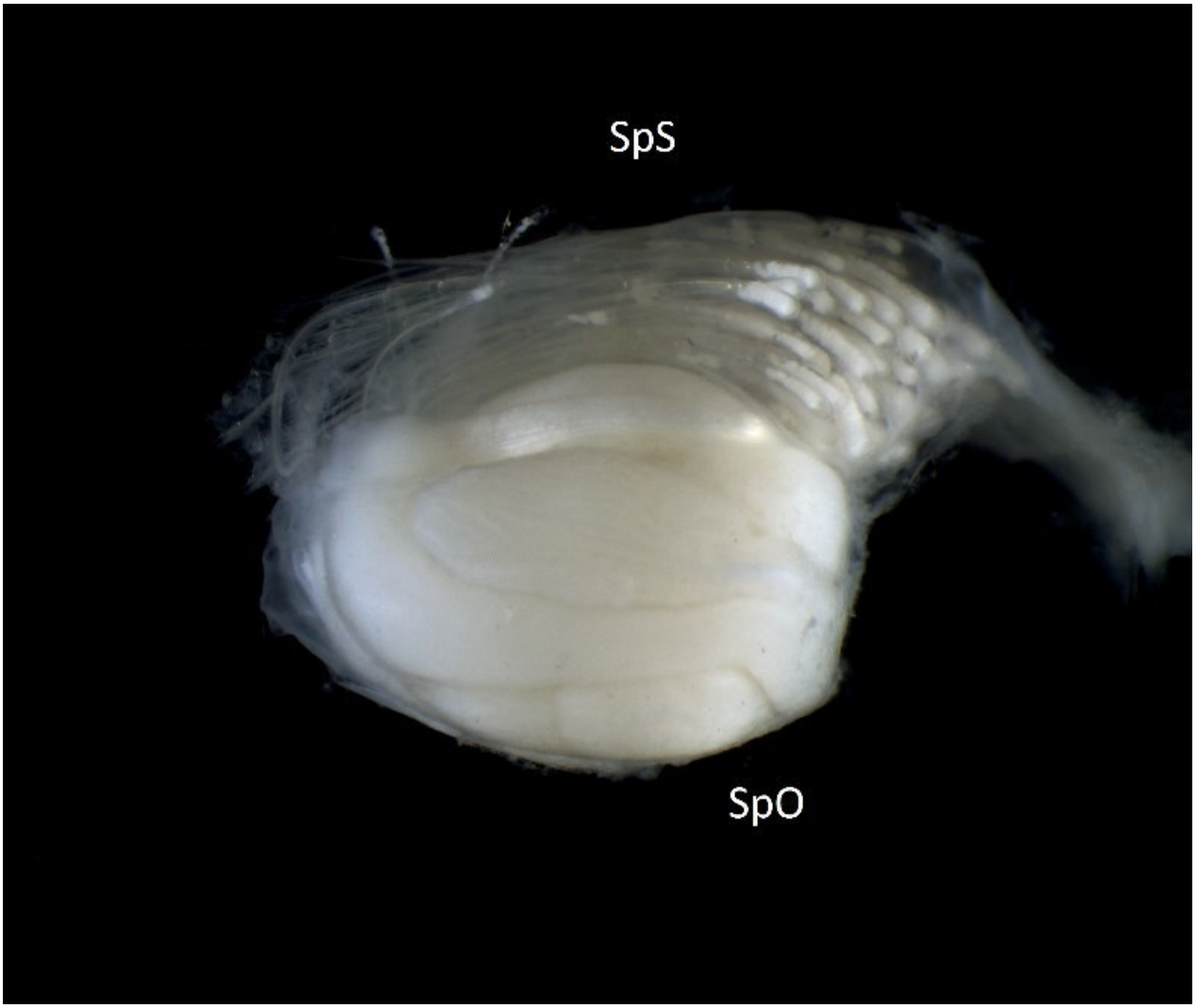


Fig 54. The spermatophoric sac (SpS) with spermatophores and, below, the white spermatophoric organ (SpO) with its glands.

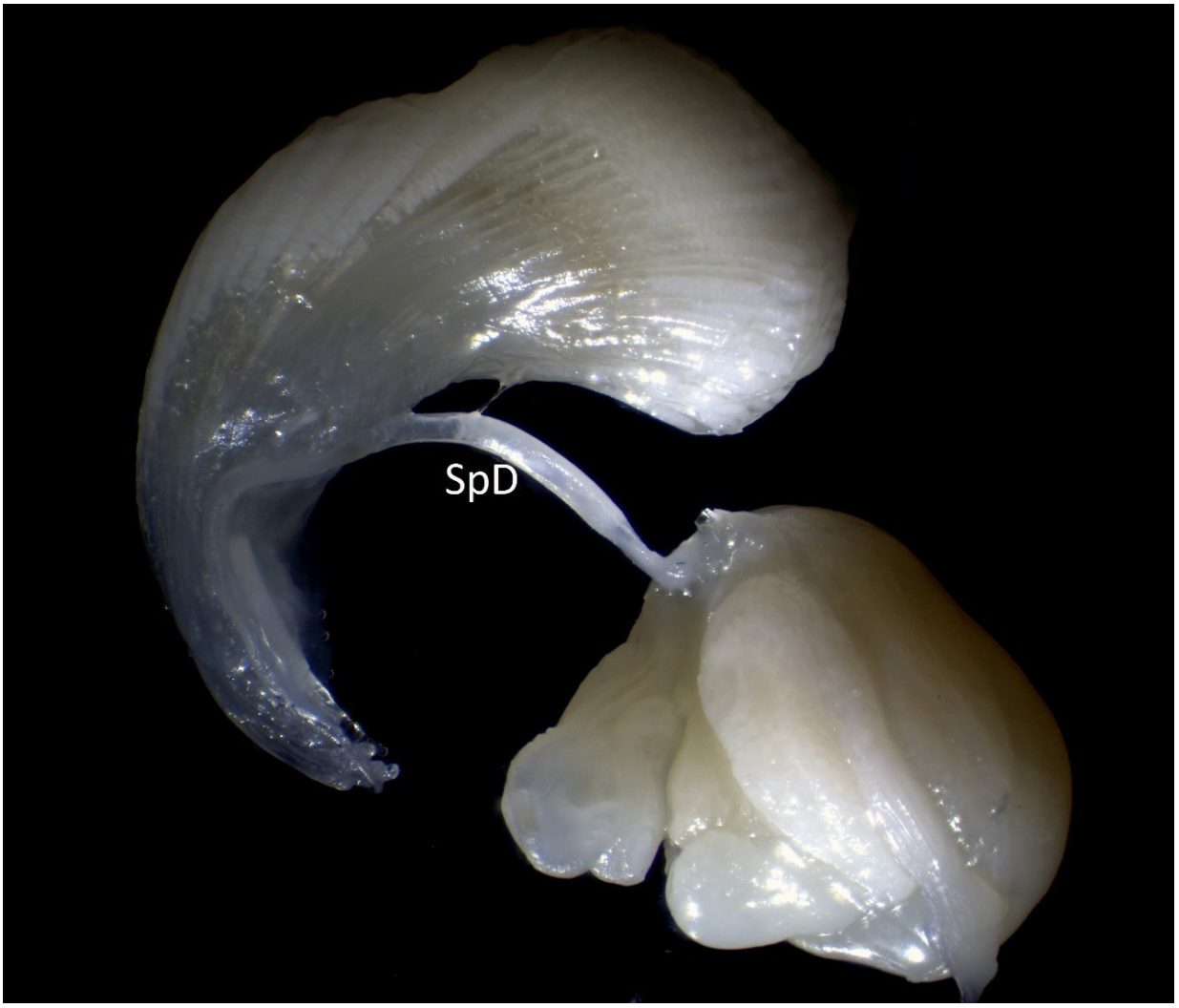


Fig. 55. Spermatophoric sac and the spermatophoric duct (SpD) connected to the last gland in the spermatophoric organ.

The glands in the spermatophoric organ are difficult to separate without damage and staining is thus advised (**fig. 56**). Slightly separate the glands (**fig. 57**), cut free the spermatophoric organ and save it in a male organ vial for any later analysis. The spermatophoric sac and the six parts of the spermatophoric organ for ommastrephid squids were described by Nigmatullin et al. (2003) and those of for lesser flying squid (*Todaropsis eblanae*) by Sabirov, et al. (2012).

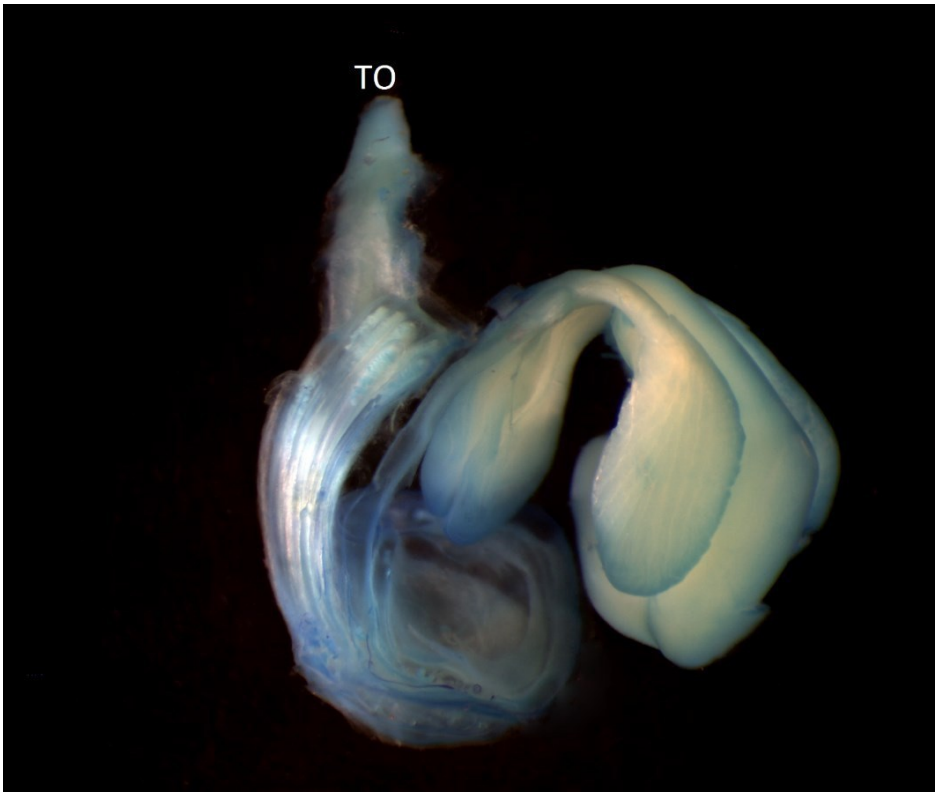


Fig 56. The outer membranes of the spermatophoric sac and the glands of the spermatophoric organ stained with azure B. TO = terminal organ.

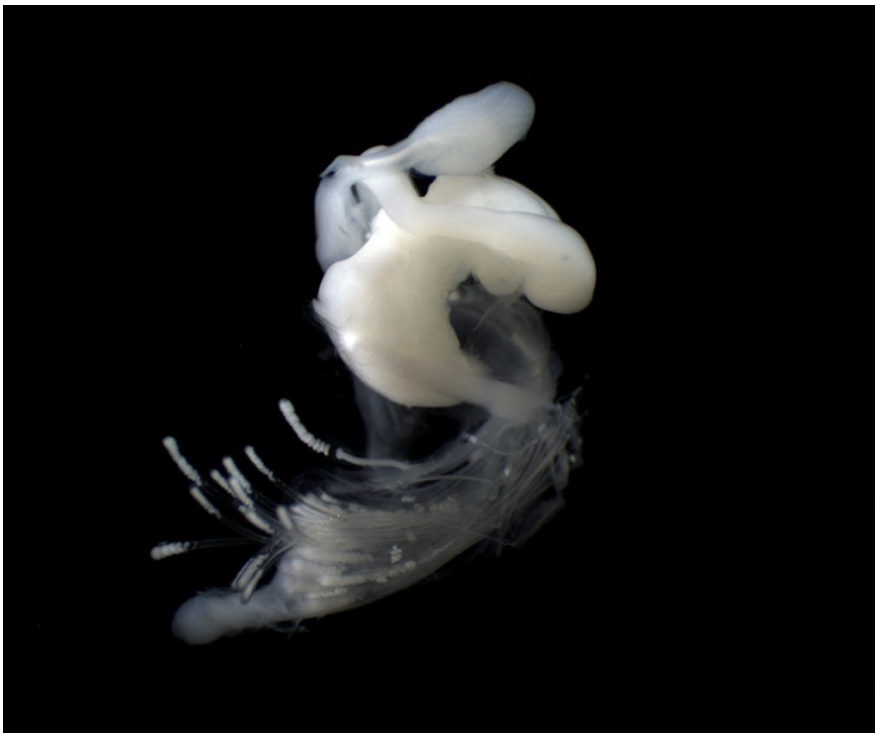


Fig. 57. The glands of the spermatophoric organ have been slightly separated and the membrane of the spermatophoric sac has been removed.

Analysing the spermatophores

Using micro scissors, cut open the outer membrane of the spermatophoric sac, from its base towards the terminal organ, so that the spermatophores come out together. Spread them out and take a photo so they can be counted (**fig. 58**). The spermatophores must be completely separated if length measurements and other analyses are needed**.**

Fig. 58. Spermatophores to be counted.

A typical spermatophore is shown in **fig. 59** and its main components in **figs 60, 61 & 62**. An interesting feature is the long thread which in *S. oweniana* can be longer than the spermatophore (**fig. 59**) and might trigger the spermatophoric reaction (Marian, 2012). The spermatophores can come in different forms, e.g., the tentative spermatophore (with no spermatozoa) and the false spermatophore (with few spermatozoa), neither of which are usually stored for any length of time in the spermatophoric sac of lesser flying squid, *T. eblanae* (Sabirov, et al., 2012). Little is known about the occurrence, morphology and function of different kinds of non-typical spermatophores in bobtail squids.

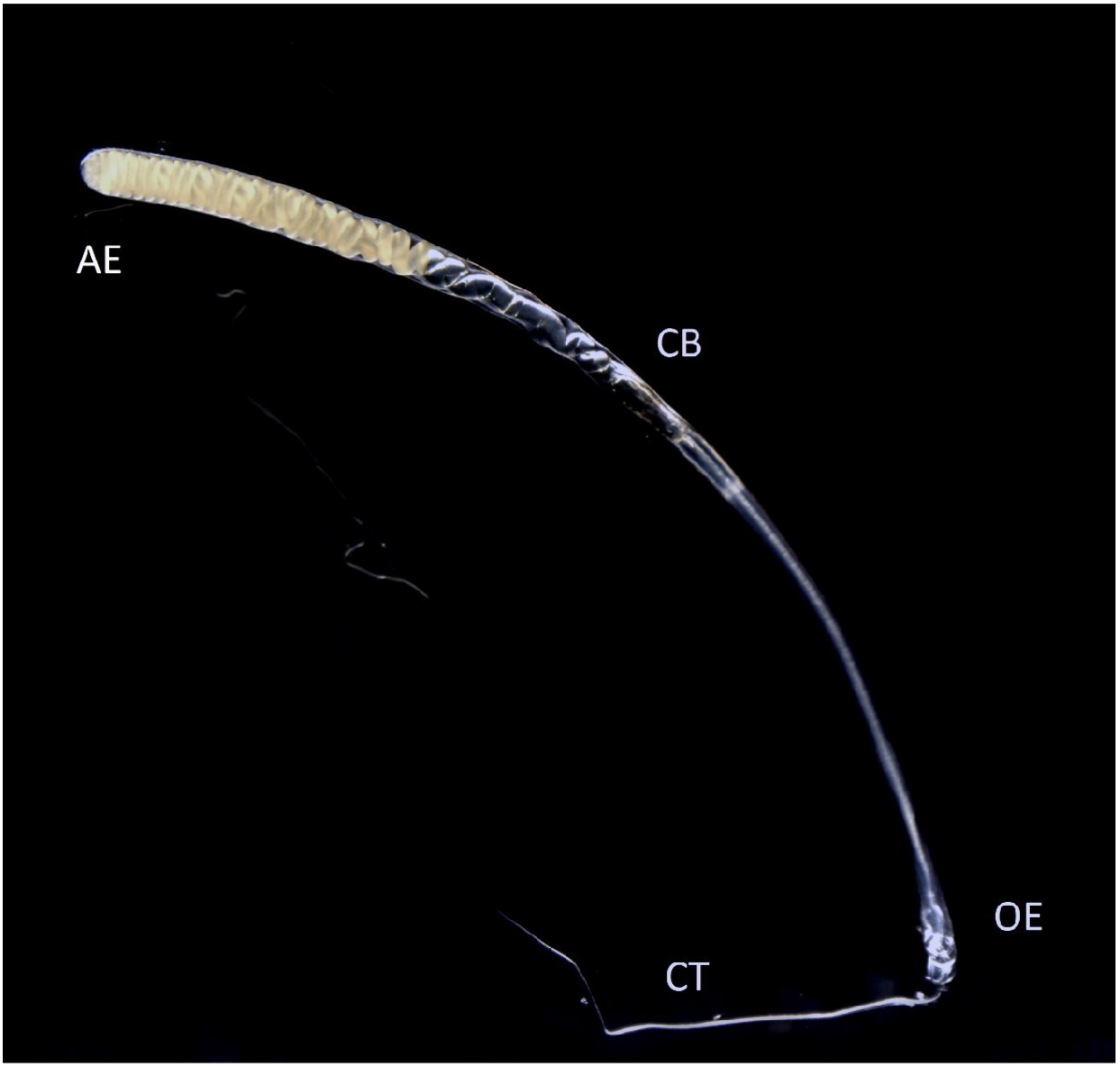


Fig. 59. The main components of the spermatophore: aboral end (AE) with the spiral coiled sperm mass; cement body (CB)**;** oral end (OE) with its ejaculatory apparatus and cap thread (CT).

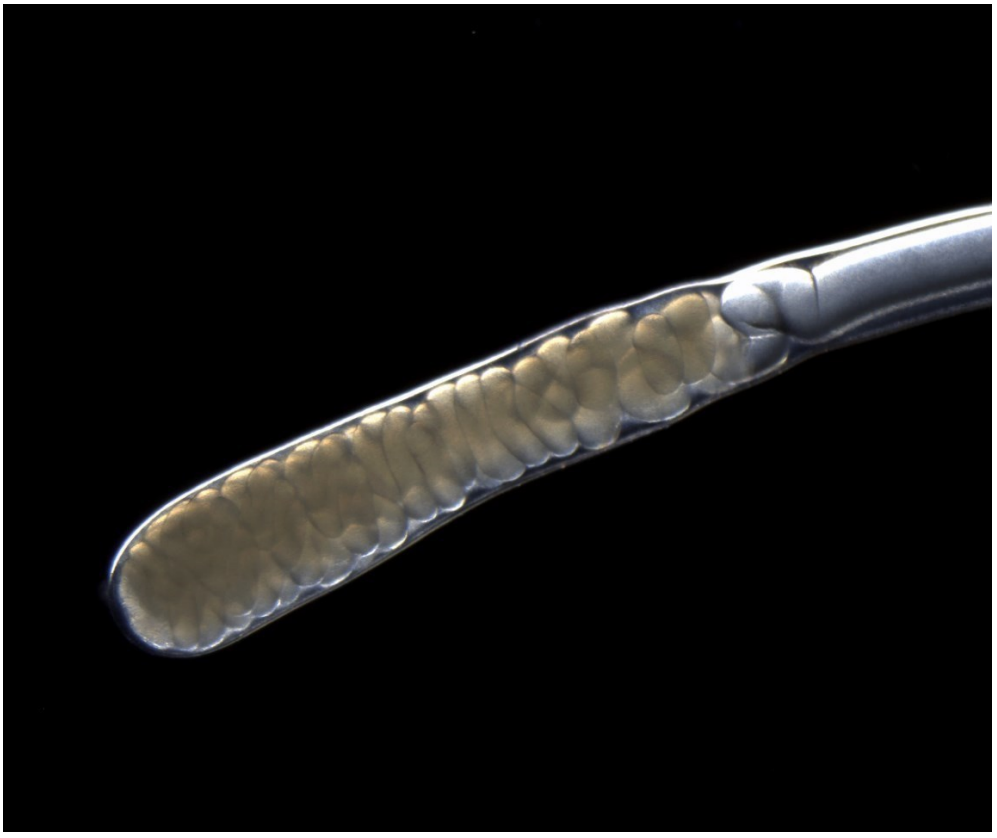


Fig. 60. The aboral end of a spermatophore with its spiral coiled sperm mass.

Fig 61. The cement body (see Salman, 2014 for details).

Fig. 62. The oral end with its ejaculatory apparatus and cap thread.

Exercise: Describe the morphology and early appearance of male organs in relation to dorsal mantle length, mass, and age (where possible). How, and how early, can the sex be determined using stereomicroscopes and compound microscopes? Will staining help?

Exercise: Cut open the different glands of the spermatophoric organs, locate the spermatophores and describe their morphology and development, as well as the function of the different glands.

Exercise: Describe the morphology of non-typical spermatophores. What (if any) function do they have?

Exercise: Try to dissect fresh male organs. Any differences in comparison with preserved ones?

Female reproductive organs

The aim is to locate and dissect the female organs, and to photograph and analyse the male spermatangia on the female bursa copulatrix, as well as the early oocytes, the reticulated oocytes and the mature oocytes in the ovary. The presence of spermatangia is clear evidence that copulation has occurred. The production of oocytes can be studied to learn about, e.g., reproductive periods and fecundity of females of different species and from different sub-populations and habitats, in order to obtain a better understanding of the factors affecting fertility, and its evolutionary consequences. The factors affecting oogenesis in bobtail squids are not well understood.

Removing the bursa copulatrix and oviducal gland

Place the female organ package into a cup with tap water until there is no smell of formaldehyde and put it into a Petri dish. Start the dissection by cutting off the membranes connecting the bursa copulatrix to the oocytes and the nidamental gland, but leave the membrane intact where it connects to the oviducal gland and the oviduct (**fig. 63)**.

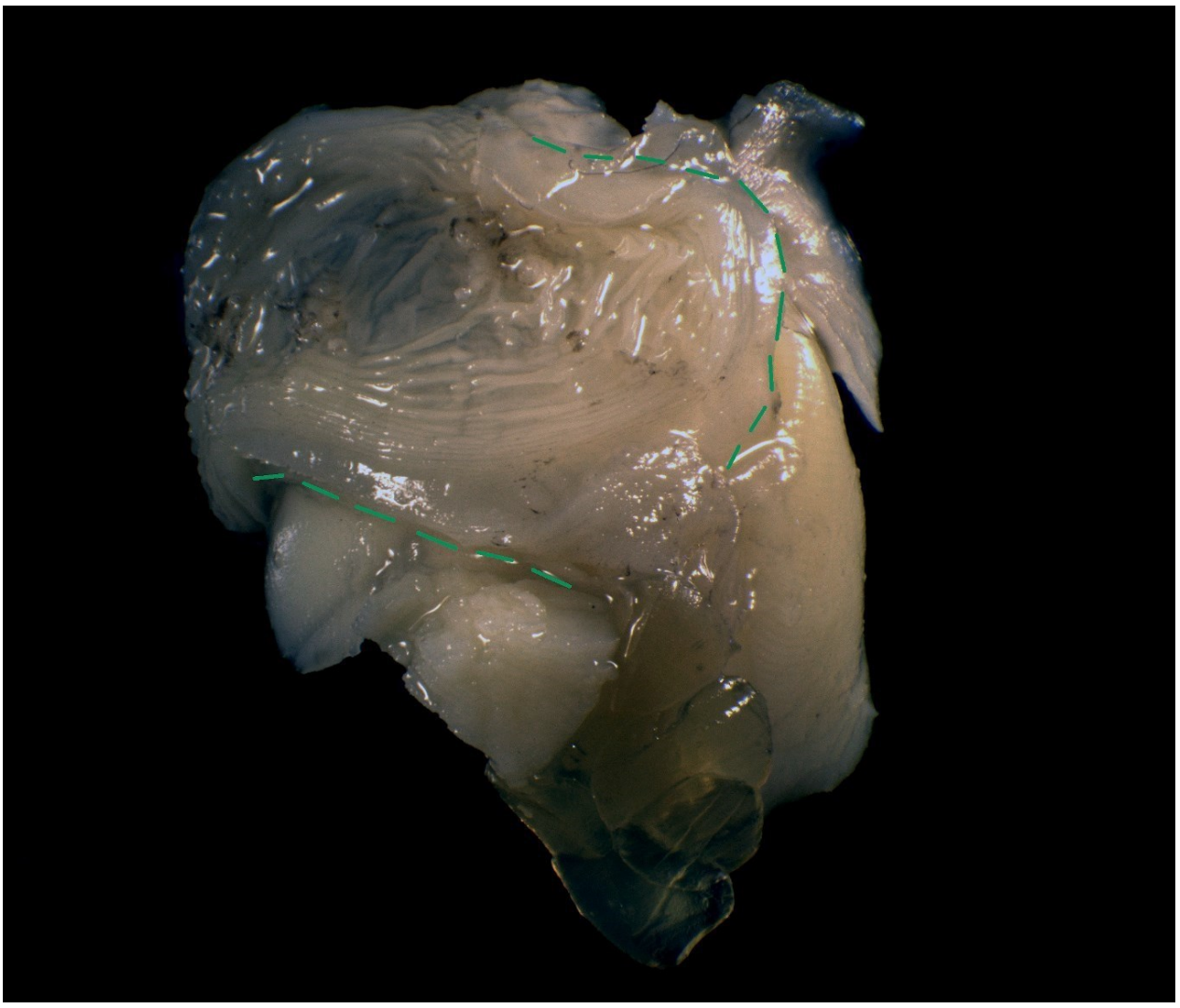


Fig. 63. The dashed green line indicate where to cut off the membranes connecting the bursa copulatrix and the nidamental glands (continue on the other side of the bursa copulatrix).

Lift the bursa copulatrix slightly while removing all the membranes underneath until it´s free. This takes some time and it is recommended to use needle scalpels and the long vitrectomy scissors shown in **fig. 1**. If needed, the membranes can be stained. The smooth underside (after removal of the membranes) of the bursa copulatrix is shown in **figs 64 &66.** The white areas are spermatangia that are embedded from the wrinkled upper side (see below).

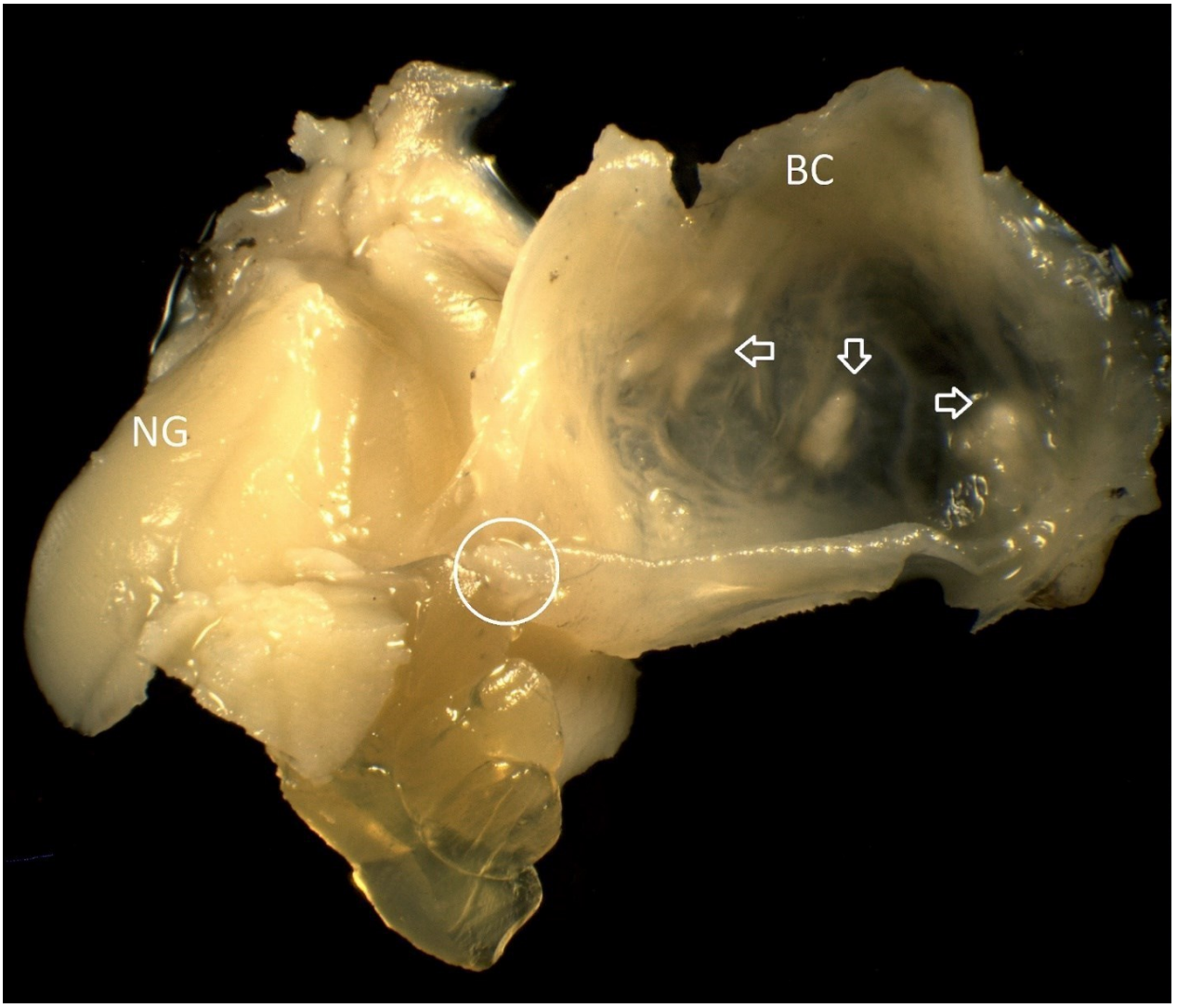


Fig. 64. The bursa copulatrix is turned over and is here connected only to the oviducal gland (circle) and the oviduct with its mature oocytes. The arrows show spermatangia embedded into the tissue from the upper wrinkled side; NG = nidamental glands and BC = bursa copulatrix.

Cut the bursa copulatrix and the oviducal gland free and move them to another Petri dish with tap water for analysis of the spermatangia (**fig. 65**). The oocytes in the oviduct should be photographed for later analysis and saved in the female organ vial.

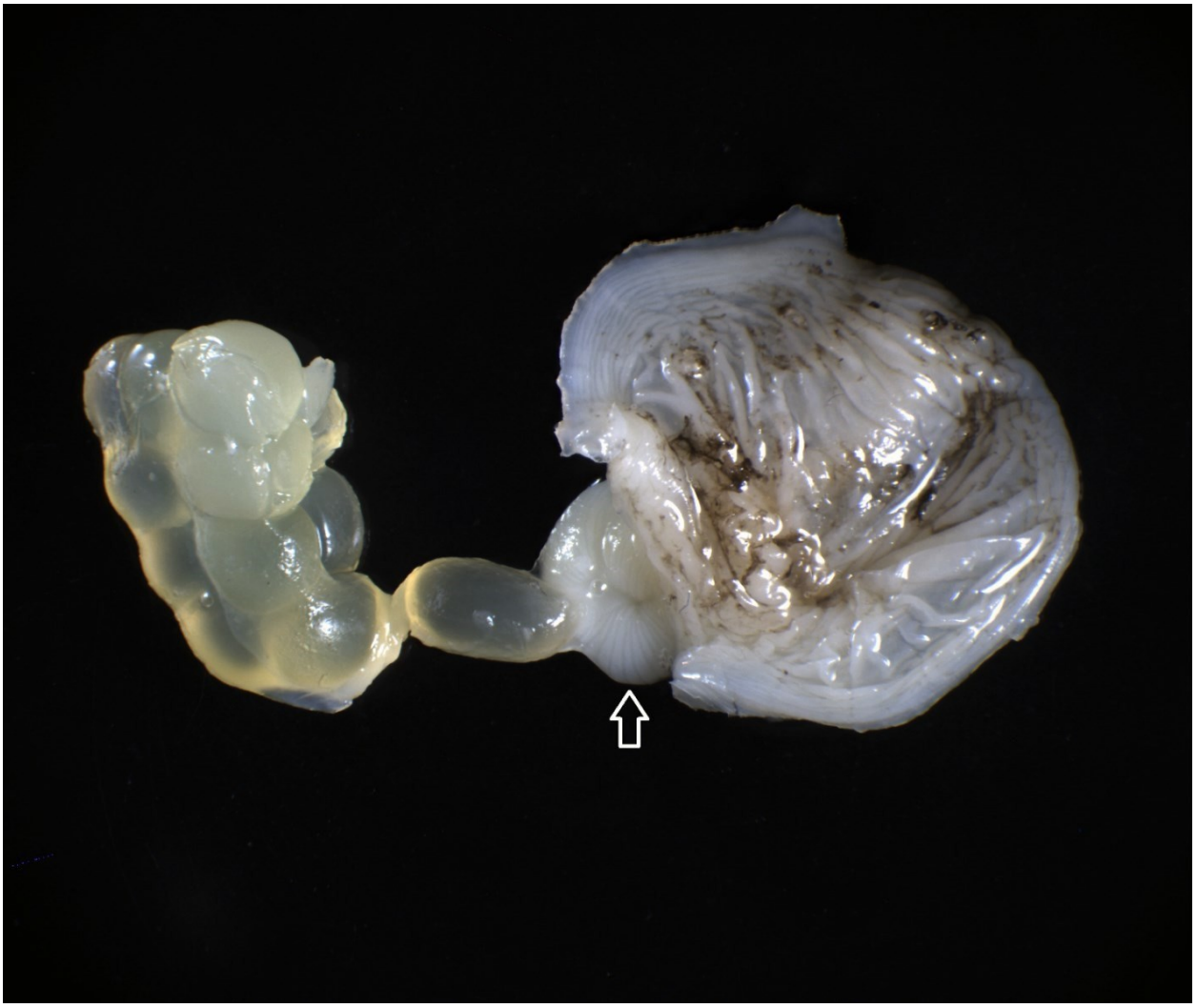


Fig. 65. The bursa copulatrix has been removed together with the oviducal gland (arrow) and the oviduct containing some mature oocytes.

Note that the oviducal gland is attached to the smooth underside of the bursa copulatrix and that there is also a hollow thick-walled duct (the bursa copulatrix duct?) connecting the oviducal gland to the edge of the bursa copulatrix (**fig. 66**). A thin pin can be put inside this duct (from the edge) all the way through the oviducal gland. We assume that at some point there would be an ovum (coated by a secretion from the oviducal gland) inside this duct, before reaching the upper wrinkled side of the bursa copulatrix where fertilization takes place.

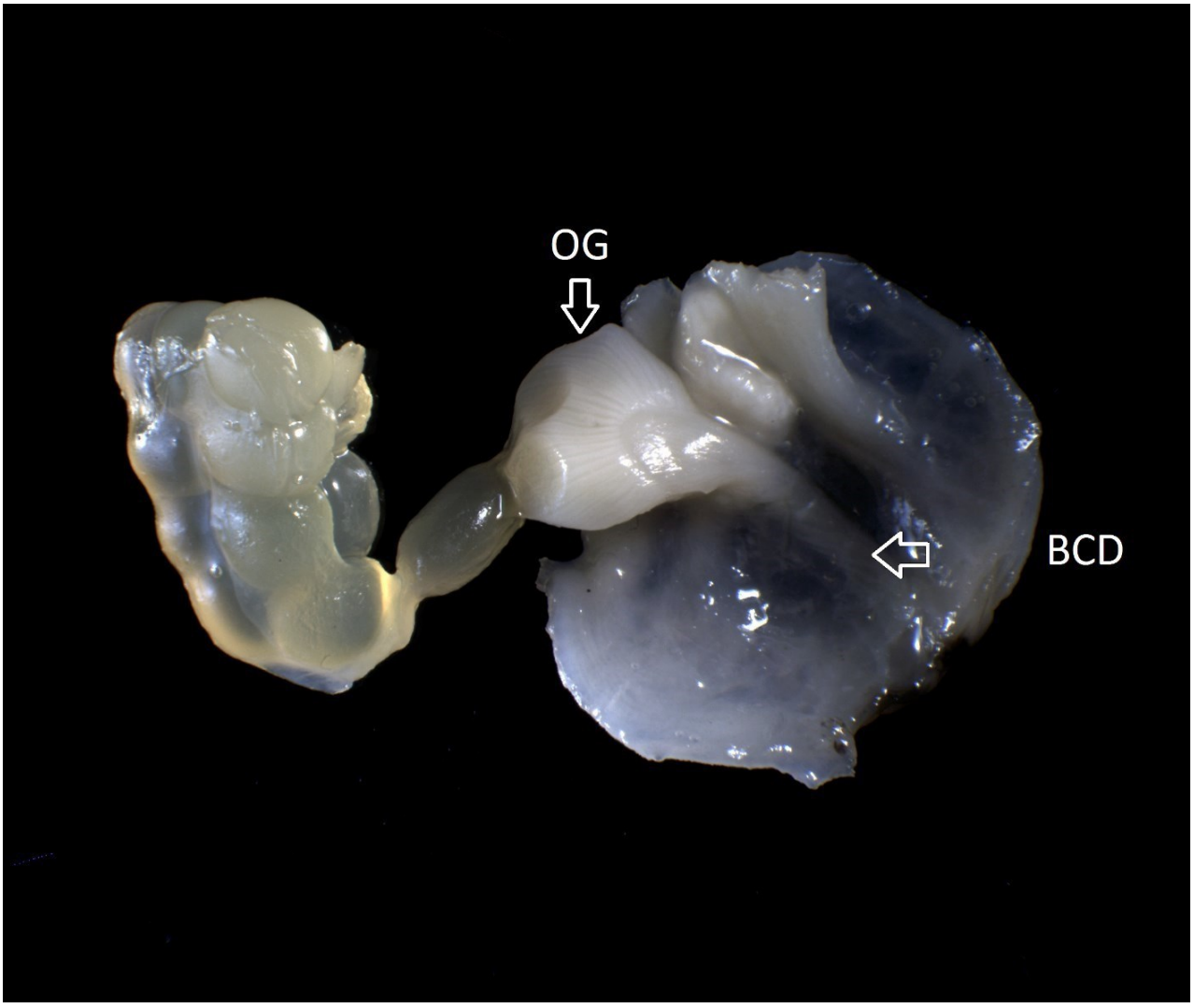


Fig. 66. The smooth underside of bursa copulatrix. OG = oviducal gland**,** BCD = **"**bursa copulatrix duct".

Analyses of the spermatangia on the bursa copulatrix

The spermatangia (which have more or less the same colour as the bursa copulatrix) should be stained with azure B so that each spermatangium can be seen and analysed correctly. Azure B is particularly well suited since the spermatangia become stained much better than the bursa copulatrix (**fig. 67**). The spermatangium consists of a "tail" (initially filled with spermatozoa) and a "base" which is, more or less, embedded into the bursa copulatrix tissue (in *S. oweniana*). Staining should be repeated if the bases cannot be detected easily but note that a partial staining of the bases can make it easier to distinguish them individually. Count/analyse both the bases and the tails. The tails will fall off once the spermatozoa have moved forward into the base and the proportion of tails to bases can therefore indicate whether or not the copulation has taken place recently. The bursa copulatrix must be cut open so that all surface areas become visible (**fig. 67**). When many (>10) bases are present, as shown in **fig. 68**, one should cut out small pieces of the bursa copulatrix with no more than 5-10 bases on each (**fig. 69**). The individual bases can then be safely separated using two curved tweezers, to enable correct counting. This dissection can take several hours. Save all spermatangia in a vial of its own.

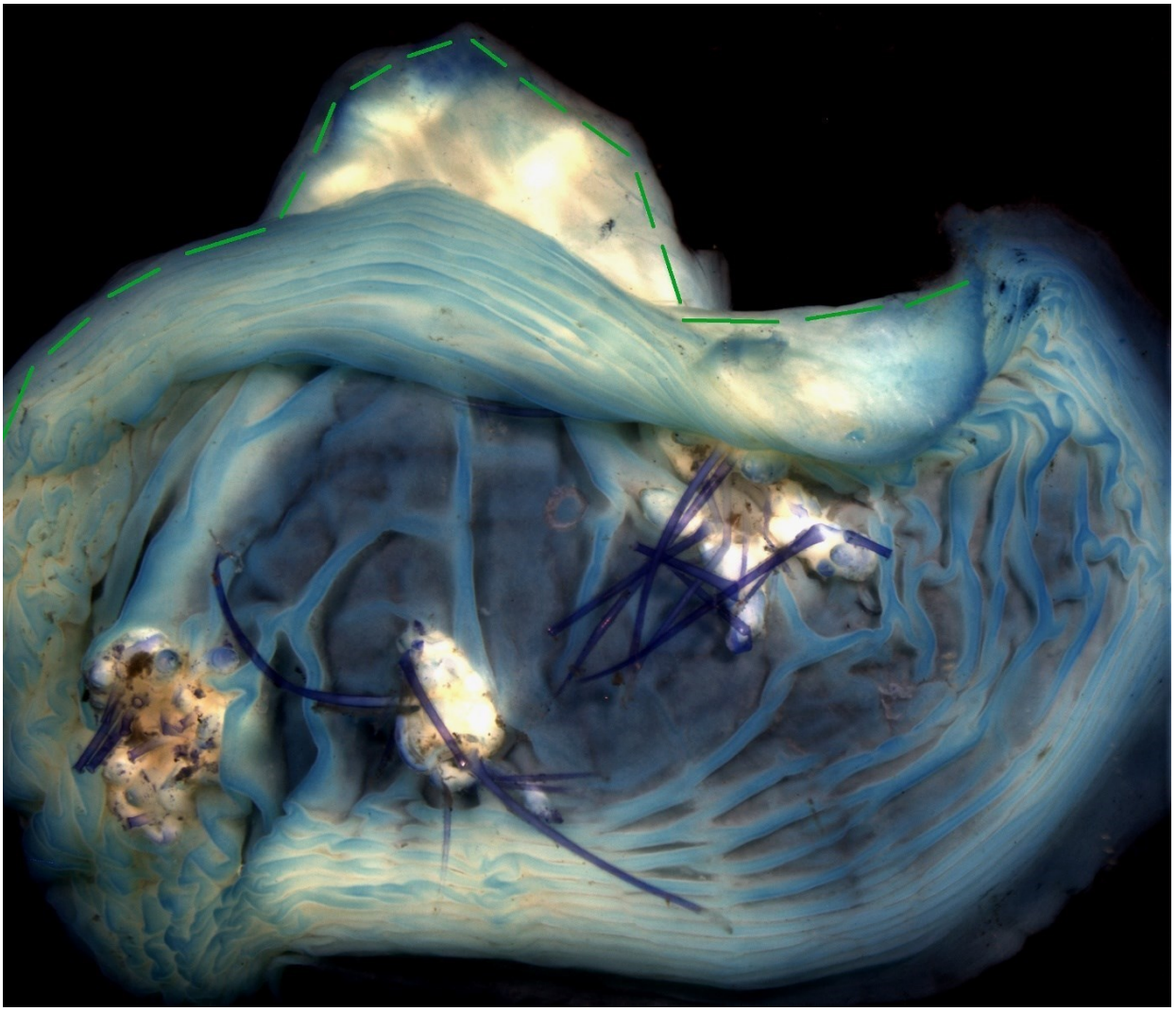


Fig. 67. The bursa copulatrix with some spermatangia tails and bases (stained with azure B). The dashed green line indicates where the bursa copulatrix should be cut open so as to see all the spermatangia.

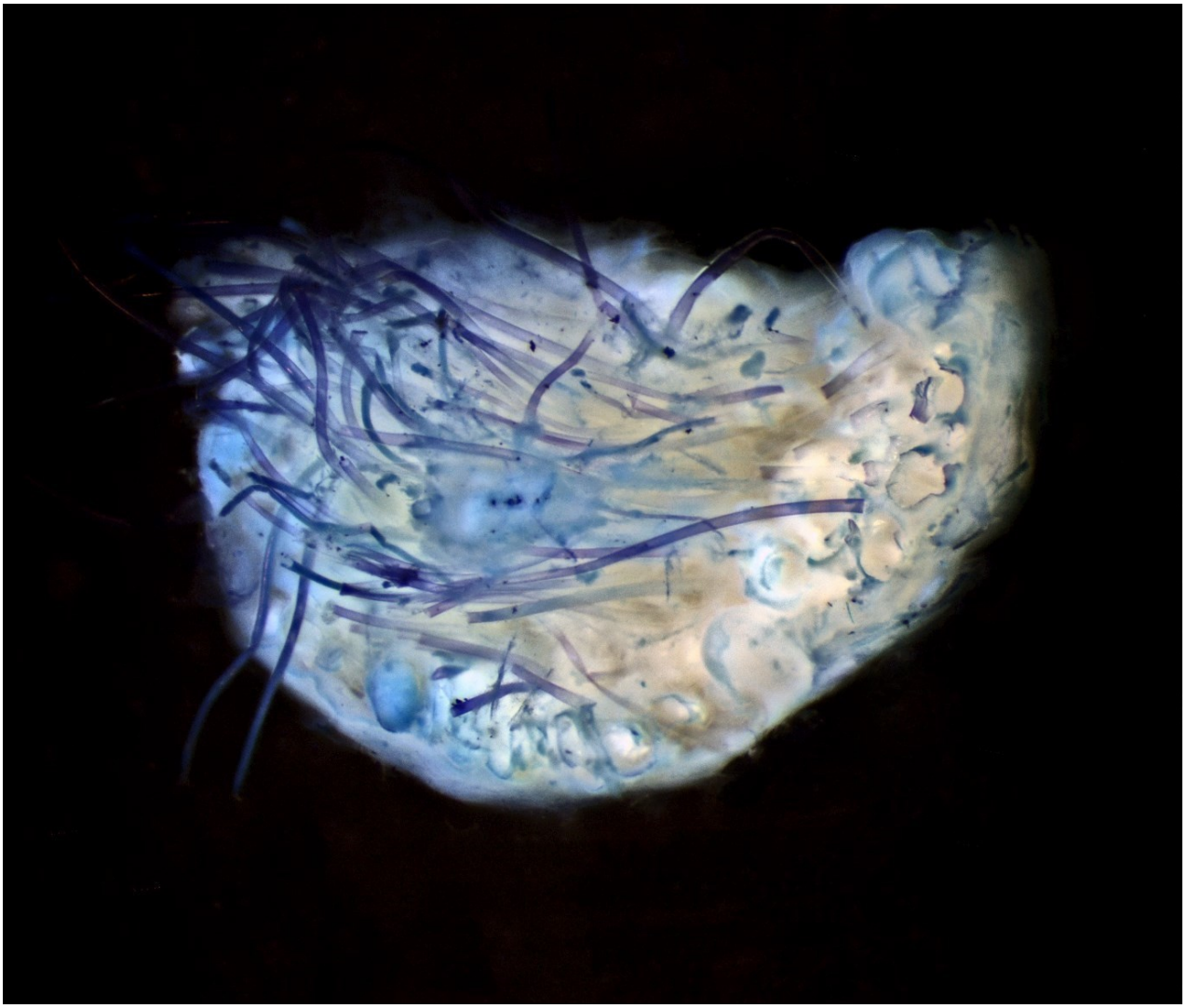


Fig. 68. The spermatangia tails and bases (stained with azure B) can occur in large numbers and cover a substantial area of the bursa copulatrix.

The bases can be seen both slightly (**fig. 69**) and more deeply (**fig. 70**) embedded into the bursa copulatrix tissue. The base can be removed from the bursa copulatrix (**fig. 71**) so the sperm mass can be dissected using curved tungsten needles (**fig. 72**) and mounted on a glass slide (**fig. 73**). Some cephalopod species have bi-flagellate spermatozoa (Coelho & O`Dor, 1984; Rodriguez et al., 2013; Laptikhovsky & Nigmatullin, 1996). Reviews of sperm morphological diversity are provided by Healy (1996) and Pitnick, et al. (2009.

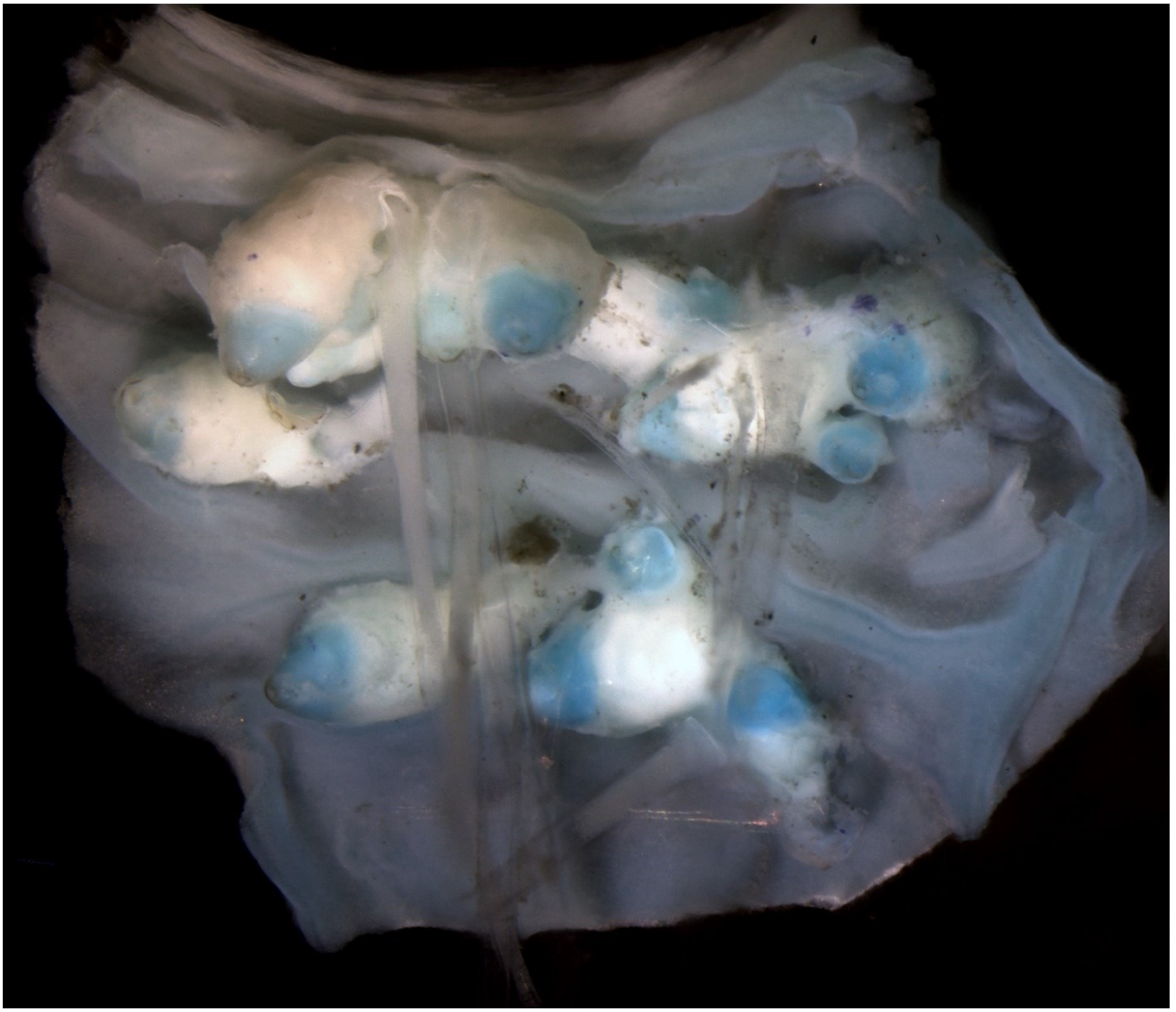


Fig. 69. A small piece of the bursa copulatrix has been cut out so the spermatangia bases can be separated and counted. Note that the bases are only slightly embedded here and partially stained with azure B so that they can be distinguished individually.

Fig. 70. A spermatangium (with spermatozoa inside its base) deeply embedded into the bursa copulatrix.

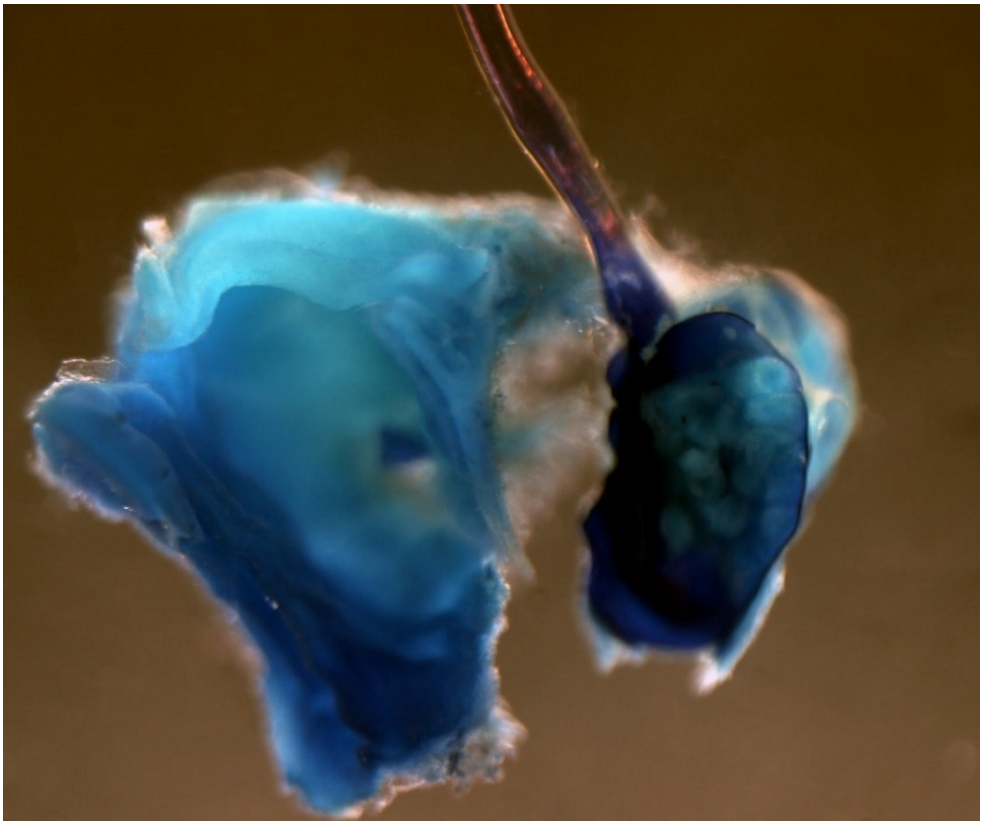


Fig. 71. A base has been removed from its embedded position.

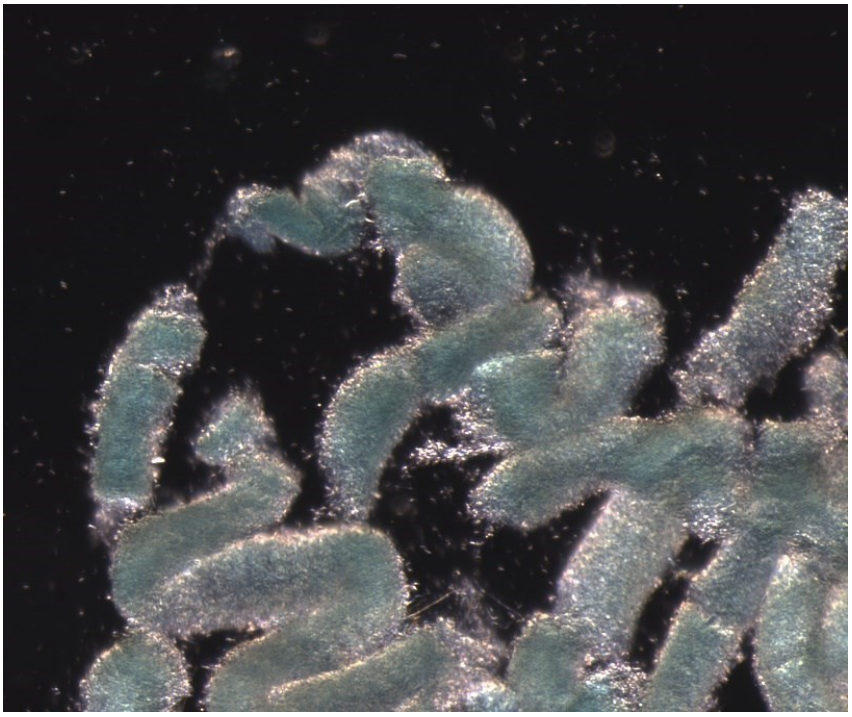


Fig. 72. A sperm mass has been dissected from the spermatangium base.

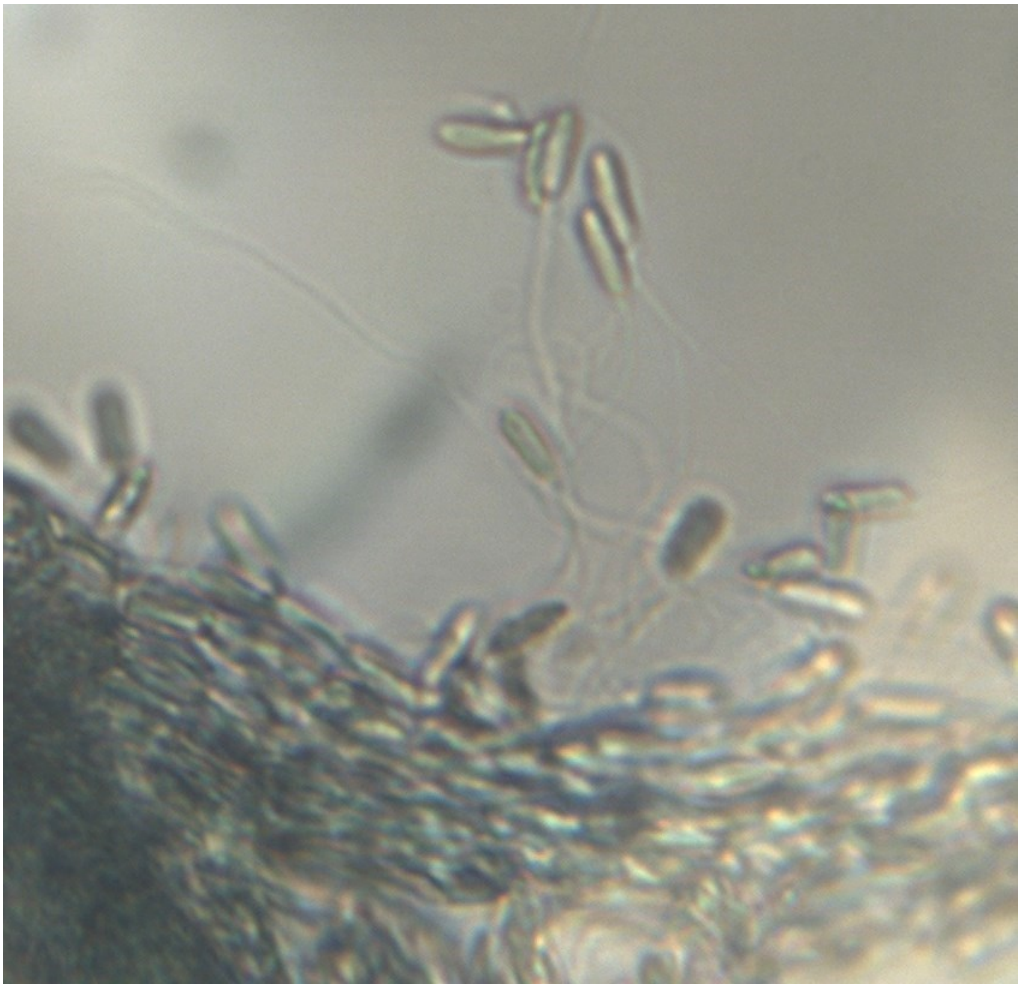


Fig. 73. Spermatozoa from the base. Are they bi-flagellated?

Removing the nidamental glands

The two whitish nidamental glands and the two accessory nidamental glands are easy to remove. The nidamental glands (**fig. 74**) are much larger than the accessory nidamental glands (**fig. 75**) which are found just posterior to the nidamental glands. Cut off the remaining membranes connecting the nidamental glands, the oocytes and other nearby organs. Note that the size and shape of the nidamental glands and the accessory nidamental glands changes considerably during their development. Save them in the female organ vial.

Fig. 74. The whitish nidamental glands of a female at maturity stage IV, with their typical lamellae structure.

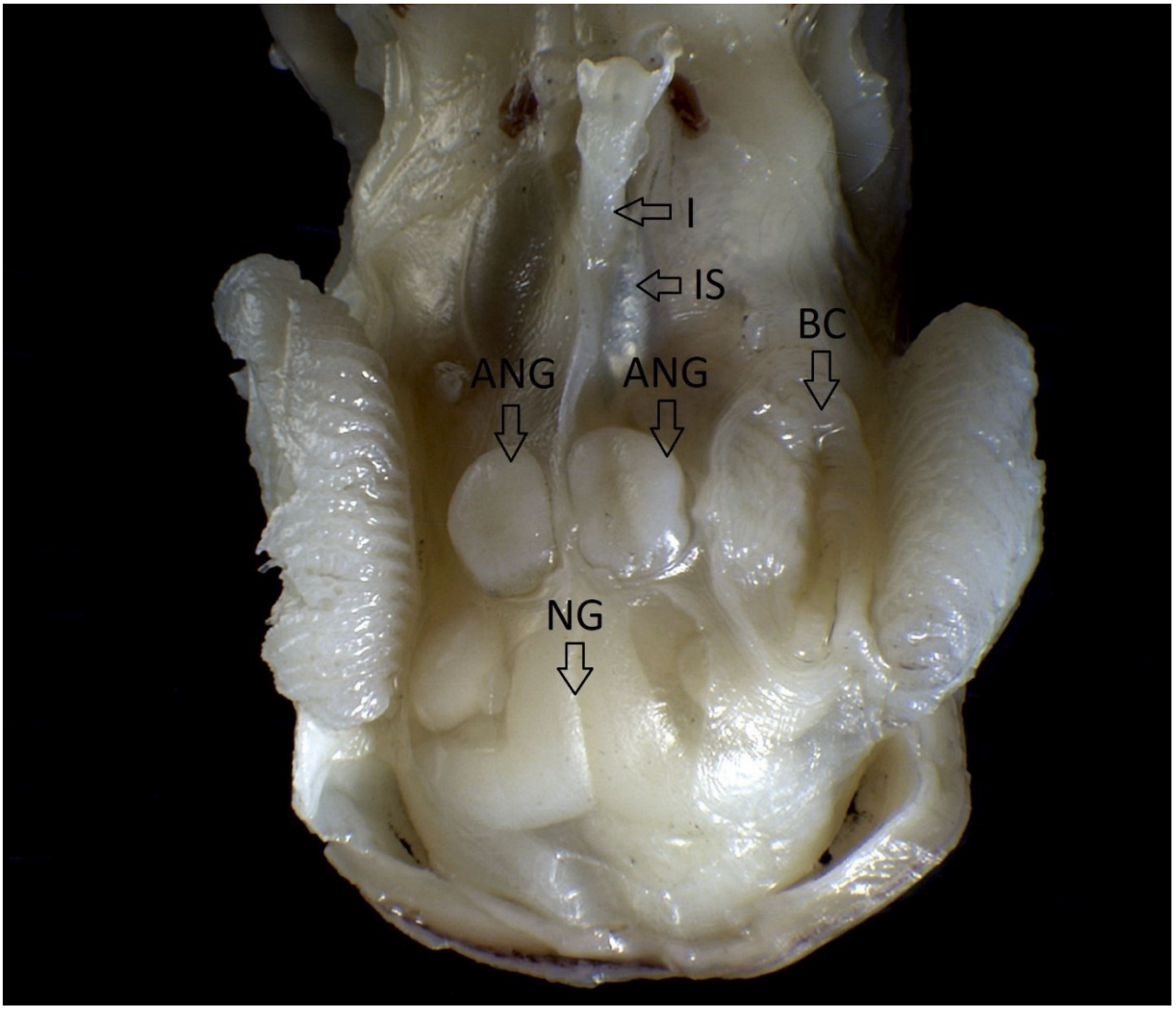


Fig. 75. The intestine (I), ink sac (IS), accessory nidamental glands (ANG), nidamental glands (NG) and the bursa copulatrix (BC) of a female at maturity stage II.

Analyses of the oocytes

Move the remaining ovary and its oocytes to a clean Petri dish. Using two curved tweezers, separate and sort the early, the reticulated and the mature oocytes (**figs 76, 77 & 78**), and take photos (**fig. 78**) of all oocytes for analysis (e.g., counting and measuring size) before saving them in the female organ vial. Note that counting and measuring of oocytes at different stages only reflect a temporal situation which changes during stages II and III. See, e.g., Laptikhovsky, et al. (2003); and Salman & Önsoy (2010) regarding potential fecundity and resorption of female gametes. We only count and measure the reticulated and mature oocytes since the early ones can be difficult in this respect.

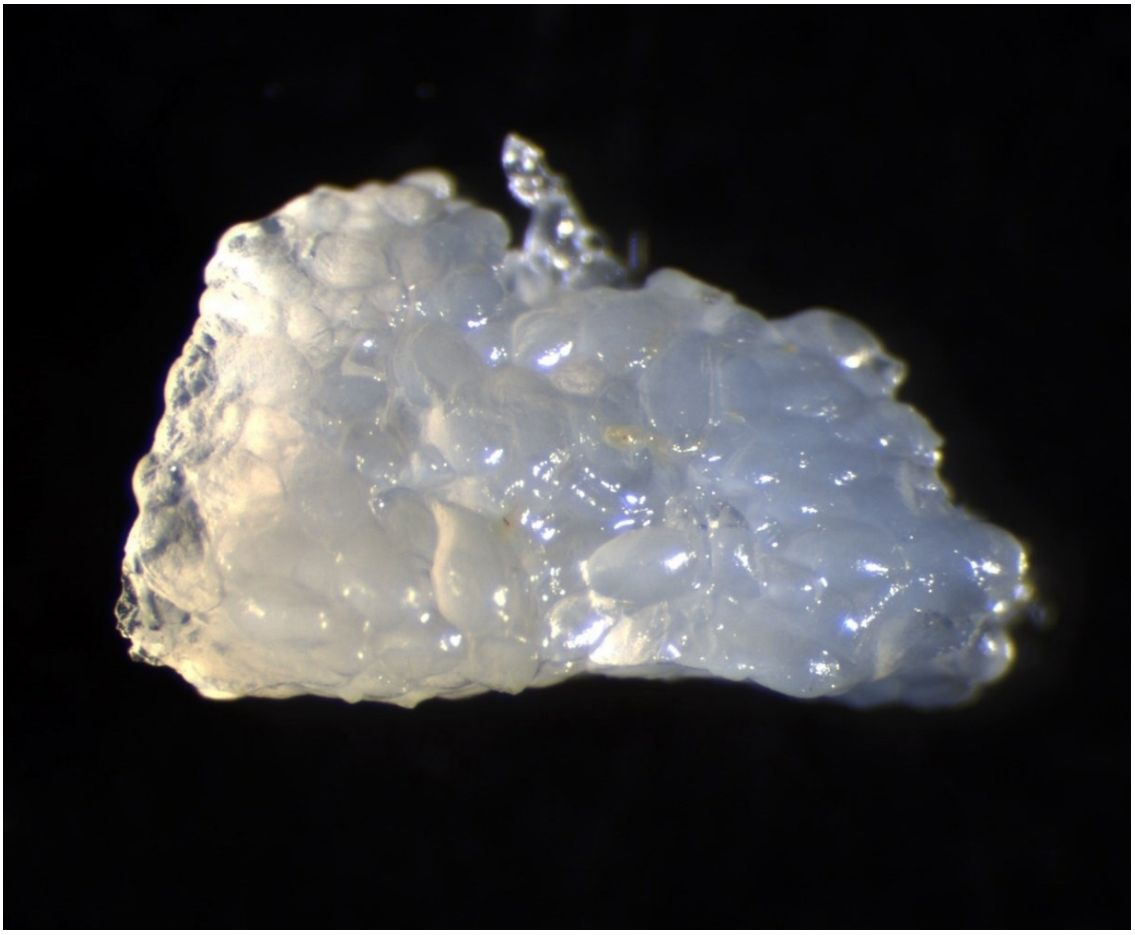


Fig. 76. Early oocytes from a female at stage IIa.

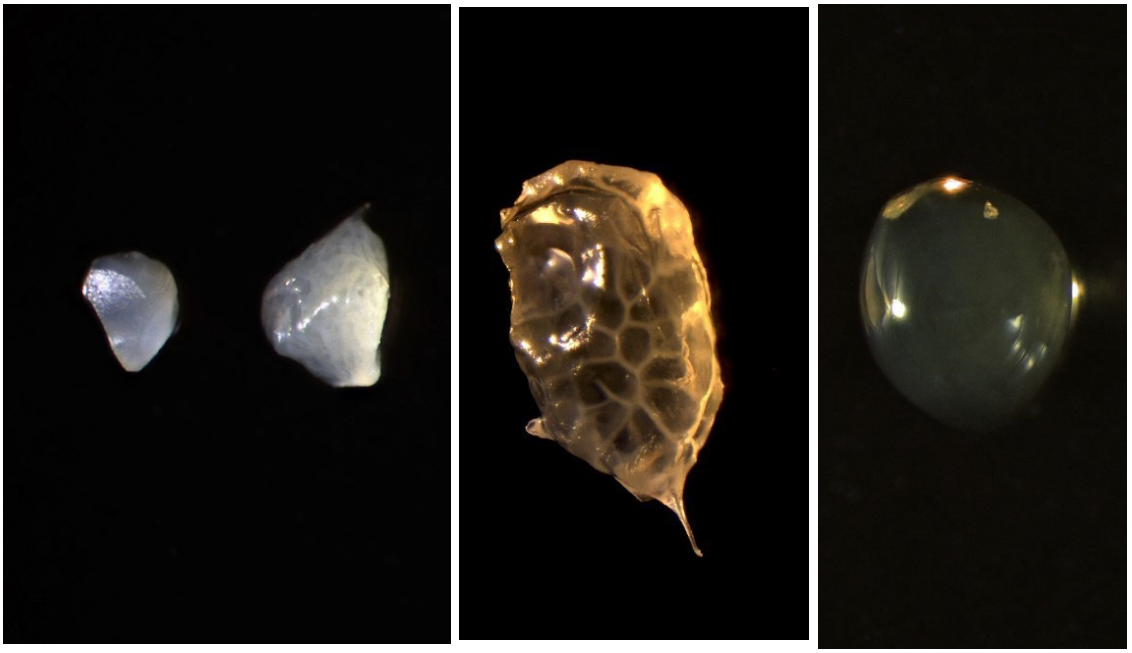


Fig. 77. Two early oocytes, one reticulated and one mature (colour of which is difficult to show correctly in a photo).

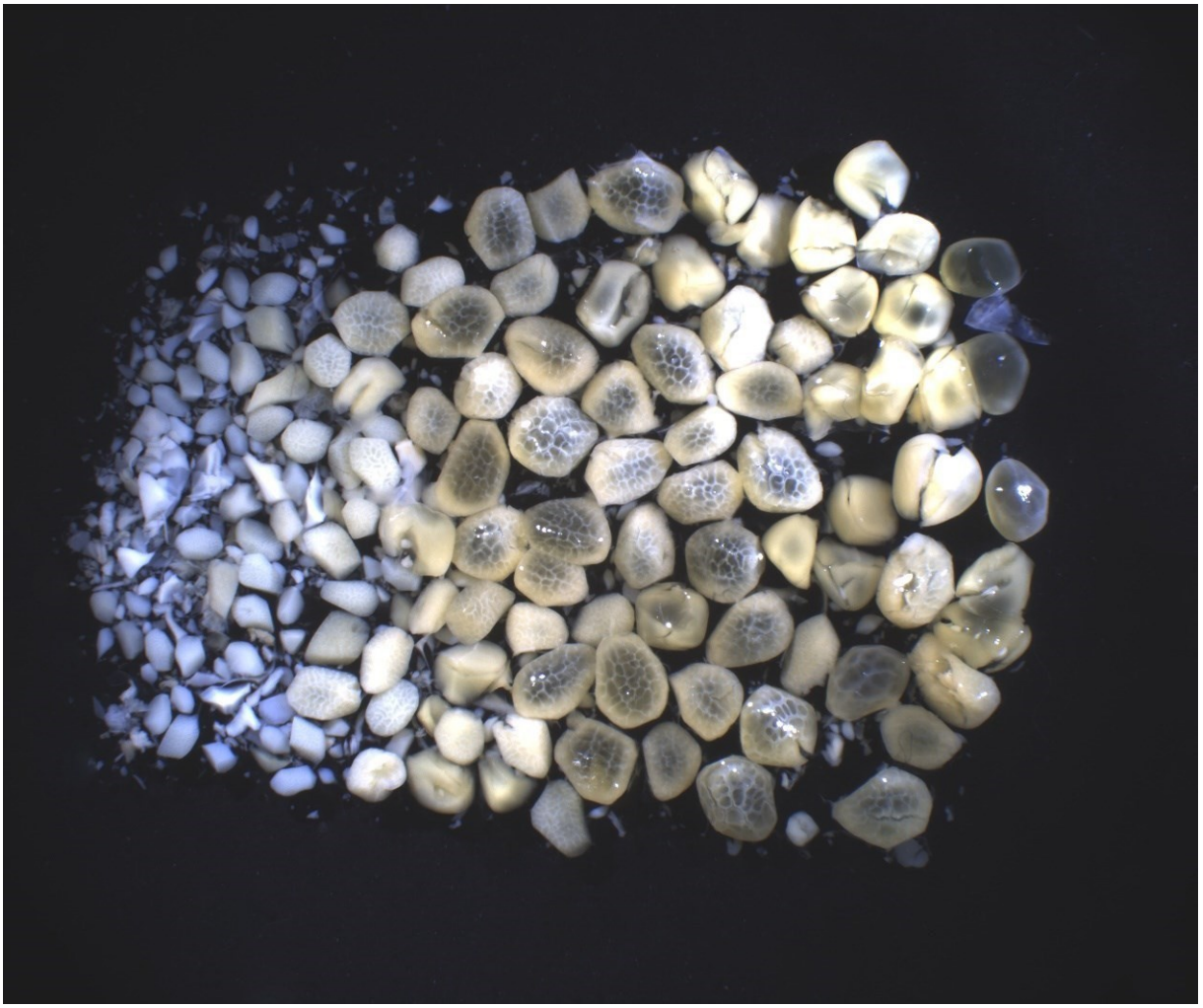


Fig. 78. Early oocytes, reticulated oocytes and a few mature oocytes.

Exercise: Describe the morphology and early appearance of female organs in relation to dorsal mantle length, mass, and age (if possible). How, and how early, can sex be determined using stereomicroscopes and compound microscopes?

Exercise: Stain different organs, using different dyes, and cut them open and stain again to discover how dyes will reveal different outer and inner structures that can improve your dissections and morphological understanding, as well as to create instructive photos. Horobin & Kiernan (2002) give a comprehensive overview of useful biological dyes and stains.

Exercise: Are spermatangia bases less embedded when found in certain areas of the bursa copulatrix ridges? Compare different species.

Exercise: Analyse spermatangia using DNA techniques. How many males can deliver spermatophores to a single female?

Exercise: Try to show how the spermatozoa are released from the embedded base of the spermatangia.

Exercise: Describe the morphology of the spermatozoa. Check if the bobtails have bi-flagellate spermatozoa. Preferably one should use TEM or SEM and check spermatozoa from the sperm duct, developing spermatophores, mature spermatophores and spermatangia. Compare fresh and preserved spermatozoa in a light microscope and make sure that the preservative does not affect their shape.

Exercise: Try to ascertain how the fertilized eggs get their coating.

Digestive organs

Research goals

The complexity of a local marine ecosystem is reflected in its food web interactions. The abundance, feeding, production, and behaviour of predator and prey developmental stages vary temporally, within their three-dimensional habitats. These, together with other direct and indirect biotic/abiotic factors, contribute to the chaotic nature of the dynamics of food webs, which explains why we still understand so little about them. This complexity also explains why feeding studies are done with different goals, from simple identification of the most commonly observed prey to, e.g., estimates of the local temporal predation impact on the population dynamics of different prey, by means of the different size classes of the predator. Consequently, the higher the research goals, the greater the need for more extensive data collection, analyses and time. See the review by Boucaud-Camou & Boucher-Rodoni (1983) and references therein for previous research on the feeding and digestion of cephalopods. There are no comprehensive studies of the diet and feeding rates of bobtail squids but see e.g., Bergström (1985) and Vafidis, et al. (2009).

DNA and stable isotope analysis are used in diet studies (see e.g., Golikov et al., 2020 and references therein). However, these methods do not reveal the number of different preys ingested (nor their size) and therefore cannot provide the data needed to estimate daily feeding rates and for preda-tion impact studies. Note that estimates of feeding rates on different prey developmental stages are needed in order to estimate daily energy intake. A significant problem which needs to be bet- ter addressed is the “food of the prey” and how it affects data interpretation. When estimating feeding rates, one needs to know the digestion time for the different types of prey and how vary- ing temperatures can affect digestion. The digestion times can be determined experimentally (seee.g., Øresland, 1987).

The daily feeding rate (in numbers of different food categories) of a carnivore can be estimated using the equation (Bajkov, 1935):

FR = Mean NPC * 24/DT

where FR = daily feeding rate (no. prey d-1) (each category of prey should preferably be estimated separately); mean NPC = mean number of a certain prey category per carnivore during a

24h period; DT: digestion time (h) for a certain category. Multiplication by 24 gives the daily feeding rate. Observe that estimates of the mean NPC require a 24 h sampling schedule (e.g., sample collection every third hour).

Diet

Apart from the complexity of food web interactions, research goals may also be affected by the fact that diet analysis can be difficult to carry out, especially if the prey is crushed into very small pieces. Consequently, it is often difficult to identify food items at the species level and developmental stage and one may be forced to settle for prey groups such as fish, amphipods, copepods, etc. However, if one is prepared to spend time and effort, one can certainly identify some items at the lowest level. (e.g., to developmental stage).

The planning of more advanced quantitative studies of food web interactions is easier if one first masters the methods for gut content analysis and gains an understanding of their limitations. One should then undertake a pilot study to investigate when, where and by which predatory stages the major prey groups are captured and how the food can be identified, and subsequently plan an appropriate field sampling programme. It is worth remembering that a variable short-term (<24 h) feeding and migration behaviour of both predators and prey can affect diet, feeding rate and predation impact estimates if a sampling programme is not optimal. As a consequence, a pilot study should be based on 24-hour field sampling, e.g., every third hour (see also "In the field" above). Diet studies that are not based on an appropriate short-term sampling should be regarded as insufficient.

The observed diet can, for many reasons, give a false picture of the real diet. Some preys have easily identifiable parts while others can be difficult to identify. Some food items are quickly digested while others take longer and the digestion time might also be affected by the amount of food to be digested. The digestive organs that can contain food items are the oesophagus, the stomach, the caecum and the intestine. Since different kinds of food items could possibly remain in the various organs for different durations, it is important to analyse all organs separately. Both small and large specimens may contain little or a lot of food and thus require different analytical methods, as shown below.

ID codes

It is essential to document the complete food analysis, and easy to use codes for individual photos, slides and data spreadsheets are necessary. How to use codes is very much a personal choice but we prefer codes that first identify the species, the sampling area, the sampling method and have a running number for each individual of the species (for each area and sampling method). We then add a prey category code and a running number for individual prey. Since we often take several photos of a prey, they each get a running letter. A photo code (= photo file name) could

be: sowegt12cop5b (= S. oweniana, Gullmar Fjord, trawling, S. oweniana no 12, copepod prey no 5, photo b).

Such codes can obviously be more precise if needed by including e.g., developmental stage, size, male or female, etc. The order of the different components of a photo code will decide upon how the photos are automatically sorted by the photo software, which should also allow for sorting based on any parts of a photo code. A list of the ID abbreviations should be attached to the computer screen.

Reference collection

When studying diet, one must compare current and previous findings with reference material of the food items available in the habitat at the time of sampling. Create a slide and photo reference collection from the potential prey collected in the investigated habitat. In shallow waters, two divers can drag a zooplankton net between them, from a few centimetres above the bottom to the surface. Bottom living, epibenthic and planktonic animals, including fish, can also be collected down to a depth of several hundred meters using light traps (Øresland, 2007). During trawl-based studies one can use a ring net and an epibenthic net operated from a boat. The sampling for potential food items should cover the habitat, feeding times and depths of the bobtail species studied. Photos should be taken of the different developmental stages of the potential prey species, and identification made, before they are dissected in PVL on glass slides in order to obtain as many useful photos of different identifiable body parts as possible. It is necessary to use a high magnification stereomicroscope with adjustable light from below (e.g., Nikon SMZ18) or an inverted microscope when dissecting and identifying small prey parts. Note that high magnification photos can reveal species and stage specific microstructures.

The mandibles of crustaceans are especially important since they can be identified as to species and developmental stage due to their morphology and width, which is measured from the outer to the inner tooth (**fig.79**). Therefore, one should have a complete mandible photo collection, with measurements and scales, from all potential crustacean developmental stages. The book series, “An account of the crustacea of Norway, 1895-1921” (16 volumes) by G.O. Sars is invaluable when making a reference collection of prey parts due to its detailed drawings of different parts of crustaceans. It is available to download for free from the net. A reference collection should also contain photos of fish otoliths, fish scales and other hard parts. Since the otoliths are destroyed by formaldehyde, one should use specimens preserved in ethanol or defrosted when studying the fish diet. For more information on fish otoliths one can use e.g., http://aforo.cmima.csic.es/., although, such data bases are not complete. The shape of otoliths changes with age and one will still need an otolith collection from relevant fish species/sizes.

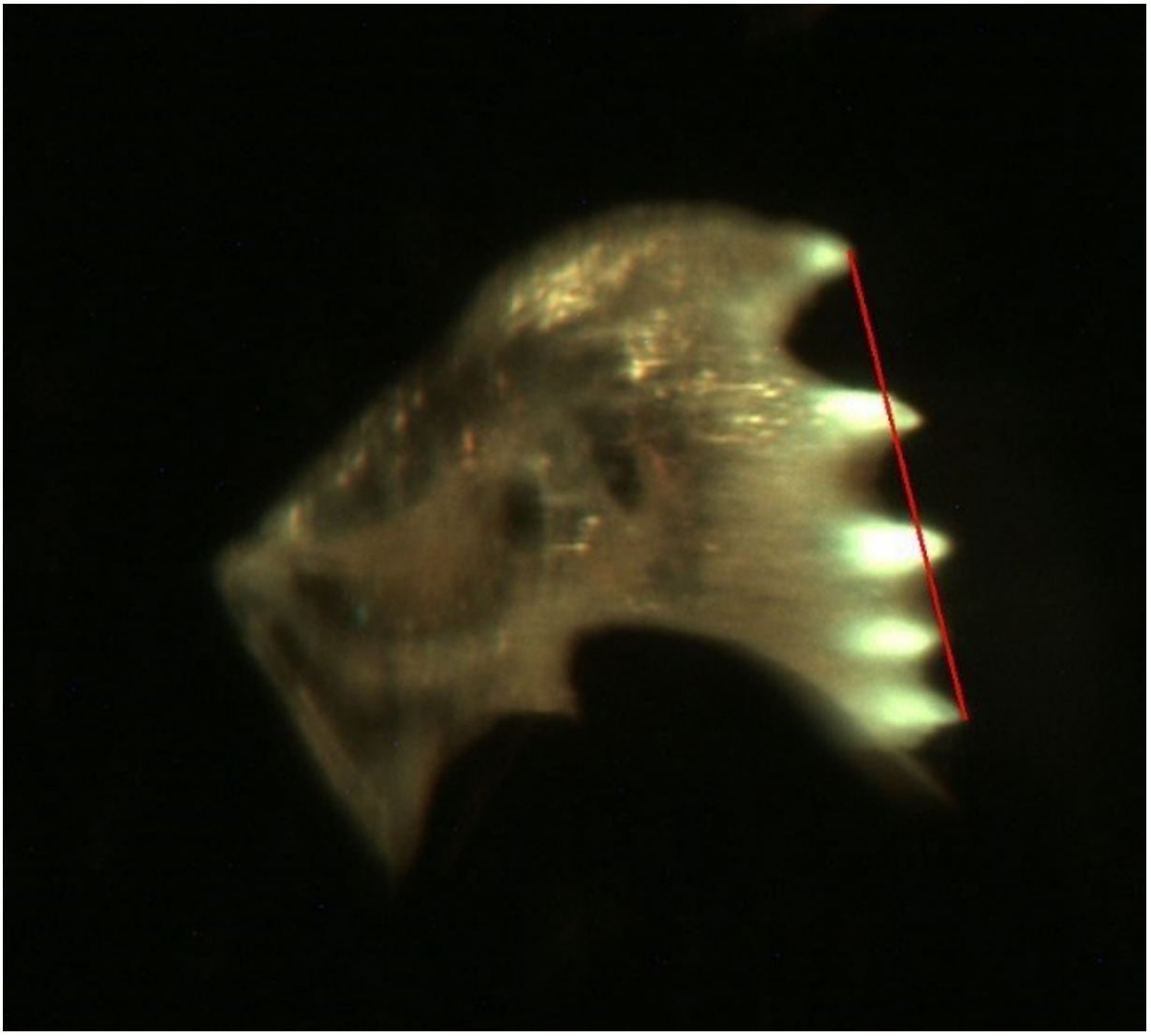


Fig. 79. A copepod mandible and a width measurement line from the outer to the inner tooth for copepodite stage determination.

It is crucial to explain how any quantitative diet estimates have been made since most identifiable parts exist as pairs (mandibles, eyes, antennas, legs, etc.). Measurements that show different sizes of these food items are helpful for keeping or separating such pairs and to determine developmental stages. Minimum quantitative diet estimates have the advantage of providing a known starting point for minimum feeding rates and predation impact estimates.

Dissection of specimens containing few food items

The aim is to examine the oesophagus, the stomach, the caecum, and the intestine to ascertain the presence of food items and transfer them to glass slides with PVL and to then photograph, identify and analyse them and to produce permanent slide preparations.

First, place the digestive organs into a Petri dish with tap water in order to remove the smell of formaldehyde and then transfer them to a new Petri dish. Separate the oesophagus from the

stomach and sprinkle a few drops of water over the remaining digestive organs to prevent them from drying out before dissection. Cut off the first two mm long section of the oesophagus and put it in PVL on a glass slide under a stereomicroscope. The PVL should be spread out to almost the same area as the cover glass. Cut the section into two one-mm pieces and turn them over so that any food items on the inside can be seen. Cut the pieces open and remove the food items (**fig. 80)** using two tungsten needles and then separate the individual items carefully so they do not cover each other. Remove the empty pieces of the oesophagus from the PVL. Adjust the positions of the individual food items and take photos from different sides of each potentially identifiable prey item before applying the cover glass. The cover glass can sometimes move the food items if the PVL has not hardened sufficiently. Code mark the slide, record it in the food analysis spread sheet and save it in a slide box. Repeat the same procedure for the remaining oesophagus, the stomach, the caecum, and the intestine. Take great care not to overcrowd the slides with food items.

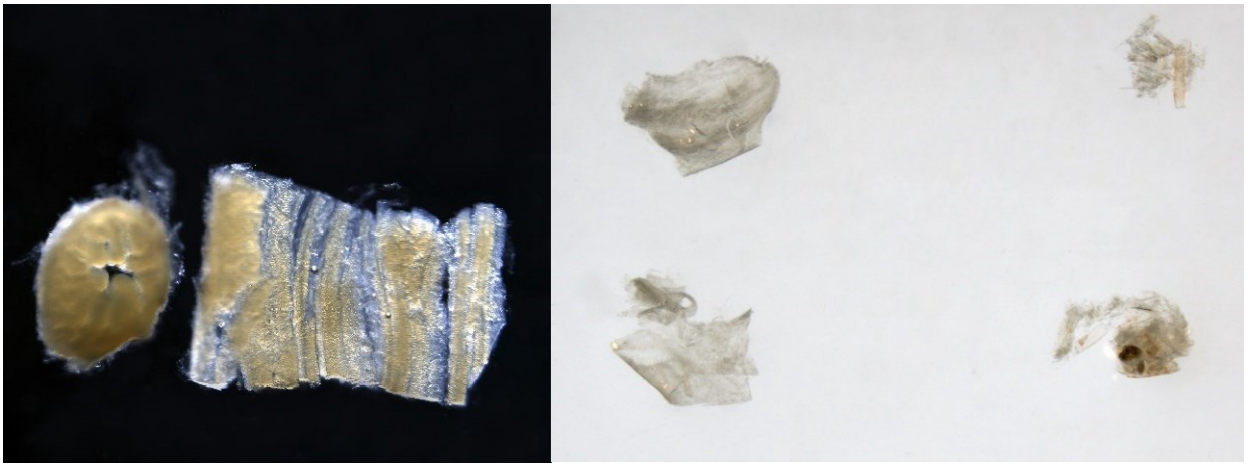


Fig. 80. The left photo shows how a 1-mm piece of the oesophagus can be cut open in PVL on a glass slide and the right photo shows that the gut content of two 1-mm pieces of the oesophagus have been moved to the right side of the glass slide.

During the dissection one should take notes of any special findings, the general amount of food present, any problems encountered and questions/ideas. Many new research projects art initiated due to such notations. The analysis of photos can be carried out later and could entail identification and estimates of, e.g., numbers of prey individuals, size, developmental stage (e.g., copepodite stage) and sex determination, including the data registration in the food analysis spread sheet. Note that the prey of bobtail squids also contains food items. How to separate prey food from bobtail food can be difficult. It is not unknown in diet studies that after further analysis unexpected findings have been found to be prey food items. Such suspected cases should be noted in the data spread sheet. Copepods are a common prey category of both bobtail squids and their prey (e.g., predatory zooplankton and fish). Diet analyses of the potential food items collected for the reference collection can provide helpful information as to when this problem might arise.

Dissection of stomachs full of food items

The bobtail stomach can expand considerably (**figs. 81, 91 & 92)** and contain many more food items than it is feasible (for time constraints) to mount directly on glass slides). Slide mounts will then be done only for food items of special interest and other items will only be photographed (if potentially identifiable).

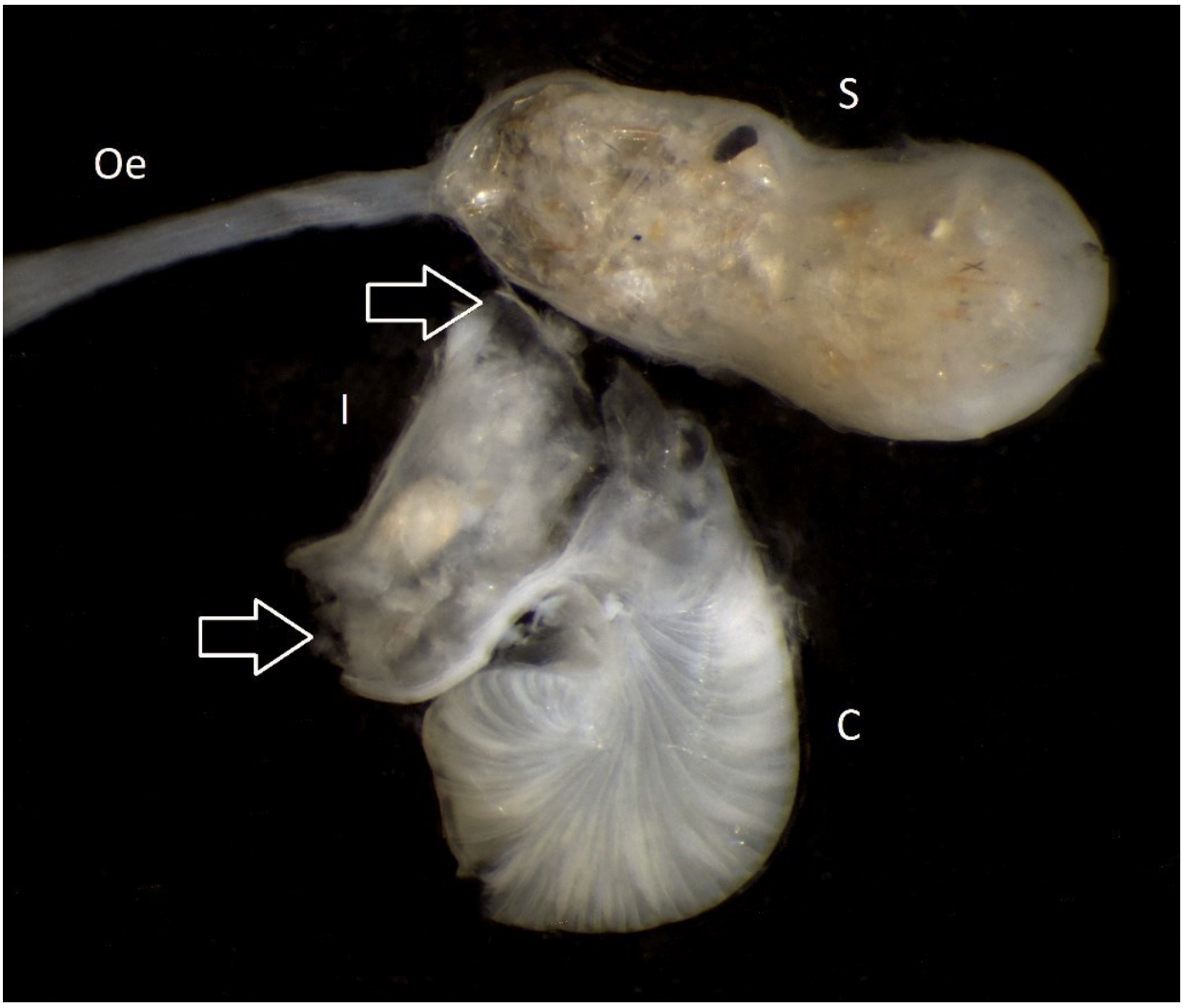


Fig. 81. A full stomach and intestine: arrows indicate scalpel cuts; oesophagus (Oe); stomach (S); caecum (C); intestine (I).

First, place the digestive organs into a Petri dish with tap water to remove the smell of formaldehyde and then transfer them into a new Petri dish and cut the stomach free from the other organs. Sprinkle a few drops of water over the remaining digestive organs so they do not dry out before being dissected in PVL on glass slides, as above. Dry the stomach on a kitchen paper, measure the wet mass and photograph the stomach for measurements. Put the stomach into a zooplankton sorting tray together with some water and place it under a high magnification stereomicroscope. Cut open one end of the stomach and, using tungsten needles, take out only a few pieces of food at a time for analysis, spreading them out as shown in **fig. 82.** After the photos and data have

been obtained and double checked one should move the stomach to the left and repeat the process until there are no food items remaining.

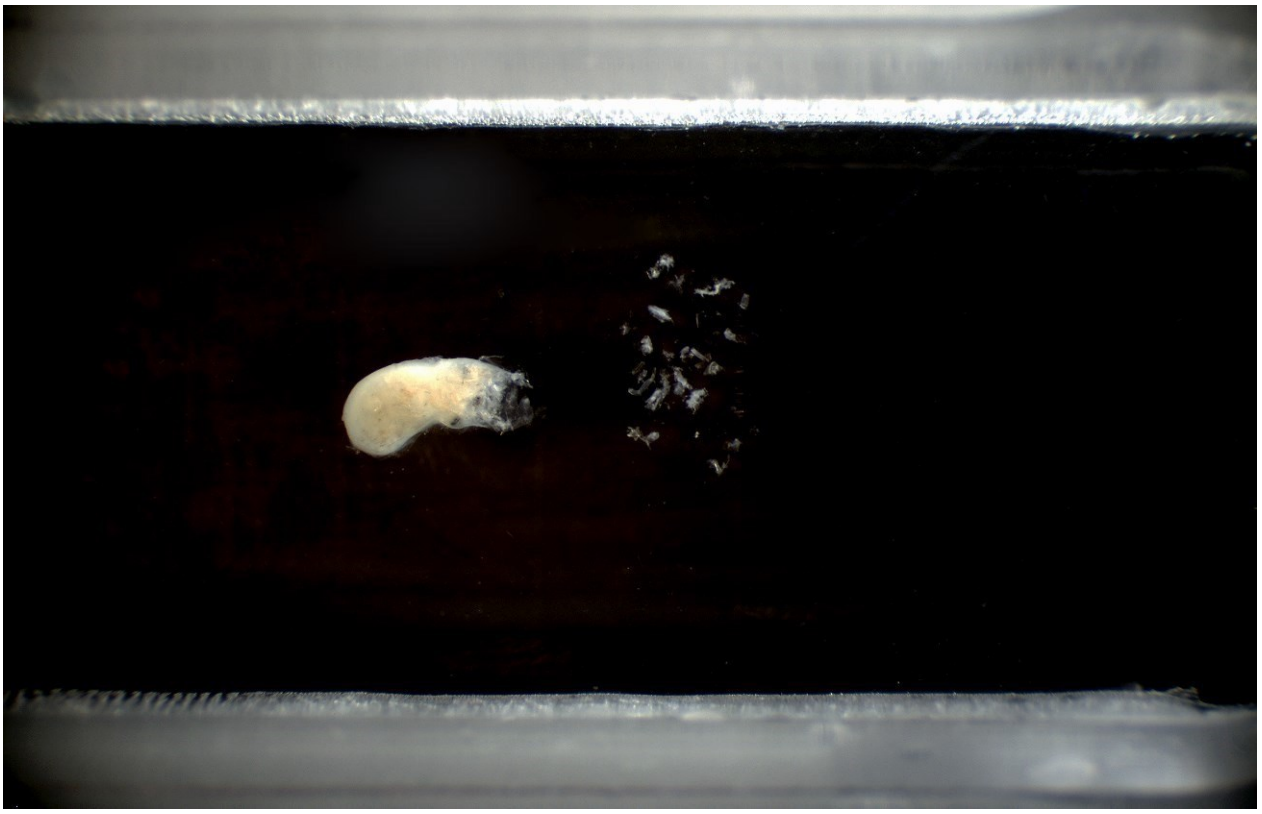


Fig. 82. Stomach and food items in a part of a plankton sorting tray.

Look for items such as eyes, mandibles, undigested and coloured parts, legs, antennae, fish scales, polychaete remains etc. that are documented in the photo reference collection. Note that fish otoliths will be destroyed by the use of formaldehyde as a preservative (use ethanol if you need the otoliths or DNA analysis of food items). Take photos of all the potentially identifiable items whilst still in the sorting tray (**fig. 83)** and make glass slide preparations of those that are especially interesting. Lifting out small items intact can be difficult. Put two bent 0.1 mm tungsten needles on a needle holder in order to move the food items to the PVL on the glass slides.

Fig. 83. An example of potentially identifiable crustacean and polychaete food items photographed in the sorting tray (fish scales were also found).

If food items are mixed with muscles it is strongly recommended to put them into household chlorine, while observing them under a stereomicroscope, to get rid of the muscles in order for the hard parts to be identified (**fig. 84**).

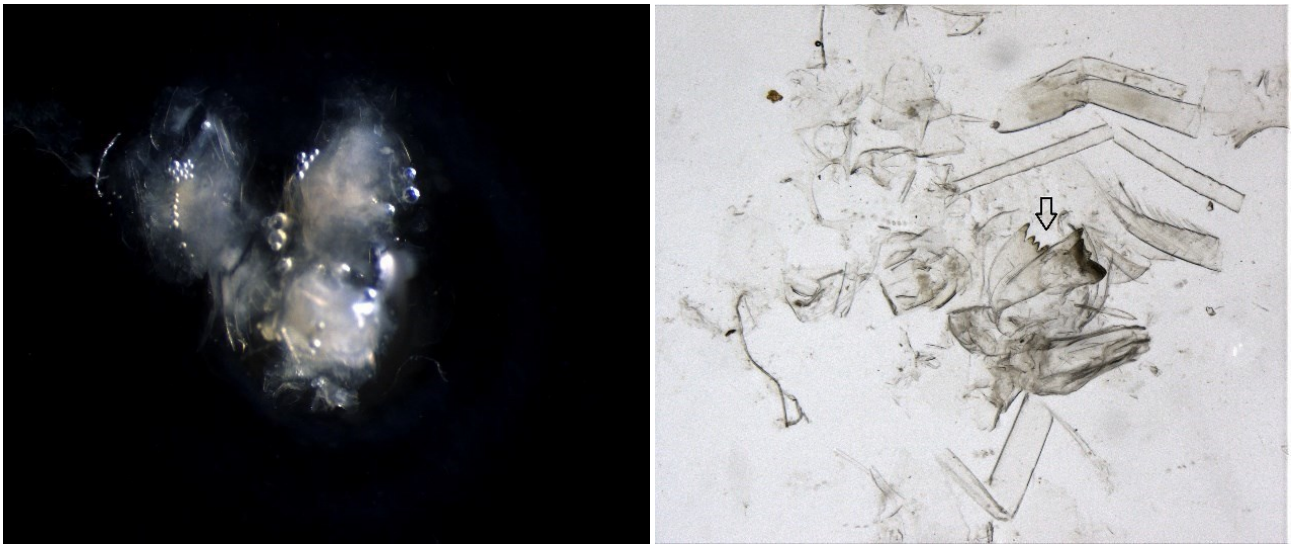


Fig.84. Food items with muscles, during (note the gas bubbles) and after chlorine treatment. The arrow shows a mandible.

Exercise: Test if staining can help can help to identify certain food items (with or without fluorescence) and whether the staining remains in the PVL and other mounting fluids.

Exercise: Identify a potential food item (e.g., a copepod or a small fish) and dissect, in PVL on a glass slide, all parts that may serve as identification. Turn the parts over to look for recognizable microstructures on both sides and take photos before and after a cover glass is put in place. Let the PVL harden somewhat before adding the cover glass. It takes some training to make good slides where the different parts stay in place and with a minimum of air bubbles.

Exercise: If fish scales are found, try to identify them as to species and age. Methods for ageing fish scales are well described in the scientific literature.

Exercise: Dissect the stomach of a potential prey species in order to identify "the food of the prey".

Exercise: Obtain newly hatched bobtails from plankton samples and identify their natural prey. This should be interesting for species used in aquaculture.

Other organs

There is a lack of photo documentation of many organs of all bobtail species and several *S. oweniana* organs are not shown here. The search for, and documentation of organs, is rewarding for students and amateur naturalists as it promotes further interest in functional biology and evolution. For example, below are shown some photos of the little-studied funnel organ (which consists of three parts) of *S. oweniana* and the flap-like funnel valve, both of which are used for species identification (Mercer, 1968; Roper & Voss, 1983). Cut open the funnel as shown in **fig. 85** and pin each side so the y-shaped dorsal funnel organ can be seen. To the left and right are two

other parts of the funnel organ. Stain all the parts, remove all surrounding membranes, and cut them out from the funnel tissue. **Fig. 86** shows the three parts of the funnel organ and **fig. 87 (**from another specimen) indicates that the form of the Y-shaped funnel organ may vary somewhat between *S. oweniana* individuals.

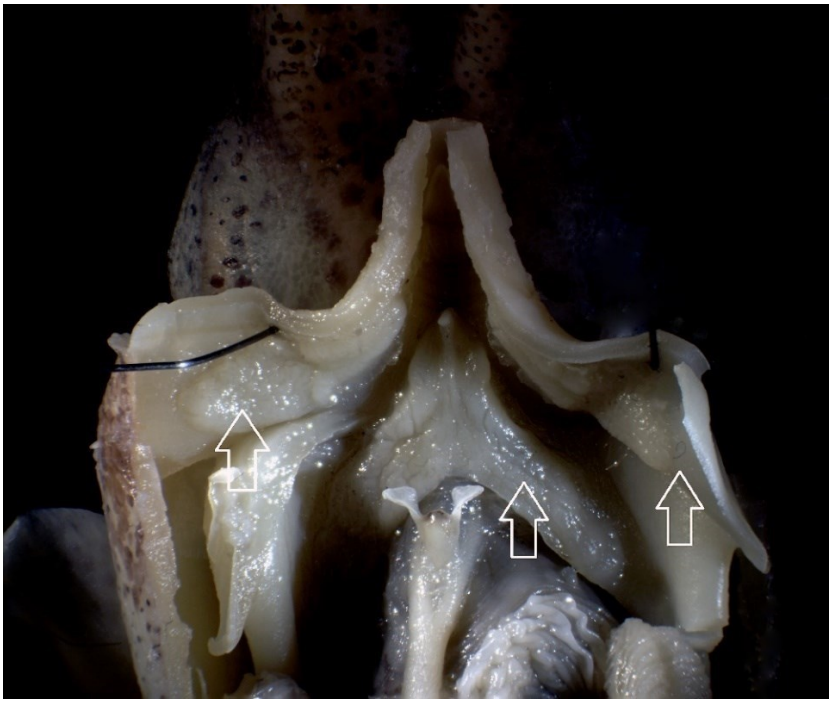


Fig. 85. The funnel has been cut open and pinned so the three parts of funnel organ (arrows) are visible. The two whitish flaps are the anal flaps.

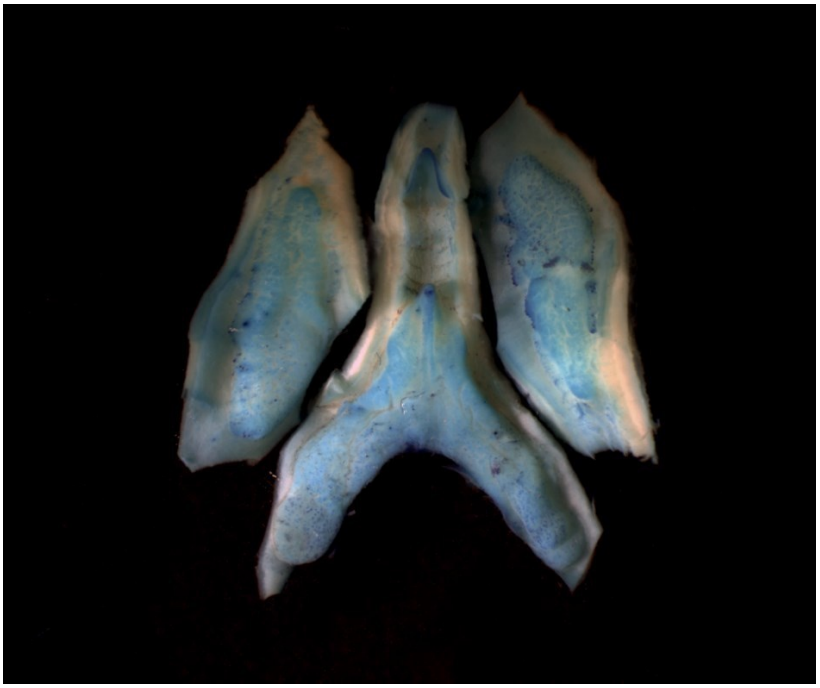


Fig. 86. The three parts of the funnel organ (and the funnel valve, see **fig. 88**) stained blue. The length of the *S. oweniana* dorsal mantle in formaldehyde was 38,8 mm.

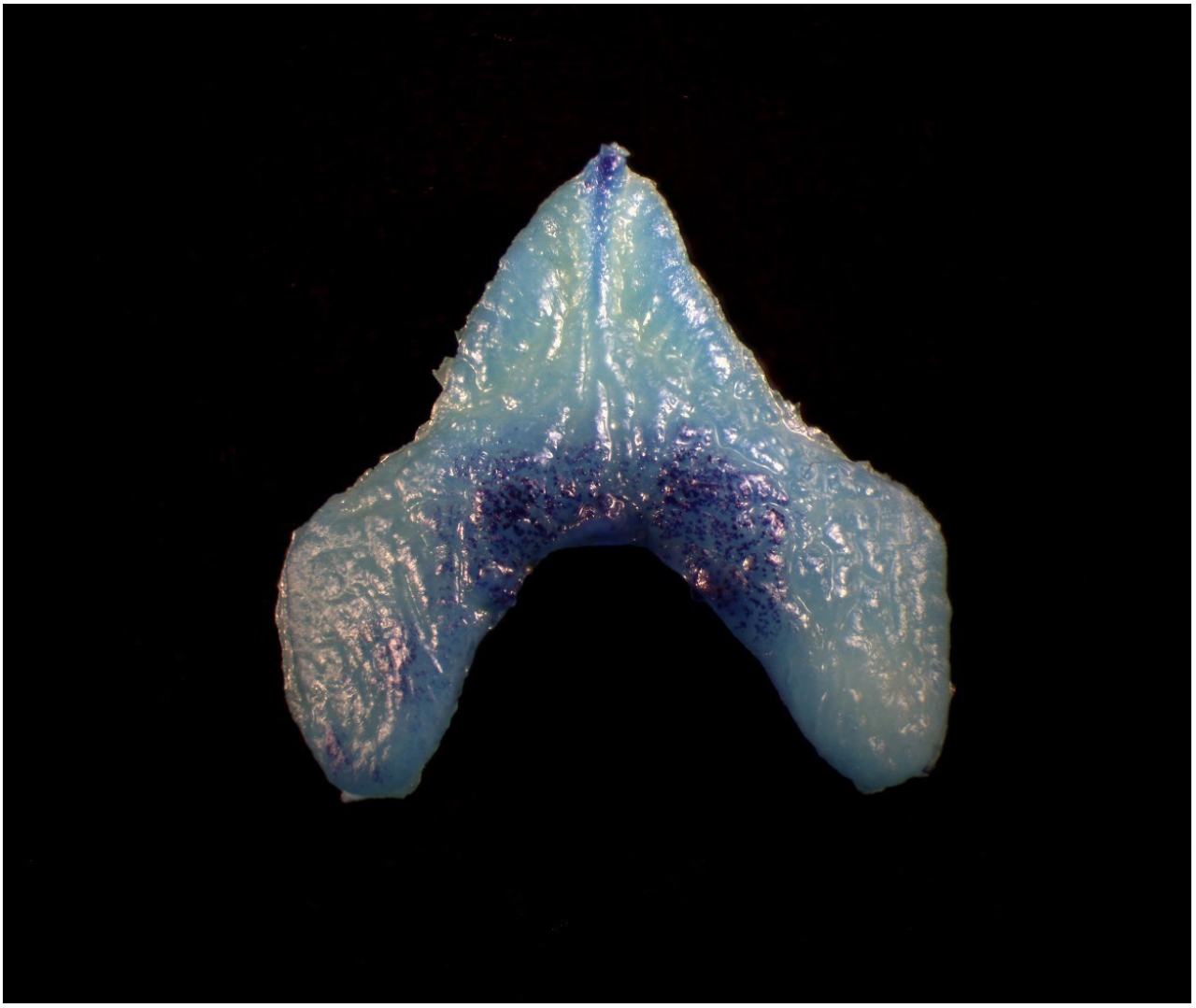


Fig. 87. A differently formed funnel organ in comparison to the one shown in **fig. 86**. The length of the *S. oweniana* dorsal mantle in formaldehyde was 30,4 mm.

When the funnel is cut open one can observe the flap like valve close to the funnel end (**fig. 88**). Some very interesting organs are the accessory nidamental glands which are ring like when undeveloped (**fig. 89**, arrows) and then develop to the shape shown in **fig. 75** and **fig. 90**. The function of these glands is briefly mentioned above (see “Reproduction biology”).

Fig. 88. The flap-like funnel valve close to the funnel end.

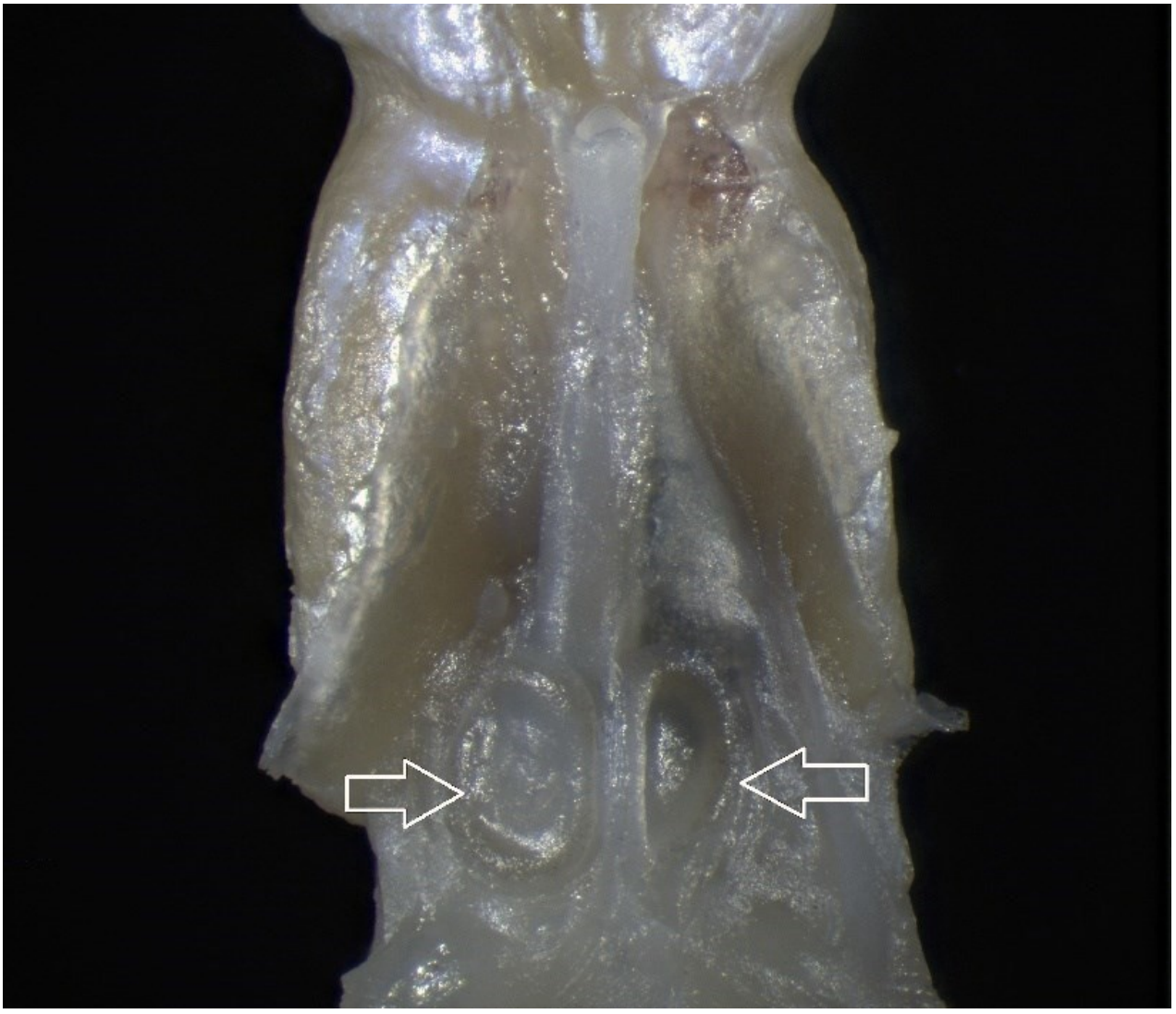


Fig. 89. The circular early accessory nidamental glands in a maturing female.

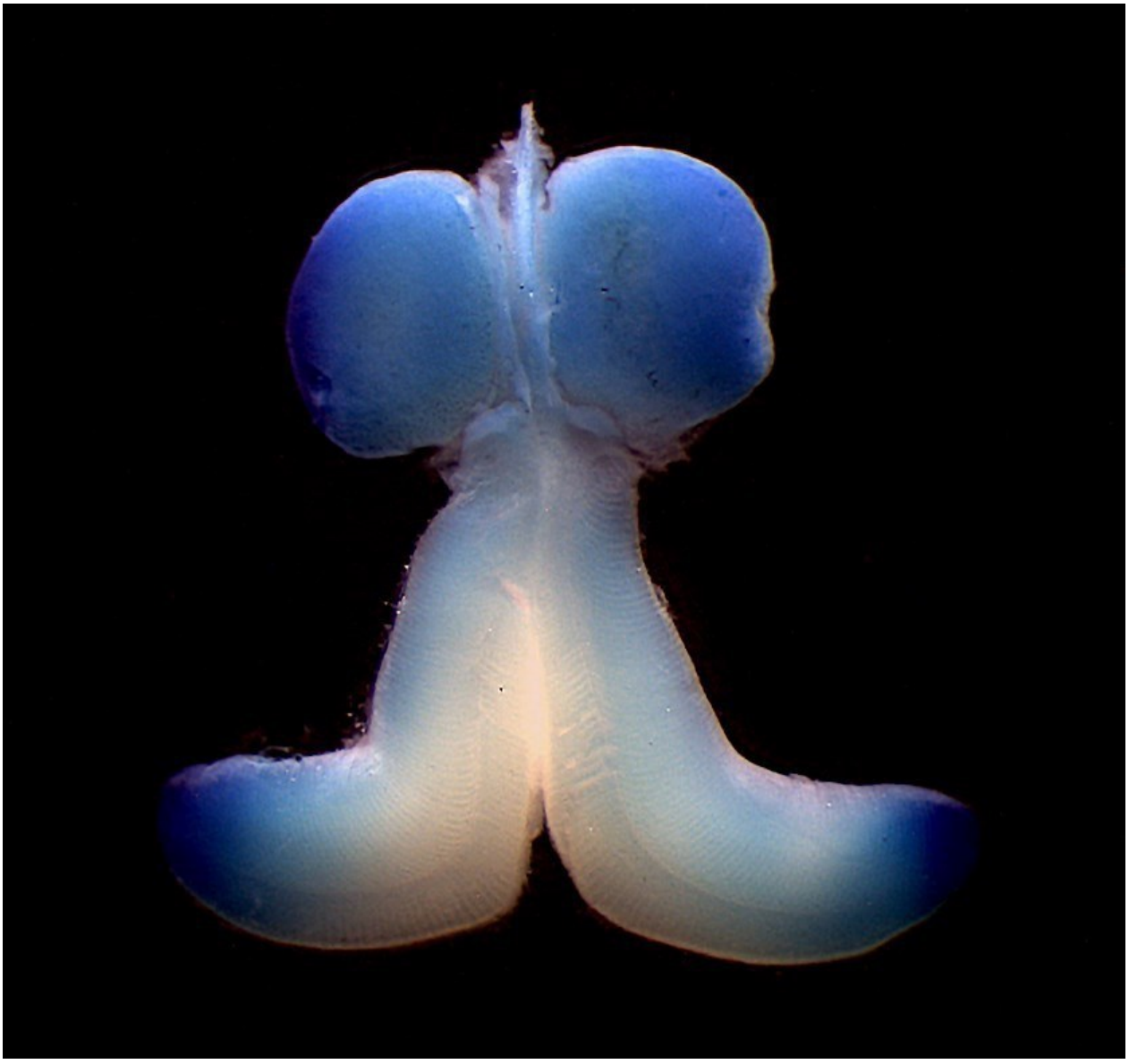
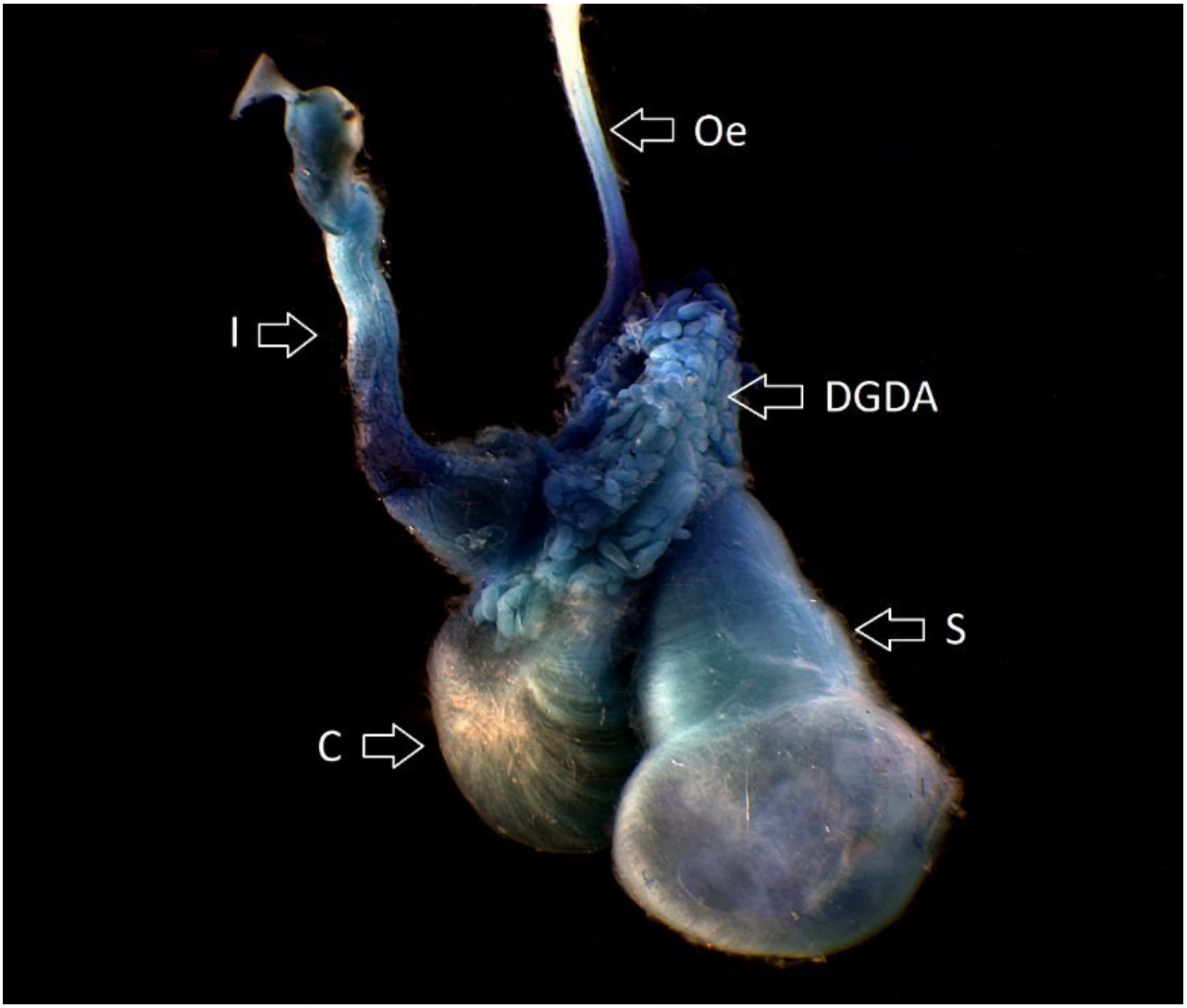


Fig. 90. The accessory nidamental glands above a pair of nidamental glands from a maturing female.

The digestive gland duct appendages (DGDA) of bobtails need to be better described (**fig. 91**). These small, whitish, oval digestive glands occur in high numbers around the digestive organs and are a challenge to dissect together in one piece. In **fig. 92** all the DGDA have been removed.



Page 95. Fig. 91. The digestive organs: oesophagus (Oe); digestive gland duct appendages (DGDA); stomach (S); caecum (C); intestine (I); the buccal mass and the digestive gland are not shown. One of the anal flaps is missing.

Fig. 92. The digestive gland duct appendages have been removed from the digestive organs.

The tentacle clubs with its suckers vary between species and are used for species identification. (**fig. 93**).

Fig. 93. Tentacle clubs with suckers. These clubs were not pinned.

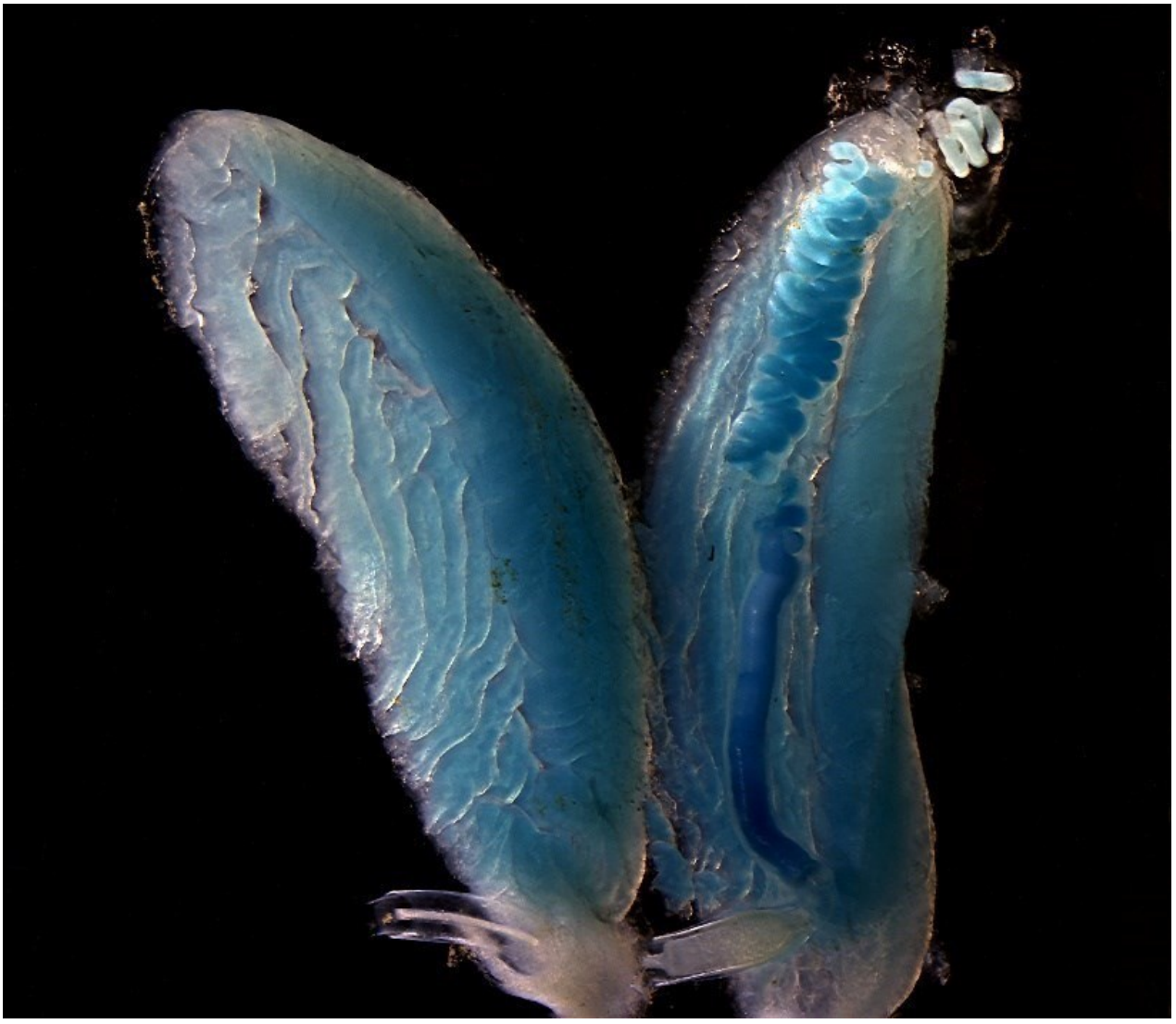


Fig. 94. One of the sac-like glands of the spermatophoric organ cut open with a spermatophore.

Exercise: In order to show how the different internal organs are positioned relative to the different muscle layers, other structures and cavities, one can cut the specimen in transverse sections, as shown in **fig. 95.** Make the first cut and take a photo; repeat the process until the posterior part of the specimen is reached. The wrinkled side of the bursa copulatrix is especially worth showing since it is in contact with sea water.

Exercise: Photo document the form of the Y-shaped funnel organ of different bobtail species and sizes.

Exercise: Make slide preparations of anal flaps and the flap-like funnel valve and compare different bobtail species and sizes.

Exercise: Describe the development of the accessory nidamental glands.

Fig. 95. Half of the posterior part of the brown digestive gland has been removed**.**

Exercise: Dissect the digestive gland duct appendages together in one piece (cut off all other organs). Describe their development over time and in relation to the development of the digestive gland.

Exercise: Pin the fresh tentacle clubs (see "Reference collection") in order to make them straight and take photos from different positions and magnifications. Test different dyes in order to increase the contrast of the suckers. Make 3D photos (see Appendix V).

Exercise: Look for small, less known, organs that lack good photo documentation (especially the early development of most organs may need documentation). One example of this is shown in **fig. 94** where one of the sac-like glands of the spermatophoric organ has been cut open to show a developing spermatophore inside it.

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Directive 2010/63/EU

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https://www.researchgate.net

https://www.sciencedirect.com

https://www.tolweb.org/tree (Tree of Life Web Project)

APPENDIX I: GLOSSARY

(Partly abridged and rewritten from Reid & Jereb, P**. (**2005) who give a comprehensive glossary with drawings)

Accessory nidamental glands - A pair of glands that are connected to the pair of nidamental glands

Anal flaps - A pair of papillae on each side of the anus

Arm - One of the appendages with suckers

Beak - Consists of two chitinous parts where the dorsal (upper) beak fits within the ventral (lower) beak

Buccal mass - A muscular bulb containing, e.g., the beak and the radula

Bursa copulatrix - An organ on which the spermatophores/spermatangia attach themselves and fertilization takes place

Bursa copulatrix duct (?) - A thick-walled duct, fused to the underside of the bursa copulatrix, connecting the oviducal gland to the edge of the bursa copulatrix

Caecum (cecum) - A digestive organ connected to the intestine near the stomach

Cephalic cartilage - A cartilage-like tissue that envelopes the posterior part of the brain and the statocysts

Digestive gland - A large, brownish gland that produce digestive enzymes

Digestive gland duct appendages - A high number of small, whitish, oval digestive glands connected to the digestive gland duct

Dorsal mantle length - A standard length measured along the dorsal midline from the start of the mantle (behind the head) to the posterior tip of the body

Egg - A fertilized ovum found for a short period of time inside the female prior to spawning and thereafter as plankton or on the sea bottom until hatching

Fin(s) - The pair of muscular flaps on each side of the body

Funnel - The large ventral tube through which water, reproductive and waste products and ink pass

Funnel organ - A flat glandular structure that consists of three parts fused to the inner surface of the funnel

Funnel valve - A small flap inside the funnel, close to its distal opening

Gill(s) - There is one gill connected to the inner mantle tissue on each side of the body

Hectocotylus - A modified arm of a male cephalopod used to transfer spermatophores to the female during copulation

Ink sac - The black, sac-like organ that produces and stores ink and is found along the lower part of the intestine into which it empties its ink, close to the anal flaps

Intestine - The portion of the digestive tract between the stomach/caecum and the anus

Lateral tooth structure(s)(?) - A pair of thin structures with teeth, close to the radula

Mantle - The sac-like structure covering the part of the body behind the head

Mature oocyte - The final oocyte stage of a female gamete before it becomes an ovum

Needham´s sac - A large male organ (also named the spermatophoric sac) for storing spermatophores

Nidamental gland(s) - One pair of large white female glands that are composed of numerous lamellae that produce a secrete for covering the eggs prior to spawning

Odontophore - A usually more or less protrusible structure in the mouths of most molluscs except the bivalves that supports the radula

Oesophagus - The portion of the digestive tract between the buccal mass and the stomach

Oocyte - A female gamete/sex cell that is not yet ready to be fertilized. The oocytes undergo several developmental stages

Ovary - A female reproductive organ that produces female gametes

Oviducal gland - A wrinkled gland between the end of the oviduct and the bursa copulatrix

Oviduct - A thin-walled duct in which oocytes are transported from the ovary to the oviducal gland

Ovum (pl. ova) - A female gamete/sex cell ready to be fertilized

Radula - The structure, with up to seven rows of teeth found in the beak. The radula is not straight but goes around the tip of the odontophore

Reticulated oocyte - A developmental stage of oocytes which is distinguished by its net-like pattern

Sperm duct (seminal duct) - The duct in males which joins the testis with the first gland in the spermatophoric organ

Sperm mass - A mass of spermatozoa within the spermatophore and the spermatangium

Spermatangium (pl. spermatangia) - An everted spermatophore (the spermatophoric reaction) from which sperm discharge

Spermatophore - A tubular structure that contains spermatozoa

Spermatophoric duct - The duct in males through which the spermatophores pass from the last gland of the spermatophoric organ to the spermatophoric sac (Needham`s sac)

Spermatophoric glands - The male, tubular and sac-like glands that make up the spermatophoric organ

Spermatophoric organ - The male organ where the spermatophores are produced, consisting of several tubular and sac-like spermatophoric glands

Spermatophoric organ complex - A unit formed by the sperm duct, the spermatophoric organ (consisting of several glands producing spermatophores), the spermatophoric duct, the spermatophoric sac (Needham`s sac), and the terminal organ

Spermatophoric reaction - The evagination of the spermatophore that takes place on the bursa copulatrix

Spermatophoric sac - The membranous organ (also named Needham`s sac) in males for storing spermatophores and directly connected to the terminal organ

Statocyst(s) - A sense organ for determining gravity, orientation, etc. that is embedded within the cephalic cartilage. There is one statolith inside each of the two statocysts

Statolith (s) - A calcareous stone inside the statocyst. The two statoliths can be used to estimate age (daily rings)

Stomach - The muscular organ of the digestive system with the function of primary digestion and food storage. Its size can increase considerably with food intake

Sucker - A circular suction structure found on arms and tentacle clubs

Tentacles - The modified pair of arms with suckers used for capturing prey

Terminal organ - A tube-like organ (directly connected to the spermatophoric sac) which stores mature spermatophores before copulation

Testis - A male reproductive gland that produces male gamete

Visceral mass - The soft, non-muscular metabolic region of the mollusc that contains the body organs

APPENDIX II: GRINDING STATOLITHS

In the chapter “Primary dissection” we show how to dissect and mount a statolith in thermo-plastic cement (TC) on a glass slide. Here we demonstrate how to grind the statoliths prior to the analysis, which is dealt with in the chapter “Secondary dissection and analysis”. **Fig. 96** shows the heater that melts the TC for embedding the statolith. Changing the objective from 0.5 to 1.0 or 2.0 X will decrease the focus distance and help fit the heater under the stereomicroscope objective. If this does not help one has to use a longer stand.

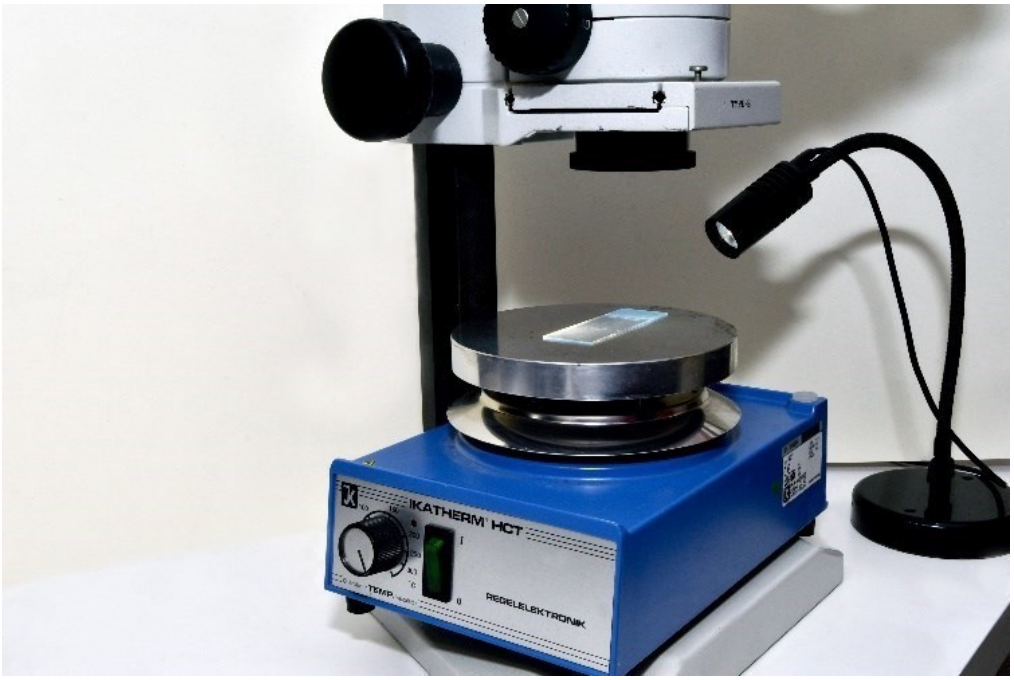

Fig. 96. A heater under the stereomicroscope objective.

A waterproof grinding box with 40, 12, 3 and 1µm 3M Imperial Lapping Film is shown in **fig. 97**. Saw two equally-sized pieces from a plastic cutting board and glue them together to form a block that fits into the grinding box. Cover the block with a damp Wettex (or any other soft material) on which the lapping films are placed. The water makes the lapping film stick to the Wettex making the film flat and neither too hard or too soft for grinding. Add tap water to the Wettex and the lapping film during the grinding process. Grinding by hand provides much better control compared with using an expensive grinding machine (often used for grinding large otoliths).

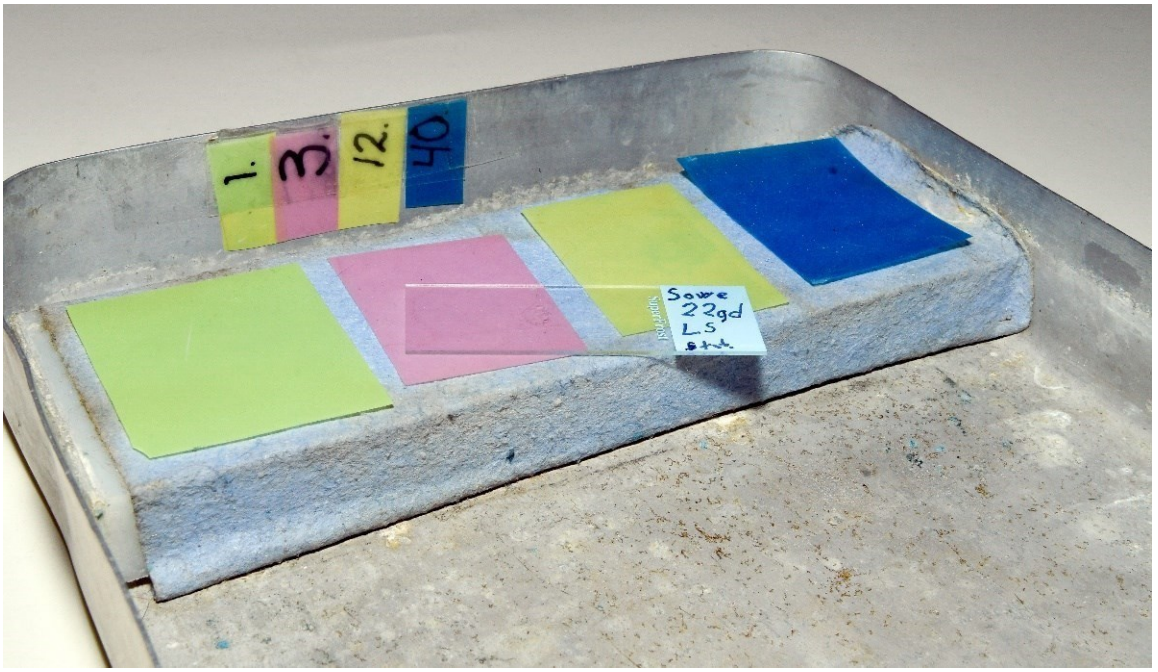

Fig. 97 A waterproof grinding box with 40, 12, 3 and 1µm 3M Imperial Lapping Film for grinding statoliths/otoliths.

Once the statolith is embedded in the TC on a glass slide one should wait at least one hour before grinding which should then be done in a circular fashion without pressing hard on the glass slide, as this could destroy the statolith. Check that the flat side of the statolith is uppermost and horizontal. Start grinding down the flat side almost to the centre and repeat this with more statoliths (to save time). Then melt the TC under a stereomicroscope and turn the statoliths over, ensuring that their convex side is horizontal and covered with a thin layer of TC. After one hour the convex side can be ground, checking in the microscope how close one gets to the centre of the statolith. At this point the statolith is very thin and fragile and the rings have become visible. The challenge is to stop grinding once both the inner rings, including the hatch ring, and the outer rings can be observed clearly. Melt the TC again and cover the statoliths with a thin layer (using a tungsten needle) which makes the ring more easily visible. At this point, take sharp photos of the rings from the centre to the edge of the statoliths for later ring counts and analysis. See the chapter on “Secondary dissections and analyses” for the analysis of statolith photos.

APPENDIX III: EQUIPMENT

An optimal work environment is a prerequisite for fast and good quality dissection. It is very important to adopt a proper sitting position (neck straight, shoulders down) and that the dissection room can be made dark during photography and fluorescence microscopy. Make sure that the microdissections can be carried out without disturbance. There should be plenty of space on the laboratory bench for a computer screen, a keyboard, low and high magnification stereomicroscopes, lighting systems, dissection tool holders, boxes for Petri dishes, vials and dyes, etc. A camera mounted on each microscope is advised in order to save time and also to avoid problems from dirt and dust inside the camera that can arise when moving it from one microscope to another.

If fluorescence imaging is needed, then NIGHTSEA® Viewing Systems is a competitive solution (**fig. 98**). This system is used primarily with stereomicroscopes, but works for inverted and other types of microscopes. We used their RB Royal blue filter, excitation 440 - 460 nm, emission 500 nm LP, for viewing oxytetracycline stained statoliths (**fig. 40**). The alternative on/off/dim switch enables one to modify the excitation intensity which is useful when viewing, e.g., stained statoliths since it can decrease blur and increase ring contrast. It is best to have two light sources in order to achieve optimal lighting from different directions. The second light source could also be used with the NIGHTSEA mobile fluorescence kit (**fig. 99**) which is very convenient when observing larger objects. Various fluorescent light sources are available.

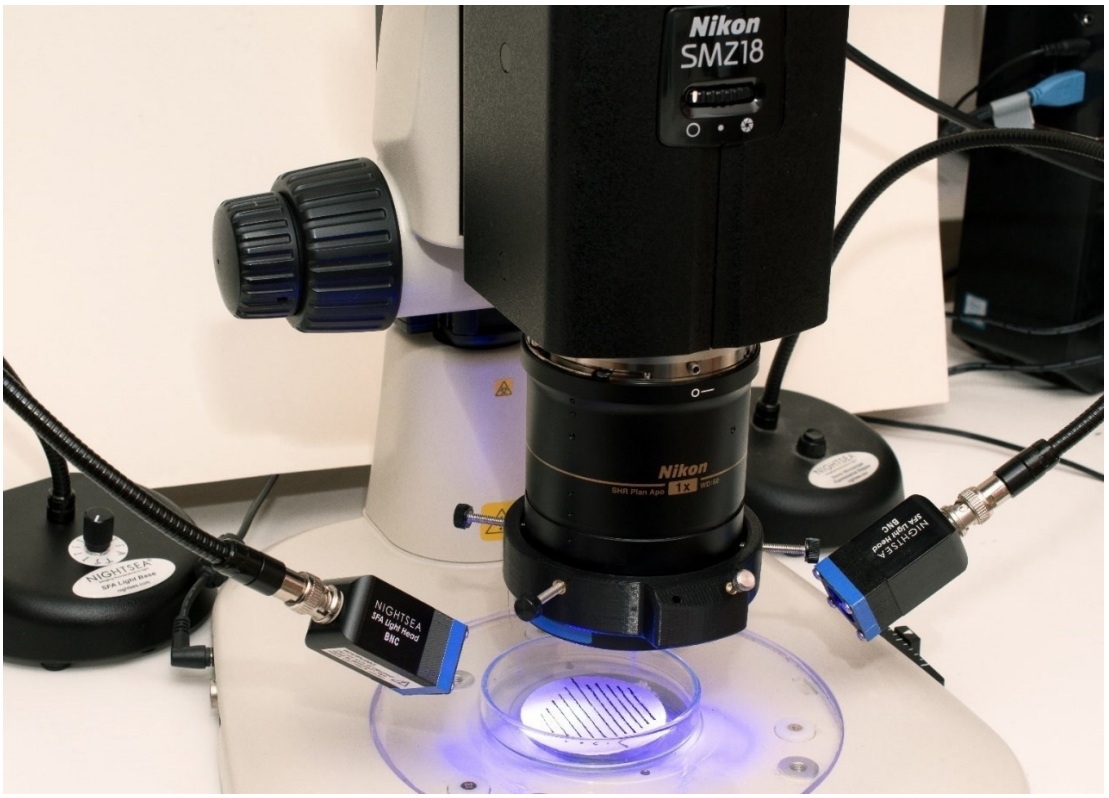

Fig. 98. The NIGHTSEA® Fluorescence Viewing System.

Fig. 99. A cephalopod viewed using the NIGHTSEA mobile fluorescence kit. Do this in a dark room.

Fluorescence can be extremely valuable for quantitative epibiont/parasite studies: a simple but effective method is given in Øresland (2019). Note that aldehydes in fixatives can induce fluorescence which may, or may not, be useful (Suvarna, S. et al., 2019). Fluorescence is also effective in the detection of surface microstructures and patterns that might be helpful for species identification and morphological studies. It is advisable to test all available fluorescent light sources and potentially good dyes (see Horobin & Kiernan, 2002; Suvarna, S. et al., 2019).

APPENDIX IV: MICRODISSECTION TOOLS

Most microdissection tools are worth the cost, if one cannot make them oneself. One example of a good purchase, even though expensive, is vitrectomy scissors. Tools should be well looked after and dissection tool holders utilized; small scissors should be kept in their original boxes. Use tweezers with the finest tips only when necessary and use plastic tip protectors. Always clean and sharpen tools after use, paying special attention to the small scissors.

Microscalpels

Even small scalpel blades are often still too large and have the wrong shape for microdissection, though they can easily be modified to meet specific needs. Use a small "pen" diamond grinder to shape the scalpel blades under a stereomicroscope (**fig. 100**). A cheap alternative is a hobby grinder with batteries. Protect the objective by taping a Petri dish to it. Blades should be sharpened under a stereomicroscope, in surgical instrument oil, on a diamond stone, a whetstone, a Black Arkansas oilstone and finally on Wettex and hard-rolled paper (**fig. 101**).

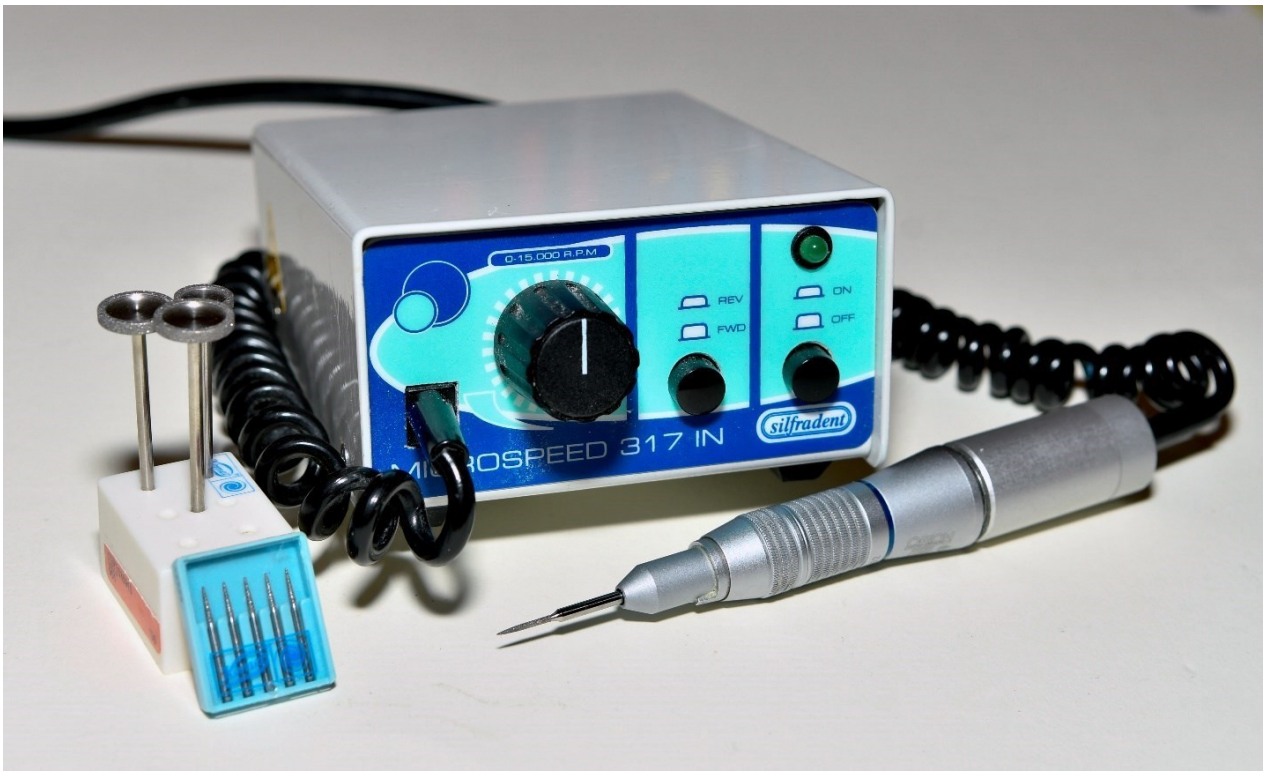

Fig. 100. A "pen" diamond grinder.

Fig. 101. Oil and sharpening tools. Note that the final sharpening is made on paper.

Needle scalpels

A short scalpel blade on a long and sometimes bent shaft is needed occasionally in order to make cuts deep in between or behind organs (**fig. 102**). Such scalpels can be made from high-quality sewing needles, choosing those with a diameter somewhat greater than the width of the blades. Cut off the needle close to its eye, ensuring that it fits into a needle holder.
Use a small diamond grinder to shape the needle blade and sharpen it under a stereomicroscope, as described above. Do not forget to protect the objective. The last step is to heat the needle over an ethanol flame until it becomes red hot. Using pliers, bend the needle into the required angle and finally cool it down in oil.

Needle scalpels usually stay sharp even after long use. Keep a variety of needle scalpels available in a dissection tool holder since they take several hours to make. They are worth the time and effort since the cost is negligible and they do a good job as each one is designed for a specific purpose; above all, they cannot be purchased ready-made.

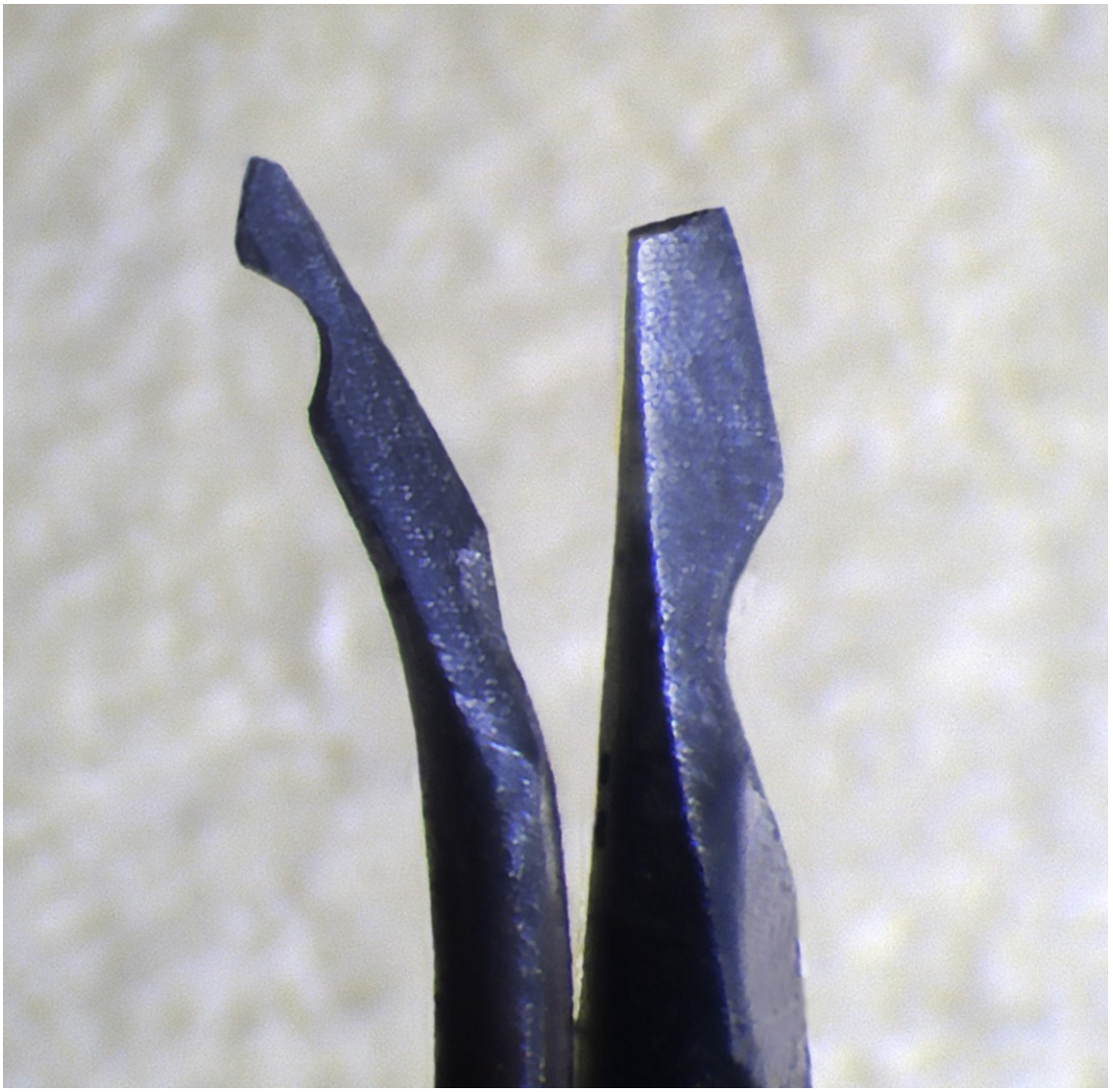

Fig. 102. Needle scalpels in different sizes and shapes.

Tungsten needles

Needles made from tungsten wire of different diameters are extremely useful since they do not bend much, can easily be sharpened and the cost is negligible. Tungsten wire with a diameter of 0.1, 0.2 and 0.3 mm covers most needs. Use wire holders with a hollow shaft that can accommodate a 10 cm long wire and leave approximately 15 mm of the wire protruding outside. The tungsten wire is sharpened by dipping it into a thin, V-shaped lab spoon containing sodium nitrite ($NaNO_2$) melted over an ethanol flame **(fig. 103)**. Avoid overheating and use safety glasses. Remove any white oxide between your wet thumb and forefinger and check the degree of sharpness under a stereomicroscope. If needed, a hook can be formed by letting the wire gently touch the lab bench. Plastic handgrips of different colours can be placed on the needle holders to differentiate various diameters (**fig. 104)**. Oil the needle holder cap so the wire can easily be drawn out at little for sharpening. This way of sharpening tungsten wire is easier, faster and cheaper than other methods.

Fig. 103. Sharpening of a tungsten wire in melted $NaNO_2$.

Fig. 104. Keep all dissection tools in separate Plexiglas® holders (or original boxes**)** that can be placed close to the microscope. Reserve one or two holders for tools that need to be sharpened.

Diathermy instruments

We have obtained good results in testing different diathermy instruments, especially for cutting off thin membranes (fig. 105), though we could not use them for cutting the mantle (scalpel cuts would, in any case, be quicker). Diathermy is the use of high frequency alternate polarity radio-wave electrical current to cut or coagulate tissue during surgery. In cutting mode, the electrode reaches a high enough power to vaporise the water content, hence it is able to perform a clean cut. It is advised to read the safety instructions especially regarding potential effects on electrically active implantations and to obtain a safety check of second-hand instruments. Some instruments have foot pedals which is an advantage.

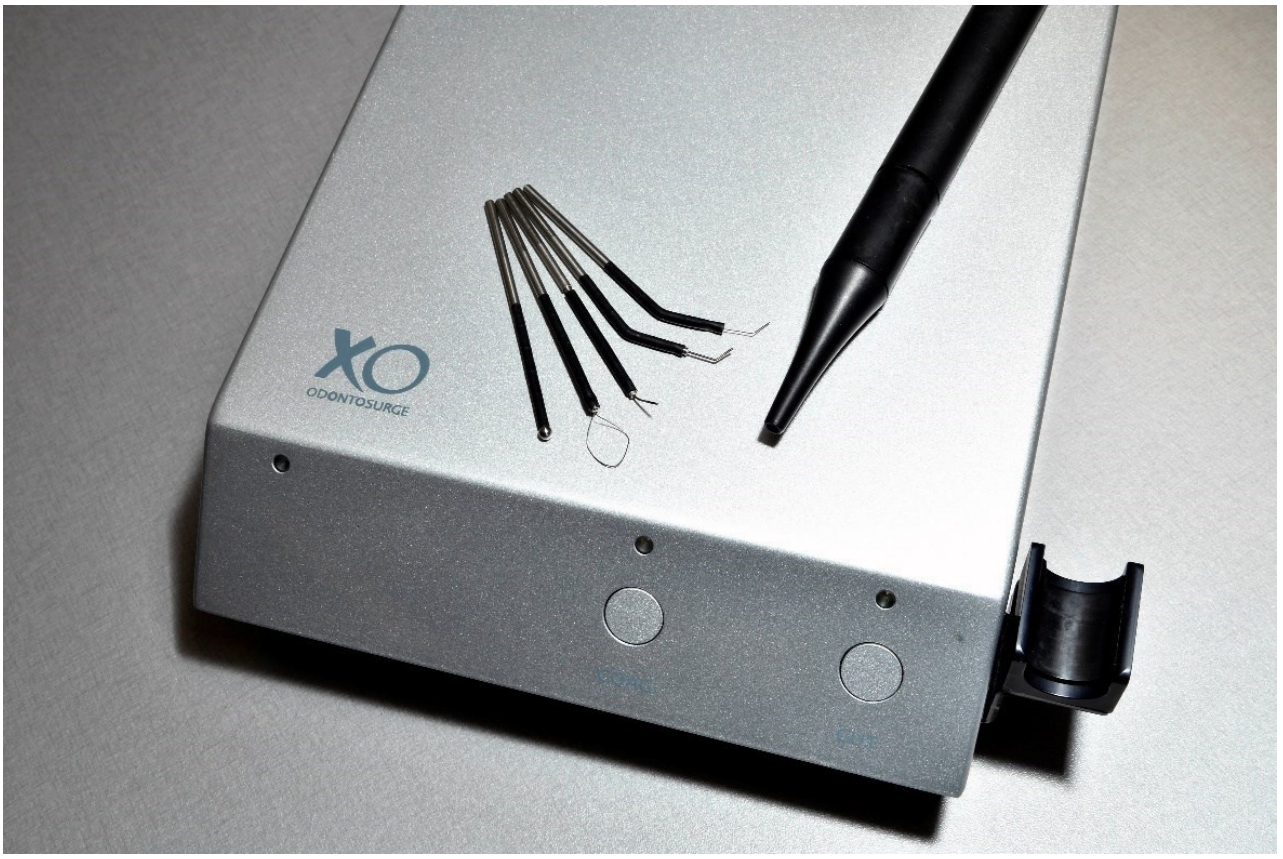

Fig. 105. This diathermy instrument has different electrodes and cuts membranes well.

APPENDIX V: PHOTO TIPS

- It is crucial that the photos of specific organs are taken from standard directions and at the same magnifications to facilitate comparison of different individuals, species and developmental stages/sizes/ages.

- If one uses a stereomicroscope with high magnification it can be difficult to move the glass slide with the precision needed for photography. An inexpensive solution is to cut a 10 x 10 cm piece of Plexiglas® and cut a square opening in its centre, on which one places a glass slide or Petri dish so that an object can be illuminated also from beneath (**fig. 106**). Since the Plexiglas® piece can be moved in all directions it is better than using an XY stage. The square opening prohibits reflexions (which may happen when one uses a glass plate).

- The choice of background colour is important, with black most often providing the best contrast, although obtaining a totally black background can be difficult. Water or ethanol between the Petri dish and the black bottom plate can help considerably. One can also print out a glossy photopaper in black and, after removing the bottom plate, place the paper under the stereomicroscope so that some distance between it and the object is achieved. Replace the bottom plate with the cut Plexiglas®. A white/grey background often provides the best contrast for dark or stained specimens, although one disadvantage is that it can show up the shadows more easily than a black one. Use a white paper and the same method as described above for black background so that the shadows will disappear.

- Uneven lighting, shadows and light reflexions are bothersome but can usually be avoided by using two to four light sources (preferably with separate controls), placing them around the object for an optimal result. A ring illuminator could also be used, although this is not always sufficient unless it can be regulated/shaded in different directions. Plastic beakers and bottles can be cut and used to modify the light in a number of ways (**fig. 106**). Use black markers to darken certain areas so that light can be limited. Dry the object with lens paper so the water does not reflect light. Often it is preferable to cover the entire object with filtered or distilled water to avoid reflexions. Use water that has been boiled for 10 minutes to avoid air bubbles on the object, and ensure that Petri dishes are scratch free.

- If one needs the photos to be completely sharp, one should choose a programme with extended depth of focus (EDF) also known as focus stacking or Z-stacking. These are quick and easy to use. However, sometimes one may wish to focus on a certain detail only, leaving the rest out of focus.

- Dirt and other unwanted particles are annoying. One can use, e.g., photoshop to remove dirt and reflexions but this takes time and might not be worthwhile unless the photo is to be published.

- Small lead supports can be put underneath an object that would not otherwise stay in the correct position for photography, or one could use a tungsten needle fastened to the bottom of a Petri dish or a piece of thick rigid polyurethane foam (very useful for 3D photography).
- The colours observed in microscopes can often differ from those in the photos. While the object is still under the microscope one can adjust the lighting and use a photo software programme to obtain more realistic colours. If describing the various shades of a colour is difficult, one should use terms such as greenish, whitish, etc.

- Objects can be viewed in 3D using 3D-software and an oblique viewing module (e.g., from Wild Heerbrugg (**fig. 107)**, or Opto, https://www.opto.de/) that is mounted under the stereoscope objective. Such modules have a variable or a fixed 30-degree view and can be rotated. The object in question should be mounted on a tungsten wire or insect needle that can be bent so that photos or videos can be taken from different angles. A disadvantage of rotating the module is that the light and background could change. In order to avoid this happening, one can make a circular platform that can be rotated whilst keeping the object centred and in focus. Maintain the same background by placing a piece of black metal behind the object and the platform (**fig. 107**). One of the arms with its suckers would make an useful test object.

- The 3D techniques are undergoing rapid development. One can also obtain 3D views via a range of other, more expensive, methods such as rotational SEM, laser technology, magnetic resonance imaging, etc., (see Xavier et al., 2015; Ziegler, et al., 2018). One should not forget that 3D printing could be useful, especially for teaching purposes. Digital 3D imaging can be found on data bases on the net (see e.g., Smithsonian 3D digitization) and in digital publications.

- Turn off all lights in the laboratory and use blackout curtains during photography and fluorescence work.

*Exercise**:*** Make an apparatus to estimate the volume of the organs (e.g., by water displacement), or use 3D software. Combining volume, mass and 3D shape might be useful when studying and comparing organ density (grams per cubic centimetre), growth and development with specimen size/age in different species and from different areas/habitats. The digestive gland is an easy organ to start with.

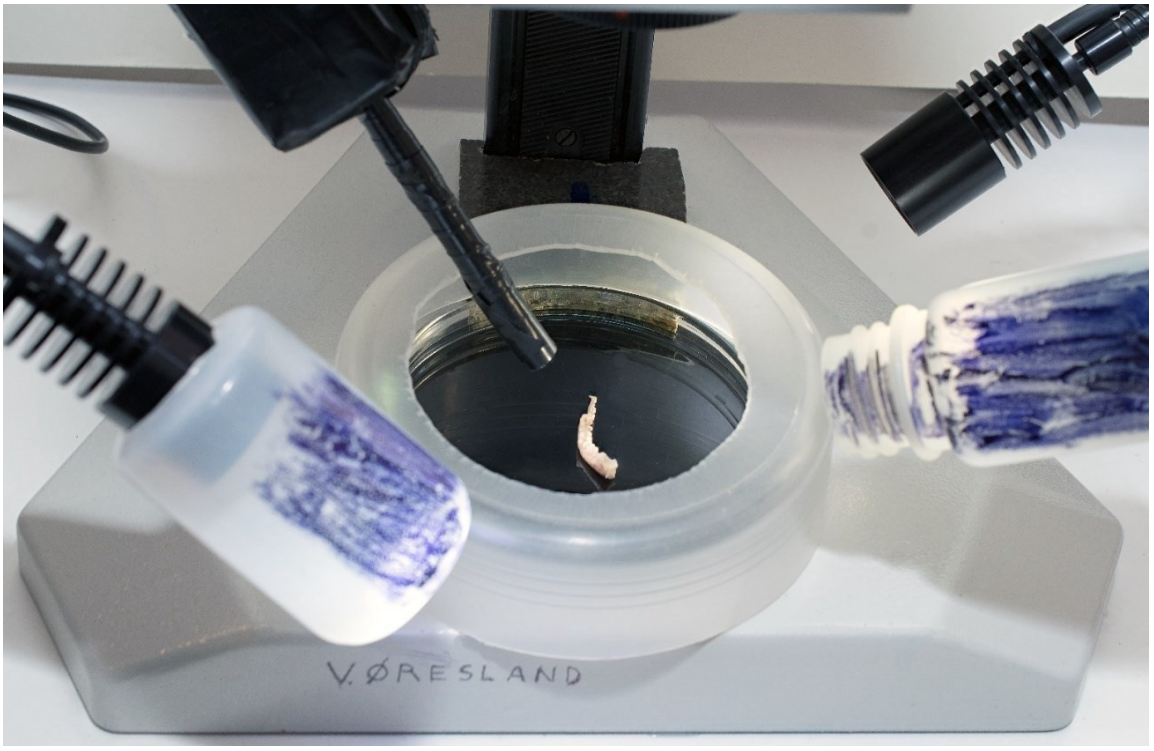

Fig. 106. Plastic beakers and bottles can be cut to fit the light source or surround the object. A Pasteur pipette and black electrical tape can be used to make different-sized spotlight which is useful to light up hollows.

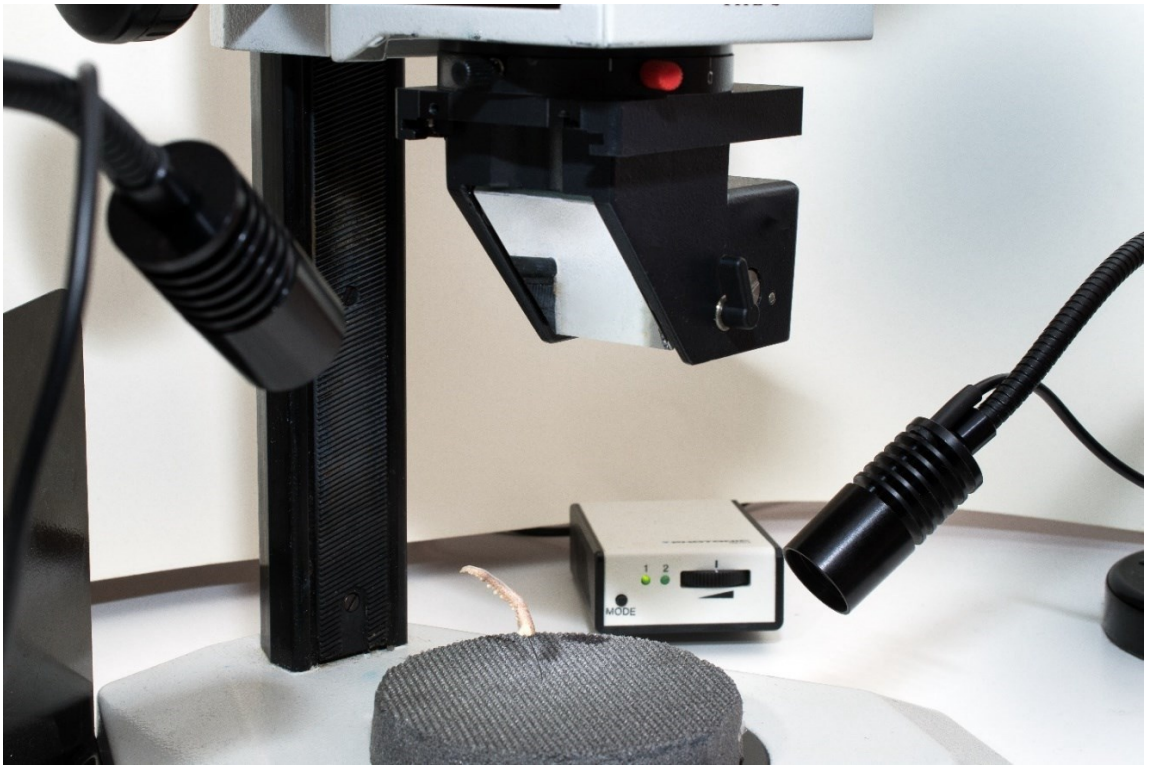

Fig. 107. A variable oblique viewer (type 404233) by Wild Heerbrugg. To the far left is a black metal background.

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SI TECH is a Swedish company focusing on development, manufacturing and marketing of innovative safety solutions for protective suits such as; drysuits, rescue suits and similar protective garments. SI TECH is rooted in the diving industry and this is still the company's core market.

NIGHTSEA develops practical, economical solutions for viewing and imaging fluorescence, for the lab and the field, both above and below water. The flagship product is a simple system that adapts any stereo microscope for fluorescence work in seconds, with 6 different excitation/emission combinations available. Variants of the system can be used with inverted microscopes and several brands of digital microscopes. NIGHTSEA products are widely used in academia for research and education, in industry, and in many other diverse applications.