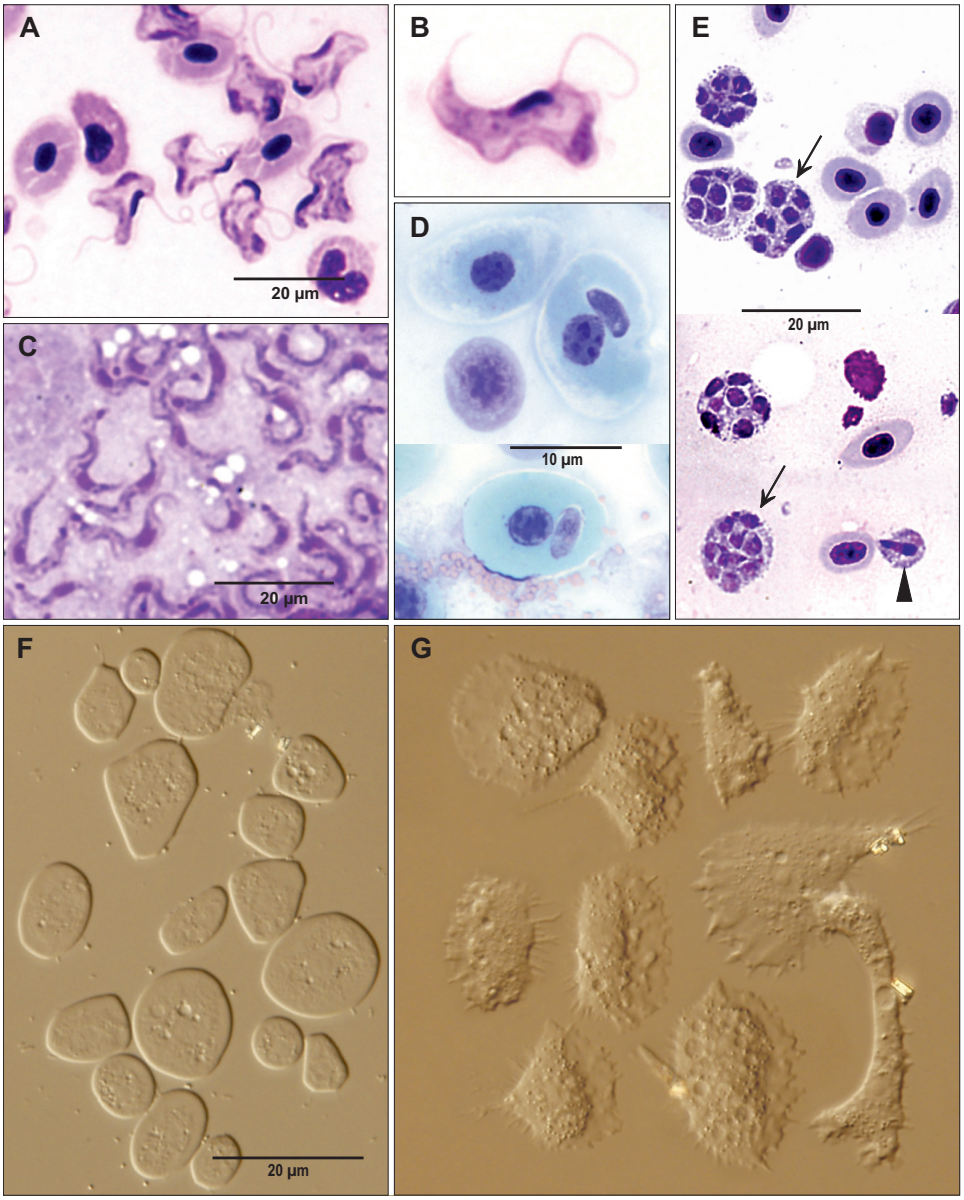

Fig. 3.3.1.3. A, B. *Trypanoplasma borelli* Laveran et Mesnil, 1901 stained with Giemsa. **C.** *Trypanosoma carassii* Mitrofanov, 1883 in Giemsa-stained blood smear. **D.** Intraerythrocytic stages of *Haemogregarina* sp. fixed and stained with Diff Quick. **E.** Proliferative stages of myxosporeans in Giemsa-stained blood smear. **F, G.** Trophozoites of an identical *Flabellula* strain seen under coverslip (**F**) and in hanging drop preparation (**G**). Scale bar F applies also to G.

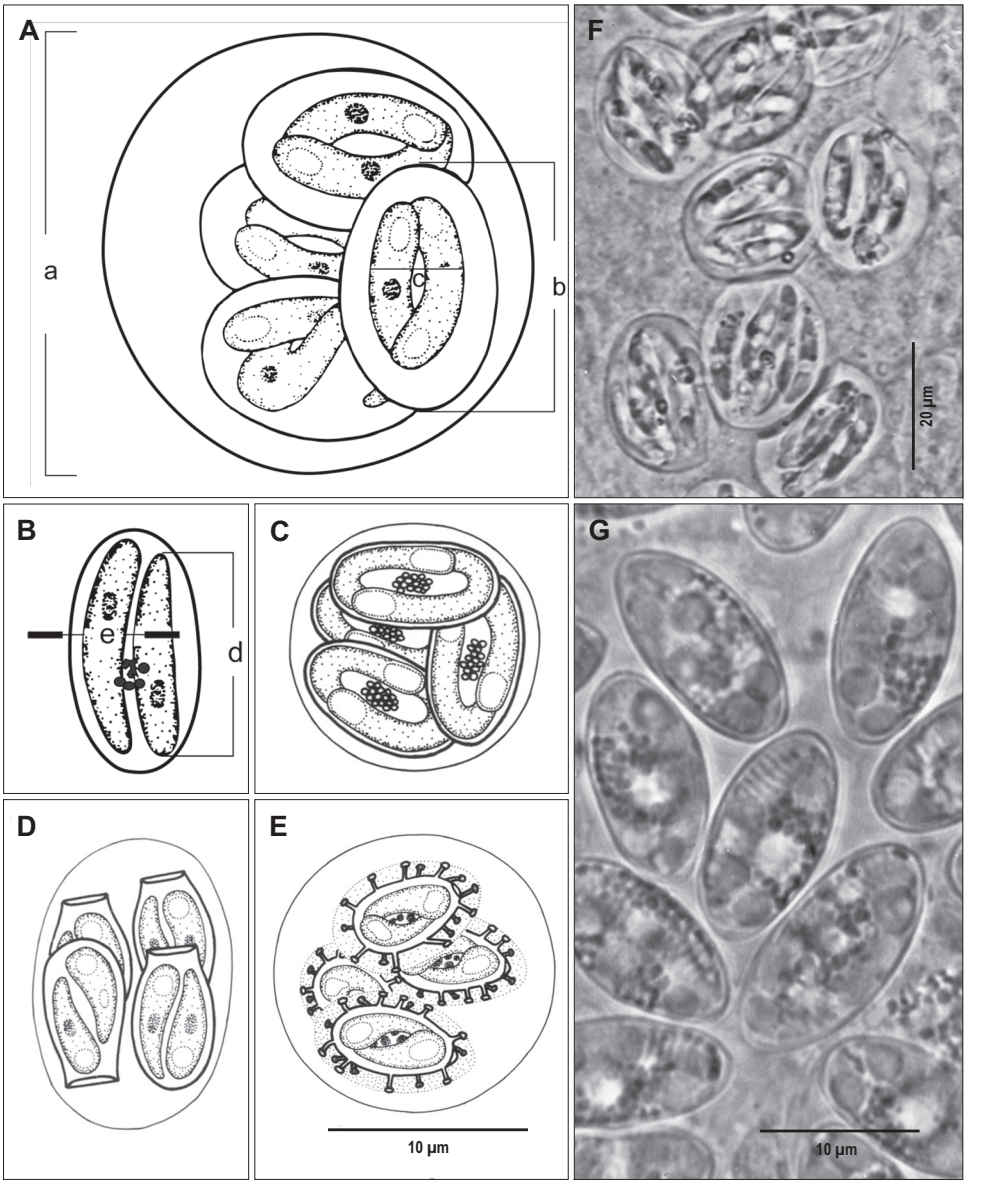

Fig. 3.3.1.4. Coccidia. Spherical oocysts (**A**), diameter (a), (length and width are measured in ellipsoidal oocysts), length and width of sporocyst (b and c, respectively). **B.** Sporozoite measurements (d, e). **C.** Oocyst of *Goussia carpelli* (Léger et Stankovich, 1921) contains sporocysts with residuum body. **D.** Oocyst of *Eimeria rutile* Dogiel et Bychowsky, 1938. **E.** Sporocyst walls of *G. deguisti* (Molnár et Fernando, 1974) bear projections (sporopodia). Scale bar E applies also to C and D. **F.** Sporocysts of *G. leucisci* (Shulman et Zaika, 1964). **G.** *G. subepithelialis* (Moroff et Fiebiger, 1905).

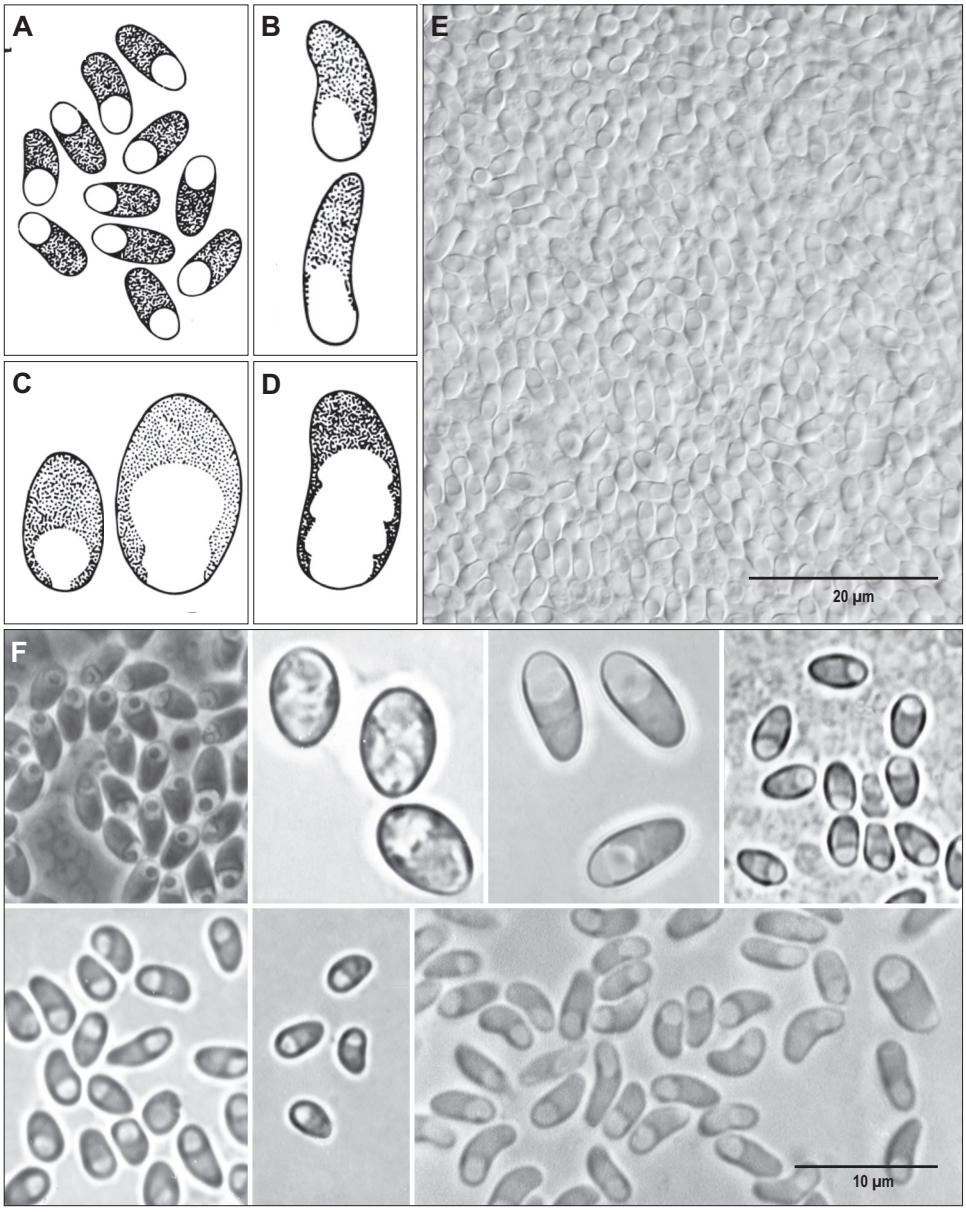

Fig. 3.3.1.5. Microsporidian spores observed in light microscope and documented in fresh state. In spores of fish-infecting species often contain conspicuous vacuole. **A-D.** Line drawings of *Microsporidium* sp., *Glugea* sp., *Pleistophora* sp. and *Heterosporis* sp., respectively. **E.** Fresh spores of *G. anomala* (Moniez, 1897). **F.** Photomicrographs exemplifying vacuoles seen in fresh smear and size differences of microsporidian spores belonging to various genera.

Screening procedure for EMs

Examination of the external surface: skin, fins, nasal pits and gills

Due to the loss of ectoparasites during capture and transport of live fish to the laboratory, the external examination is the most problematic part of the screening for the presence of EMs. Care has to be taken to preserve the outer surface of fish in an undisturbed condition.

PROCEDURE

1. Remove fish from the water using a small dip net and in accordance with relevant national legislation.
2. Scrape mucus from the skin and gills, using a coverslip, either while the fish is still alive or after pithing (anaesthesia is not recommended for the purpose of external examination as it may affect skin parasites).
3. Scrape the gills gently to prevent excess blood in the sample.
4. Spread mucus obtained on a slide and examine the fresh/wet mount for the presence of ectoparasites, at 40x to 1000x magnification (screen large area at low magnification first, then magnify; fix with methanol and store one smear for detailed observation if necessary).
5. Examine scrapings from both sides of the body, fin bases and the belly because the distribution of ectoparasites on the host may not be uniform.
6. Inspect also scrapings from the inner sides of the gill opercula as well as samples from the nasal pits, a special niche for some EMs.
7. Examine macroscopic, cyst-like structures or haemorrhagic areas following the detailed instructions given below.

Blood sampling, detection of blood parasites in fresh blood, blood smears

Venipuncture is the best method to withdraw blood from small fish (immediately after euthanasia). Blood is collected with a heparinised syringe inserted directly into the caudal vein in the area of the peduncule. Samples can also be used for blood chemistry, immunology, etc. Clotting time for fish blood is much shorter than for mammalian blood so always rinse syringes with heparin before use. Haemoflagellates and mobile proliferative blood stages of myxosporeans make themselves apparent by their vigorous movement in fresh blood mounts. Blood flagellate infections of extremely low intensity can be detected if several ml of blood are allowed to clot in a centrifuge tube placed overnight in a refrigerator or by using a haematocrit centrifuge. The following day, the flagellates can be found wriggling in the serum above the blood clot (tube)/compacted cells (haematocrit tube) while myxozoan blood stages occur intermixed with fish leukocytes (top layer after centrifugation). If necessary, the haematocrit tube is cut immediately above the compacted cell layer and the material transferred to a slide, using a micropipette.

PROCEDURE

1. Collect blood from the caudal vein with a heparinised syringe (the size of the needle should correlate with the size of the fish); the needle has to pass through the skin and muscles until it enters the vessel just below the spine.

2. Prepare several blood smears prior to examination of a drop of fresh blood under a coverslip (at a 400x magnification); stained blood smears are a prerequisite for detection of haemoflagellates, haemogregarines and proliferative stages of myxozoans.

3. Stain smears with Giemsa or Diff-Quik for subsequent detailed microscopical examination.

Examination of internal organs and muscle in fresh mounts

PROCEDURE

1. Inspect the internal organs after the body cavity has been opened by an incision made ventrally from the anal opening extending forward to beneath the heart, followed by the removal of one side of the body wall.

2. After macroscopic inspection of the organs, examine fresh mounts (see below).

3. Compress a piece of tissue about 1-2 mm in diameter between slide and coverslip; the coverslip is pressed after placing another slide on top to exert an even pressure over the whole coverslip, then it is removed.

4. Examine the samples under a compound microscope, first at a 100x magnification and then magnifying to 1000x; the number of samples examined from each organ depends on the size of the organ inspected.

5. During routine examination, include gill filaments, liver, spleen, kidney (*i.e.*, trunk kidney and head kidney), gonads, heart, swim bladder, the gall and urinary bladders and their contents (see point 6), muscle and brain.

6. Collect a sufficient quantity of bile and urine from the respective bladders (glass pipette) into a small vial and then examine several drops only for the presence of parasites (thus the bulk of material, if positive, is saved for further processing).

7. Cut open the digestive tract, separate its contents from the tissue and examine scrapings of the stomach, anterior, middle and posterior intestine (and from pyloric caeca, if present).

8. Examine also the rete mirabile at the back of the eyeball.

Examination of organs by histology

Simultaneously or prior to the examination of fresh mounts (squash and scrape preparations) tissue samples should be fixed to ensure adequate structural fixation for histological examination of fish organs infected with EMs (see also Chapter 4.4.). We recommend Davidson's as the best fixative for a well-defined

cell architecture in histological sections. However, neutral buffered formalin is also good and, furthermore, allows for parasite DNA detection by *in situ* hybridisation. A guide to the identification of fish protozoan and metazoan parasites in stained tissue sections is available from: https://www.researchgate.net/publication/6911910_Guide_to_the_identification_of_fish_protozoan_and_metazoan_parasites_in_stained_tissue_sections.

Storage of fresh materials

Since the study of living EMs is time consuming and may interfere with the examination of fish brought into the laboratory for routine necropsy, it can be postponed in some cases by storing the fresh sample for later examination. Fresh mounts can be stored for some time in the refrigerator, either in a wet chamber (containers holding slides and some moist tissue) or if the edges of the coverslip are sealed to the slide with nail varnish. Myxosporean or microsporidian spores can be stored in distilled water at 4ºC for up to 12 months.

To prevent bacterial growth, the amount of host tissue debris in the sample should be kept to a minimum. 'Clean' spores from large 'cysts' can be recovered by puncturing cysts with a capillary tube. Small cysts can be separated from the surrounding tissues using dissecting needles or scissors. They may then be teased open and crushed, releasing the spores, which can then be stored for a limited period of time. As an emergency measure, myxosporean spores collected during long field trips can be studied in a preserved state, either fixed in 10% neutral buffered formalin, or in semipermanent mounts, *e.g.*, glycerol gelatine or glycerine ammonium-picrate.

Storage of material for extraction of DNA

The introduction of DNA-based taxonomy has advanced the identification of EMs as well as the understanding of their phylogenetic relationships. Molecular taxonomy and phylogeny have become an integral part of the EM research. The fixatives used for morphology/histology frequently damage DNA. The negative effects of formalin can be partly reduced if a neutral-buffered formalin solution is used instead of unbuffered or acidic formalin solutions; nevertheless, extraction of good quality DNA cannot be expected, especially after a long-term formalin fixation. Ethanol (95% or higher concentration) is routinely used for DNA preservation. The ratio of any fixative to sample should be at least 10 : 1 to ensure optimal fixation.

Identification of EMs detected and description of new species

The information collected from fresh mounts is of paramount importance; however, the organisms detected in fresh mounts can usually only be assigned to some of the major groups of fish-infecting EMs. The morphology of some of them allows assignment to a genus. Species identification and description of new species require detailed study using methods specific to each organism group, including molecular analyses (see Table 3.3.1.1 and references).

Table 3.3.1.1 Survey of basic methods used in the identification and description of species of EMs

Group	Principal method for morphology	Staining of smears/sections	Additional desirable techniques	DNA-based identification
Ciliates	fresh smears	Giemsa, silver nitrate, protargol	SEM[1], culturing	18S rDNA COI
Blood flagellates	stained slides	Giemsa, Diff-Quik	culturing	18S rDNA gGAPDH
Haemogregarines	stained slides	Giemsa, Diff-Quik	-	18S rDNA
Amoebae	hanging drop (live)	-	TEM[2], culturing	18S rDNA ITS
Coccidia	fresh smears	Giemsa, Diff-Quik, Gram	flotation method	18S rDNA
Microsporidia	fresh smears, TEM sections	PAS, Gram	-	18S rDNA ITS
Myxozoa	fresh smears	Giemsa, Diff-Quik, Gram	-	18S rDNA

[1] Scanning electron microscopy; [2] Transmission electron microscopy

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3.3.2. MYXOZOA

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Introduction

The present text is focused on myxozoan parasites of freshwater fishes, which can be found on the host surface as well as in internal organs. It is necessary to examine fresh fish individuals because, if dead, the host's tissues that potentially harbour parasites undergo fast degradation and parasites become unsuitable for subsequent studies, especially for transmission electron microscopy and histology. Data on the host species, sampling locality (if possible with GPS location), sampling and fish dissection date, the collector's name, fish condition (alive/dead), and fish total and standard length and weight should be recorded. It is highly recommended to transfer this information to spreadsheets, best as Excel files.

PROCEDURE

1. Assign a unique code to the fish individual examined and write it down in the dissection (field) notebook. If possible, it is highly recommended to perform fish dissection in teams of two, so that one person dissects the fish and fixes the material and the other examines the slides under the light microscope and takes pictures.
2. The dissection starts with the inspection of the fish surface; specifically, skin and gills are target infection sites for myxozoans. Evaluate both organs macroscopically; if you see cyst-like structures (usually whitish in colour), collect them carefully and squash them between a glass slide and coverslip. Scrape the fish skin mucus using a coverslip, flip it over onto a glass slide and prepare a squash slide by gently squashing the sample with another glass, thus equally distributing the pressure on the tissue sample.
3. Anaesthetise the fish using a clove oil solution or MS-222 for a few minutes before it is humanely euthanised (see Chapter 3.2).
4. Cut a small piece of gill filaments (maximum 0.5 cm large) and prepare a squash slide as described previously.
5. If interested in myxozoan extrasporogonic (blood) stages (*e.g.*, *Sphaerospora* spp.), take blood from the caudal vein using a heparinised syringe. Place the blood in a 1.5 ml microtube and collect it in a glass microhematocrit capillary tube which is then centrifuged in a microhematocrit centrifuge at 4000 RPM for 4 minutes. Break the capillary above the white blood cell (WBC) layer which may contain blood stages and collect this layer with a micropipette. Examine the fresh wet mount which is prepared by placing the WBC fraction and a small amount of fish serum onto a glass slide and covering it with a coverslip. For example, *Sphaerospora* blood stages can be distinguished from the host cells by their morphology (Lom & Dyková 1992) and by their specific twitching movement (Hartigan *et al.* 2016).

6. Open the fish by ventral incision starting from the anal opening following the midline of the body to the space beneath the heart. Make another incision from the starting point of the ventral incision close to the anus, and cut upwards to the top of the body cavity. Be careful not to damage the internal organs. Remove the lateral body wall on one side by cutting along the top of the body cavity.

7. Continue with the inspection of fish internal organs among which the kidney, gall bladder, muscles, liver and spleen are the most important locations for myxozoan parasites. The gall bladder must be carefully extracted from the rest of the organs and cut above a 1.5 ml microtube (or larger if necessary) to collect the clean bile, which is then transferred by pipetting a small drop onto a glass slide to be covered by a coverslip. Do not forget to clean the used dissecting tools between the dissection of different fish individuals or even between organs of a single fish individual by washing them under running tap water and subsequently in 70% ethanol or preferably in a 10% hydrogen peroxide solution, to avoid contamination.

8. Observe each sample under a light microscope at 400× magnification. If a parasite is detected, observe the same sample under a higher magnification using immersion oil and (if available) Nomarski differential interference contrast at 1,000× magnification.

9. Take microphotographs of all parasite developmental stages and spores observed immediately. Document at least 10 spores for each myxozoan species to enable later calculation of spore size variations and include a scale bar with each picture. Alternatively, continue with the fish dissection and photograph the parasites later. In the latter case, keep the slides with infected sample(s) in a wet chamber (a large Petri dish with wet tissues inside) in the fridge for a maximum of 24 hours to avoid drying out of the sample. In case the plasmodia or myxozoan blood stages move, a video can also be taken. Later on analyse the spore measurements (see Fig. 4.3.2 in Chapter 4.3) using ImageJ (Wayne Rasband, http://imagej.nih.gov/ij) or another software package.

10. Immediately after microscopic examination, fix a piece of infected tissue in cacodylate buffered 2.5% glutaraldehyde for further processing for transmission electron microscopy (TEM) (Glauert & Lewis 1998). The same fixative is applied for the preparation of samples for scanning electron microscopy (SEM) (Jirků & Bartošová-Sojková 2014); before fixing spores for SEM, separate them from the surrounding tissue on a dextran-polyethylene glycol gradient (Jirků & Bartošová-Sojková 2014), mix them with water or PBS (phosphate buffered saline) and place them on a grease-free poly-d-lysine coated coverslip. Glutaraldehyde-fixed samples can be stored for 24-48 hours in the fridge. Afterwards, the samples should be post-fixed in a 1% osmium tetroxide solution, followed by dehydration in a graded acetone series (in the case of TEM embedded in Spurr resin).

11. For histopathology, fix a sample (maximum size 1 × 1 cm) of the host organ in Davidson's fixative for 24 hours and transfer it to Davidson's stock solution, in which samples can be stored at room temperature for a longer