code to every Petri dish with individual organs to avoid any subsequent confusion about the host. Check the body cavity of the fish (some helminth larvae can be present there).

3. Examine the surface of the internal organs (heart, liver, spleen, gall bladder, digestive tract, gonads, kidney, swim bladder) for parasites. Then separate the organs into Petri dishes with saline. Examine parenchymatous organs after teasing them apart into small pieces using scissors or forceps.

4. Open the intestine by cutting its wall longitudinally with small sharp scissors, preferably from the posterior part (anus).

5. Observe the content of the intestinal lumen and organs, preferably under a dissecting microscope or at least magnifying glass (good illumination is crucial for dissection; a good headlamp can be useful in the field when electricity is unavailable). The intestinal content should also be gently scraped with a scalpel and observed in a Petri dish with saline under a dissecting microscope.

6. Remove worms carefully (they are usually whitish or pale-white moving organisms) from the intestinal lumen and other organs with the aid of dissecting needles, a brush or a soft (entomological) tweezer or pipette. To detect (and reliably count) tiny worms, it is also possible to press the intestinal content and teased organs between two glass plates after their previous thorough observation.

7. Carefully place the worms in a small Petri dish with saline and wash them gently by flushing with saline using a fine pipette to take out mucus or host tissue. Use decantation (washing and sedimentation of the content in saline) for voluminous gut contents.

8. If time allows, observe the worms when alive (small endohelminths under light microscope), *i.e.*, their shape, movement, presence of structures not observable in fixed worms such as flame cells, *i.e.*, the terminal part of the osmoregulatory system of flatworms. Take a picture or video with a digital camera, make sketches of taxonomically important characteristics or record this information in your field notebook. Keep correct labelling and magnification to each document.

Fixation of endoparasitic helminths

The worms found should be fixed as soon as possible after their isolation and proper cleaning from host tissue or intestinal content. Adults of all endohelminths, except for acanthocephalans, are fixed in a similar way, whereas the fixation of their larvae (metacercariae and metacestodes) requires some modifications. Trematodes and tapeworms should never be flattened because fixation under pressure affects their shape and changes their size. Exceptions are a few special cases such rostellar hooks in larvae of gryporhynchid cestodes or the circumoral spines in trematode metacercariae, which are more visible after flattening. The present authors have found fixation in hot formalin the best choice for morphological studies of trematodes, tapeworms and nematodes including histological sections and scanning electron microscopy (SEM). If heating formalin is not possible, an acceptable alternative is to use hot saline or hot tap water to keep worms in natural shape, not deformed or contracted (see Justine *et al.* 2012). Specimens for morphological studies should afterwards be placed immediately into 4% formalin and samples for DNA sequencing into molecular-grade ethanol.

All fixed samples must be labelled with a unique number/code. We strongly recommend simple codes, preferably unique, consecutive numbers after the country code (*e.g.*, Sud304 for fish No. 304 examined in the Sudan), with small letters (a, b, c, etc.) as subcodes that enable you to distinguish individual samples found in the same host and avoid any confusion.

It is also recommended to use some specimens for both morphological (light microscopy, histology and SEM) observations and genotyping (DNA sequencing), *i.e.*, as hologenophores (see Pleijel *et al.* 2008 for terminology). If there are presumably conspecific worms in the same host, several specimens can be fixed for morphological observations (these are paragenophores), whereas the others should be fixed in 96-99% molecular-grade ethanol for molecular studies.

Fig. 3.3.4.1. Examination of endohelminths. **A.** *Malapterurus electricus* with host code and ruler; **B.** Internal organs removed from *Bagarius bagarius*, India; **C.** Opened intestine of freshly killed *Clarotes laticeps* with alive tapeworm *Proteocephalus sulcatus* (Klaptocz, 1906), Sudan; **D-F.** Examples of differences in fixation of tapeworm *Monticellia amazonica* de Chambrier et Vaucher, 1997 from *Calophysus macropterus*, Peru; **D.** Unnaturally contracted worms fixed after long time of relaxation; **E.** Unnaturally contracted worms fixed in 'cold' fixation (formalin solution); **F.** Worms properly fixed in hot fixative (hot water). (Photographs by R. Kuchta and T. Scholz).

Fixation for morphological, histological studies and SEM

PROCEDURE

1. Place clean worms in a beaker, Petri dish or plastic heat-resistant vial with a small volume of saline (just to avoid drying out).

2. Heat 4% formaldehyde solution (*i.e.*, mix 36-38% aqueous solution of formaldehyde in water or saline in ratio 1 : 9) or water/saline to its boiling point, with bubbles coming up from the bottom. The volume of the fixative should considerably exceed (at least 10 times) that of the saline in which worms are placed to ensure that the worms are heat-fixed.

3. Pour the hot fixative over the worms in saline. Well-fixed worms should be straight immediately after fixation, not contracted or deformed.

4. Once the fixative has cooled, transfer the fixed worms to a vial with the fixative at ambient temperature and add a label with the unique field number of the host (or write the worm's unique code with ethanol-resistant pen on the vial, not its lid).

5. After 1-2 weeks, transfer the worms to 70% ethanol before further processing (long-term storage of worms in formalin makes them hard and fragile).

6. Acanthocephalans are placed, after thorough cleaning (especially of the hooks on the proboscis), in a Petri dish with tap water and are maintained at 4°C for 1-15 hours until the proboscis is everted. Once the proboscis is everted, the worms are fixed with 70% ethanol (suitable for DNA sequencing, even though 96-99% molecular-grade ethanol is preferred) or 4% formalin. Some worms can be flattened between two glass plates and fixed in formalin.

7. Helminth larvae (except gryporhynchids) are usually difficult or impossible to identify based on their morphology and it is recommended to simply place them into a vial with molecular-grade 96-99% ethanol for subsequent molecular identification.

Fixation for molecular study (DNA sequencing)

PROCEDURE

1. Thoroughly rinse the worm (or its tissue sample) in saline to remove all possible traces of host tissue.

2. Place the worm directly in a vial with molecular grades 96-99% ethanol (*i.e.*, non-denaturated ethanol suitable for DNA sequencing). Check that the worm is actually in the vial, not still stuck on the dissecting tools.

3. Place a label with the unique host code (see above) in the vial and keep a morphological voucher (hologenophore or paragenophore – see above) of the same individual or species with the same unique code.

4. If possible, keep samples in ethanol in a refrigerator or freezer until further use.

Processing of fixed endoparasitic helminths

For morphological research, parasitic flatworms (trematodes and cestodes) and sometimes acanthocephalans are stained with carmine or haematoxylin to visualise their internal structures and organs. Following staining, they are dehydrated in an increasing ethanol series, cleared (best with eugenol – clove oil) and finally mounted as permanent preparations (whole mounts), preferably in Canada balsam, which has the best optical properties and does not crystallise as do some of the cheapest synthetic mounting media. These whole mounts are suitable for a deposition in museum collections as vouchers (or type specimens if a new species is described).

In contrast, parasitic nematodes cannot be stained and are observed as temporary mounts after clearing with glycerine (glycerol), which makes it possible to see their internal organs beneath the cuticle. Specimens stored in vials with 70% ethanol are placed on a slide and covered with a coverslip. Thereafter, a mixture of ascending concentration of glycerine: water (1 : 20, 1 : 10, 1 : 5, 1 : 2, pure glycerine) is added at each step after the water has evaporated on a histological heating plate to make clearing gentle. After examination, the nematodes are transferred back to vials with 70% ethanol for further storage.

Other specialised techniques such as gut washing, observation of the anterior end of nematodes (*en face* view), fixation of metacercariae, etc., are described in the specialised literature (Anderson 1958; Jones 1990; Moravec 1994, 2013; Scholz & Aguirre-Macedo 2000; Scholz *et al.* 2004; Cribb & Bray 2010; Oros *et al.* 2010; Justine *et al.* 2012 – see references below).

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3.3.5. PARASITIC CRUSTACEA

Martina DÁVIDOVÁ & Nico J SMIT

Introduction

Parasitic crustaceans (PCs) are very abundant, utilise an extraordinary broad range of hosts (fish primarily) and occupy a similarly broad range of microhabitats on their hosts. Most of these fish parasites are ectoparasites, being found all over the body surface of the host as well as in more sheltered microhabitats that are directly connected to the external environment, including the external nares (nostrils), eyes, oral and branchial cavities, gills and cloaca. A smaller number are mesoparasitic, living with their anterior (cephalothoracic) end embedded in host tissues and their posterior trunk protruding from the host's body surface.

The diversity of morphological forms of PCs (especially parasitic copepods), life cycles and host associations are enormous. Parasitic crustaceans of African fresh- and brackish water fishes belong to three distinct taxonomic groups: Copepoda, Branchiura and Isopoda (see the key in Chapter 4.9). Several genera of African parasitic copepods, branchiurans or isopods also occur outside Africa but other genera, such as *Dysphorus, Lernaeogiraffa* or *Chonopeltis*, are endemic to the African continent. Fryer (1968) recorded 48 species of copepods, 28 species of branchiurans and 3 species of isopods in Africa. Since then, more species have been described, providing better information about the richness and diversity of the parasitic crustaceans on this continent (see Chapter 4.9 for an updated list).

Screening for and collection of parasitic crustaceans on/in fish

The collection of fish hosts, host sedation and euthanasia and external surface examination follow the same protocol as that described in Chapter 3.3.3. Information on the host is vitally important in studies on PCs and every effort must be made not to mix host species following capture, because parasites may be transferred by accident while in the net (Boxshall *et al.* 2016). If it is necessary to transport the fish, they should be stored individually in plastic bags, because of the possibility of ectoparasites being dislodged. In the laboratory, the bag or container with fish should be screened for detached parasites. Although it is always best practice to collect material directly from freshly euthanised hosts, fish markets and fish donated by local fishermen can also be a good source of PCs. However, ectoparasites can be lost during the capture and handling of fish obtained from markets and local fishermen. These losses will affect results for prevalence and intensity of infection.

To find PCs, a macroscopic examination of the external body surface (including fins), mouth cavity, gills, opercula and nasal pits is necessary. It is also important to inspect for PCs first, before scraping for eukaryotic microorganisms (protists and myxozoans) as scraping may damage PCs, especially mesoparasites.

The fish should be examined externally in the following sequence (Kabata 1985):

1. Examine the skin, fins, eyes and nares.
2. Look for signs of external parasites, such as lesions, subcutaneous haemorrhages and missing scales (some copepods produce pouch-like invaginations by burrowing under scales along the side of the host, or into the walls of the alimentary canal, often in the rectal area – Boxshall *et al.* 2016).
3. Nares should be opened and examined as they are a favoured microhabitat for PCs, such as species of the Ergasilidae.
4. Open the mouth and examine the upper buccal cavity and space around the tongue and teeth.
5. Soaking the body in saline for 30 minutes can dislodge small ectoparasites copepods; the sediment from soaked fish should be then examined under a dissecting microscope.
6. Following macroscopical screening and removal of all PCs found, the gills should be screened again under a dissection microscope for small parasitic copepods, which are not always visible to the naked eye (see Fig. 3.3.3.2).
7. Entomological forceps, fine needles and Pasteur pipettes are required for the manipulation of PCs (*e.g.*, removing, cleaning from host tissue and transferring into fixative).
8. Ectoparasitic copepods are typically attached by clawed appendages which are of taxonomic importance. Therefore, care must be taken not to break off the claws when removing the parasite from its host.
9. Mesoparasitic PCs, *e.g.*, members of the Lernaeidae, typically have large metamorphosed females that live with their heads embedded in the muscles of their hosts, forming branching, anchor-like structures. According to Boxshall *et al.* (2016), the best way to extract a mesoparasite with its cephalic holdfast intact is to excise a large portion of the muscle tissue of the host, sufficiently large to enclose the full estimated extent of the holdfast, and place it in 50 ml of saturated potassium hydroxide. Cover it so that it cannot evaporate and leave for one or more days at room temperature, checking every day. The hydroxide digests host tissues surrounding the holdfast so that it can be teased away using dissecting needles. This process also digests the internal tissues of the copepod but the empty exoskeleton is intact and can be used for taxonomic study.

Fixation of parasitic crustaceans

Parasitic crustaceans can be fixed in different ways. For morphological studies, 4% formaldehyde is the most commonly used fixative. To avoid the negative effects of long-term preservation in formalin, specimens should be transferred in 70% ethanol for storage. Ethanol (70-95%) is also a good fixative for morphological evaluation and identification, and for molecular analyses. Davidson's AFA fixative (mixture of 10 ml of 37% formaldehyde, 50 ml of 95% ethanol, 5 ml of glacial acetic acid and 45 ml of distilled water) is recommended for histological sections.

Samples for molecular analysis should be frozen or fixed in 95% or absolute, molecular-grade ethanol. Such material should not be exposed to formalin, which contains methanol.

Processing of fixed material of parasitic crustaceans

The taxonomy of parasitic crustaceans is based mainly on external morphology; therefore, it is necessary to observe the details of the integument. Before identification, the material should be cleared in 90% lactic acid or glycerine to reduce visual interference from internal structures. Lactic acid gives excellent contrast. It is also possible to stain the integument. A good light stain for use with lactic acid is a few drops aqueous solution of lignin pink, added either to the undiluted acid or to 50% aqueous solution. Further information about stains for small crustaceans is available at http://invertebrates.si.edu/copepod/techniques.htm.

The choice of dissecting medium depends on the eventual mounting medium. It is often convenient to dissect the specimen in the eventual mounting medium rather than to attempt transfer of small parts. Dissection is accomplished most easily in glycerine or lactic acid using either fine entomological pins mounted in wooden holders, tungsten needles or a micro-scalpel. The most frequently used mounting media are glycerine, glycerine jelly or lactophenol. The latter medium was recommended by Huys and Boxshall (1991) for type specimens to be deposited in museum collections. Canada balsam can also be used, as for other groups of metazoan parasites. For mounting crustaceans in glycerine jelly or lactophenol, it is also possible to apply procedures used for parasitic nematodes (Ash & Orihel 1991; Moravec 2013). More information on mounting media and procedures for mounting PCs is available at http://invertebrates.si.edu/copepod/techniques.htm.

Preparation of glycerine jelly

Dissolve 10 g gelatine in 60 ml distilled water using moderate heat.

Add 70 ml glycerine and 0.5-1 ml of phenol to the gelatine solution and mix well.

Pour the liquefied glycerine jelly into glass bottles and store in a refrigerator.

Preparation of lactophenol

Mix 20 ml glycerine, 10 ml lactic acid, 10 ml phenol and 10 ml distilled water.

Store the solution in the dark at 2-25°C.

Identification of parasitic crustaceans

Parasitic crustaceans are usually identified using a stereomicroscope and/or a light microscope equipped with differential interference contrast. Taxonomy of PCs is based mainly on their external morphology. For their identification, features such as shape of the body and its individual parts, characteristics of segmentation, size of individual parts of the body, structure of head and thoracic limbs, characteristics of attachment apparatus, etc., are used (see Chapter 4.9).

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Chapter 3.4.

HISTOLOGICAL TECHNIQUES

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Introduction

Histology can play an important part in research on fish parasites and parasitic diseases as long as its objectives and limitations are recognised. Histological examination is used mainly for diagnostic purposes, in screening for the presence of parasites in fish hosts and in evaluating their pathogenicity. However, it can also be used in research on specific structures of parasites, including diagnostic characteristics used to distinguish higher taxa, such as families of caryophyllidean and subfamilies of proteocephalid cestodes (see section 4.6).

The aim of histological techniques is to obtain thin sections of tissue samples of interest with as few artefacts as possible. To obtain satisfactory results, some degree of experience and insight is required. Histological techniques have been perfected for years to reach the point of an almost complete automation of sample-processing. This is important in big diagnostic and research centres, however, the prerequisites and individual steps of processing are the same whether automated or performed manually. These basic prerequisites and individual steps with their pitfalls are outlined below. More detailed instructions and recipes can be found in numerous histology manuals and websites, some of which are listed under the references.

Sampling for histology

Correct sampling for diagnostic purposes requires taking samples from freshly killed or moribund fish. To understand pathological processes caused by parasites, macroscopically visible lesions need to be sampled together with the surrounding, presumably intact, tissue. In order to avoid misinterpretation of artefacts, the fragile consistency of parenchymatous organs should be taken into account when tissue samples are extracted by forceps or other instruments. Tissue samples should be large enough to provide good quality information but small enough to be fixed (preserved) well. In the field, fish sometimes cannot be examined while fresh. Fixed tissue samples can be stored in 70% ethanol for a relatively long time (weeks) to be processed and examined later. Then an essential screening for the presence of parasitic infections can be based on histological sections (Figs 3.4.1-3.4.6).

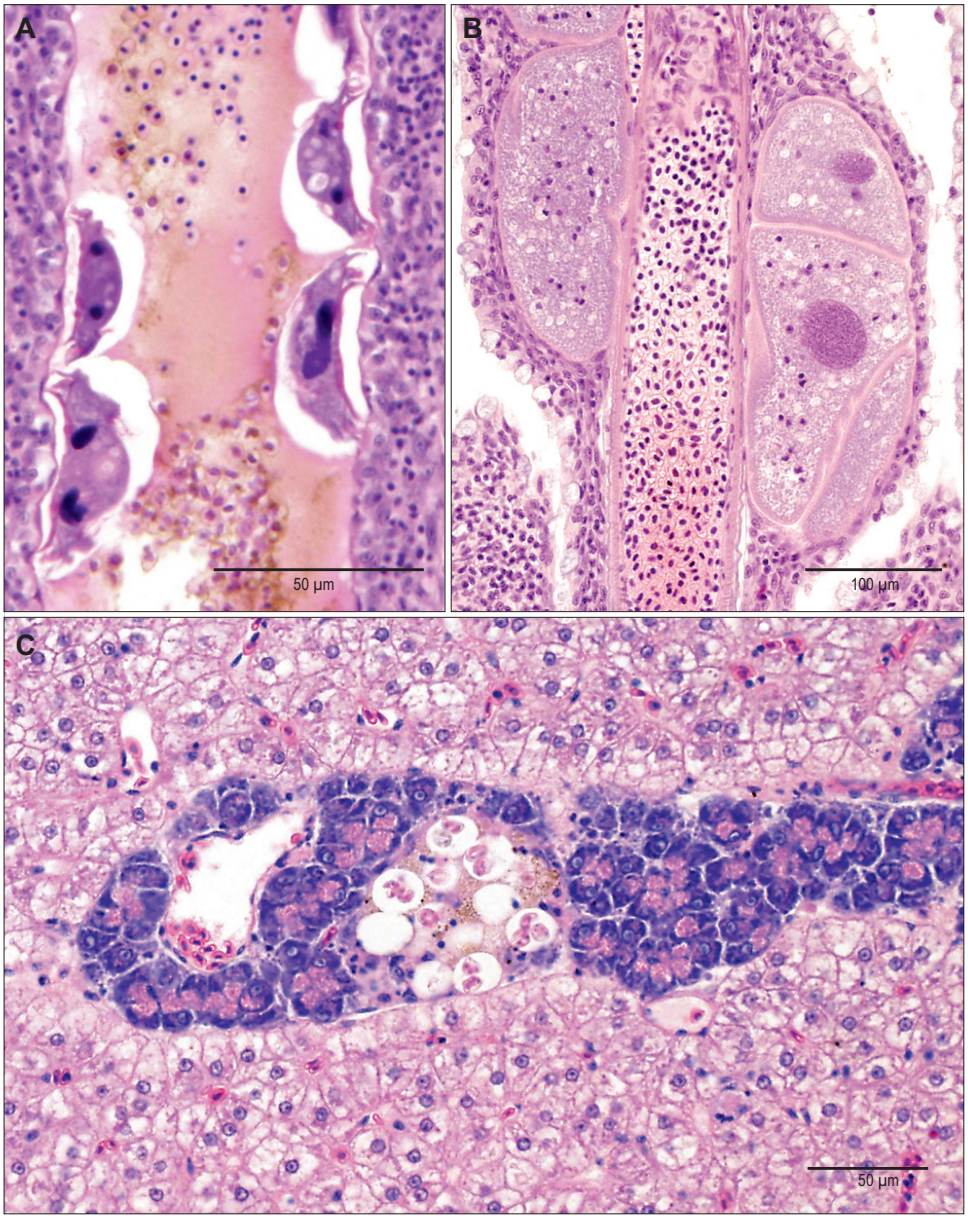

Fig. 3.4.1. A. Trichodinid ciliates seen on the surface of gill filaments of cichlid fish; **B.** Histophagous ciliate *Ichthyophthirius multifiliis* Fouquet, 1876 in the gill filament tissue of *Pseudotropheus* sp.; trophozoites with prominent macronuclei and host cells in the cytoplasm; **C.** Thin-walled oocysts of coccidia in hepatocytes surrounded by pancreatic tissue of *Haplochromis* sp. contain eosinophilic sporozoites. All haematoxylin & eosin. (All microphotographs by I. Dyková.)

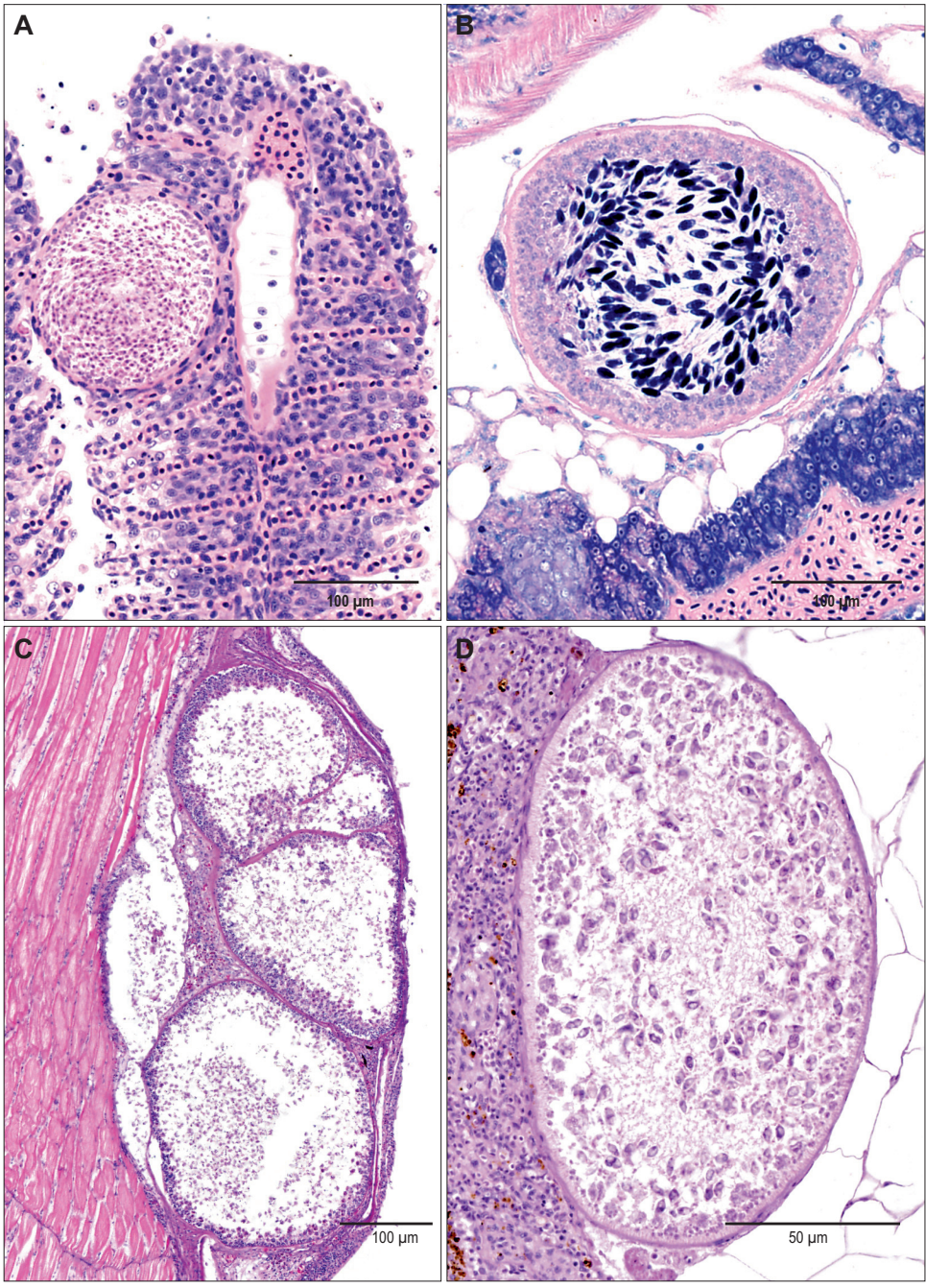

Fig. 3.4.2. A. Polysporic plasmodium of a myxosporean in the gill tissue of *Cichlasoma* sp. H & E; **B.** The plasmodial stage of a myxosporean species localised in the body cavity of *Leporinus* sp. contains intensely stained myxospores. Giemsa stain; **C.** Four myxosporean plasmodia localised subcutaneously in *Haplochromis* sp. H & E; **D.** Myxosporean plasmodium developing in the spleen of *Haplochromis* sp. H & E.