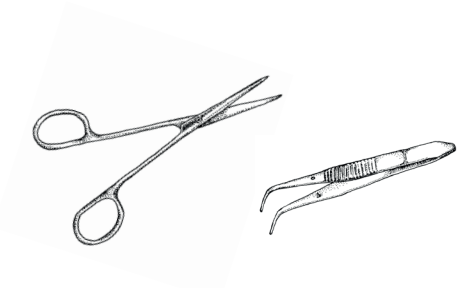
PART 3

BASIC METHODS TO STUDY FISH PARASITES



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Chapter 3.1.

IMPORTANCE OF SAMPLING DESIGN: HOW TO COLLECT DATA ON FISH PARASITES

Milan GELNAR, Nico SMIT & Maarten P.M. VANHOVE

Introduction

There is no doubt that the importance of fish parasites is related directly to the importance of fish they may affect (Hoffman 1999). It is well known that fish are an excellent source of complex proteins, they provide an important recreational asset, both for sport fishing and as one of the attractions of nature. In addition, a lot of fish species are also very important for development of various types of aquacultures, and finally, fish and their parasites also represent an important and interesting subject for science including ichthyoparasitology investigating parasites as potential causative agents of various fish diseases and also in ecotoxicology and evolutionary ecology (*e.g.*, Woo 1995; Khalil & Polling 1997; Hoffman 1999; Scholz 1999; Alvárez-Pellitero 2008; Eiras *et al.* 2008a,b; Sitjà-Bobadilla 2008; Buchmann *et al.* 2009; Leatherland & Woo 2010; Woo & Bruno 2011; Woo & Buchmann 2012).

Many years ago, Lester (1984) has reviewed methods for studying the effect of parasites on feral and cultured fish. Before fish parasitic diseases are effectively treated and controlled, the study of fish should follow a logical pattern:

- identify the parasite;
- obtain a thorough knowledge of its life history, which may be simple (direct or monoxenous) or very complicated (indirect or complex);
- learn the ecological requirements of the parasite, such as host specificity, optimum temperature, pH, nutrition, and other metabolic requirements;
- map the geographical range of the parasite;
- determine effect of immunological mechanisms of the host on the parasite, and *vice versa*;
- study control and treatment methods.

Hierarchical structure of parasitology

Parasitology and especially evolutionary ecology of parasites can be studied at three hierarchical levels: (1) organism, (2) population and (3) community (see Fig. 3.1.1). The smallest scale of study in parasite ecology is the individual parasitic organism, but parasitologists also deal with populations of parasite individuals of the same species, and with communities made up of several populations of

different species (*e.g.*, Kennedy 1976; Esch *et al.* 1990; Esch & Fernández 1993; Rohde 2005; Poulin 2007).

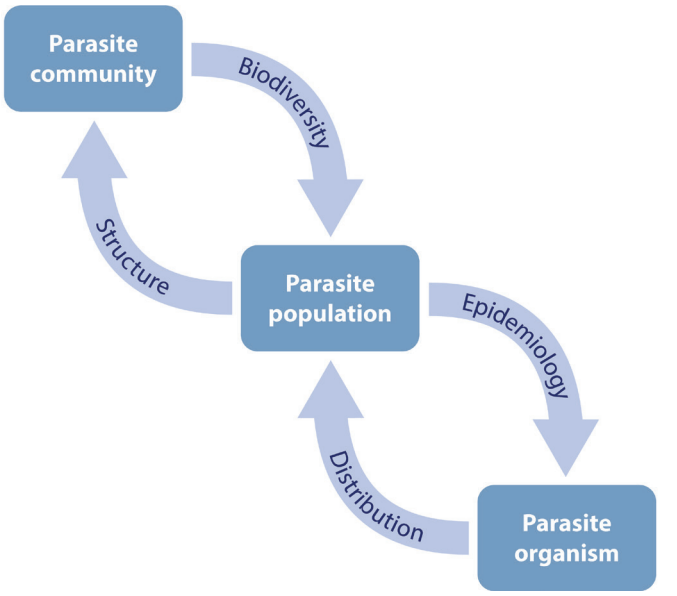

Fig. 3.1.1. A schematic representation for the three hierarchical levels of organisation of parasite-host associations. (Illustration by M. Luo and M. Gelnar.)

Sampling of parasitic organisms

Correct diagnosis is essential not only for parasite species identification but also for effective treatment and control of any fish disease. This means that there needs to be a consensus on the names and terms used in the identification process. Therefore, before we begin to consider a specific parasite, it is necessary to have an understanding of how the taxonomic system works and its relevance to parasitology (*e.g.*, Gussev 1978, 1985; Halton *et al.* 2001; Pugachev *et al.* 2010; Gunn & Pitt 2012). Those who study the classification of organisms are called taxonomists and they arrange organisms into hierarchical categories to reflect their assumed relationships.

Table 3.1.1. Taxonomic hierarchy with specific reference to the monogenean parasite *Paradiplozoon homoion homoion*

Taxonomic division	Taxon name	Common name
Super kingdom	Opisthokonta	
Kingdom	Animalia	animals
Subkingdom	Bilateralia	
Branch	Protostomia	
Phylum	Platyhelminthes	flatworms
	Neodermata	
Class	Monogenea Carus, 1863	
Subclass	Oligonchoinea Bychowsky, 1937	
Order	Mazocreaidea Bychowsky, 1957	
Suborder	Discocotylinea Bychowsky, 1957	
Family	Diplozoidae Palombi,1949	
Subfamily	Diplozoinae Palombi, 1949	
Genus	*Paradiplozoon* Akhmerov, 1974	
Species	*Paradiplozoon homoion* (Reichenbach-Klinke, 1961) Akhmerov, 1974	
Subspecies	*Paradiplozoon homoion gracile* (Bychowsky et Nagibina, 1959) Akhmerov, 1974	

Note: not all taxonomists agree with the same classification scheme. For example, some specialists prefer to divide the Monogenea (or Monogenoidea according to other authors) into different subclasses:

- Monopisthocotylea (= Polyonchoinea) and Polyopisthocotylea (excluding Polystomatidae and Sphyranuridae = Oligonchoinea) – Bychowsky (1957)
- Polyonchoinea, Polystomatinea and Oligonchoinea – Lebedev (1989)
- Polyonchoinea and Heterochoinea (including two infra-subclasses Polystomatoinea and Oligonchoinea) – Boeger & Kritsky (2001)

Selection of proper morphometrical characteristics and effective laboratory techniques

There is no doubt that the usage of selected morphological/anatomical characters and some metrical parameters represents the most important step in parasite species identification (*e.g.*, Rubbi 1994; Rizzuto & Fasolato 1998; Lacey 1999).

As an example, the following morpho-anatomical characteristics can be recommended to be used for the identification of monogeneans (Gussev 1978, 1985; Pugachev *et al.* 2010).

- Shape and size of the body and haptor
- Structure of the anterior end; presence or absence of lobes, lappets, suckers and their number
- Structure of the tegument, its thickness and presence or absence of folds, scales or thorns
- Presence or absence of eyes, their number and structure
- Shape, number, arrangement, orientation and size of haptoral structures
- Structure and size of the copulatory organ and vaginal armament
- Structure of the intestine
- Number of testes
- Shape and arrangement of the ovary
- Relative position of the ovary and testes
- Number, shape and position of the gland reservoir of the copulatory organ
- Course of vas deferens and shape of the seminal vesicle
- Position of the genital and vaginal pores, course and armament of the vaginal duct and seminal receptaculum (if present)

It should also be pointed out that correct identification of the fish host is extremely important. Erroneous identification of hosts or infection site may result in misleading conclusions. It is therefore recommended to always take a picture of the host and to fix a small piece of its tissue (fins, liver or muscle) in molecular-grade ethanol for DNA-based identification, or to fix and preserve the entire host specimen as a voucher.

Sampling of parasite populations

Parasite populations vary in size over short and long-time scales and are affected by biotic and abiotic environmental factors. Some of these factors cause changes in parasite numbers, whereas others reduce the amplitude of fluctuations around an equilibrium population size.

Parasite populations are invariably fragmented into as many subgroups as there are infected individuals in a host population. For practical reasons, it is easier to consider only a single parasite life stage, such as adult parasites only, when defining a population (*e.g.*, Esch et al. 1990; Esch & Fernández 1993; Hanski 1999; Šimková *et al.* 2002; Poulin 2007). Thus, a parasite population consists of all adult parasites in all individual hosts of a host population; it is subdivided into numerous infrapopulations of unequal size, each inhabiting a different host individual. Infrapopulations are ephemeral groups, lasting no longer than the host's lifespan. Offspring issued from different infrapopulations have the opportunity to mix outside hosts and reassemble in new combinations to form new infrapopulations in new

individual hosts. The infrapopulation fragmentation is thus temporary and changes continually from generation to generation (for a schematic illustration of factors affecting parasite populations, see Fig. 3.1.2).

To date, the population biology of parasites has been investigated on three different fronts (Poulin 2007):

1. The dynamics of parasite populations can be modelled mathematically, usually with a few simplifying assumptions (*epidemiological approach*).
2. Empirical studies of field populations have highlighted the many density-dependent and density-independent mechanisms acting to regulate parasite abundance over time in specific systems (*ecological approach*).
3. Genetic structure among infrapopulations and among populations allows us to determine transmission processes and estimate the frequency of exchange of individuals among populations (*genetic approach*).

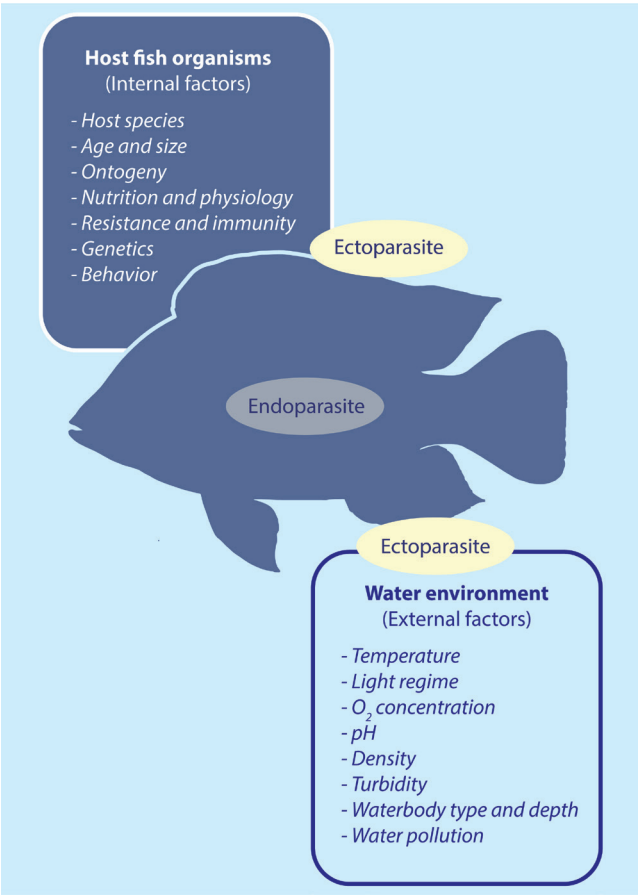

Fig. 3.1.2. A schematic representation of parasite-host interactions in an aquatic environment. (Illustration by M. Luo and M. Gelnar.)

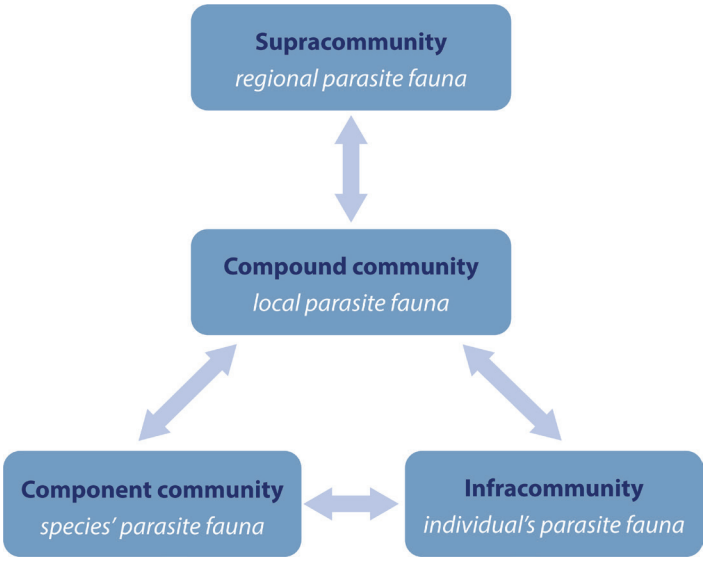

Fig. 3.1.3. A schematic representation of the hierarchical organisation of parasite supracommunity, compound community, component community and infracommunity. (Illustration by M. Luo and M. Gelnar.)

Sampling of parasite communities

The assemblage consisting of all parasites of different species in the same host individual, whether they actually interact or not, forms an infracommunity (*e.g.*, Esch *et al*. 1990; Bush *et al.* 1997). Infracommunities are subsets of the component community, which consists of all parasites exploiting the host population. In theory, infracommunities can range from highly structured and predictable sets of species, to purely stochastic assemblages of species coming together entirely at random (see Fig. 3.1.3 for a schematic illustration of parasite community structure).

Interactions among parasite species are one of the main forces that can shape infracommunity composition and structure and give it a non-random structure. In isolationist parasite communities, where interactions are negligible either because of very narrow niches or small infrapopulation sizes, the co-occurrence of species in hosts is not expected to deviate from that expected by chance (*e.g.*, Esch *et al.* 1990; Esch & Fernández 1993; Rohde 2005; Poulin 2007).

Recommendations for parasite community sampling design

The vast majority of available studies on parasite community ecology are based on the examination of patterns observed in one or a few samples of host individuals, patterns existing among different infracommunities sampled at one point of time. These provide a snapshot of what the parasite infracommunities looked like at the time of sampling, but no information on their development through time, starting from the moment the first parasite arrived on a host. Very few investigations

have attempted a longitudinal survey of parasite infracommunities, beginning with uninfected hosts, either young individuals or animals reared in captivity, that were allowed to recruit parasites under natural conditions (*e.g.*, Poulin 1996a,b; Poulin & Rohde 1997; Bagge & Valtonen 1999; Poulin & Valtonen 2002; Šimková *et al.* 2002, 2004; Vidal-Martínez & Poulin 2003). For hypothetical determinants of parasite community structure in real environmental conditions (see Fig. 3.1.2).

Collection of data

Parasitologists, like ecologists and other biologists, collect data to be used for testing hypotheses or describing nature. Modern science including parasitology proceeds by conjecture and refutation, by hypothesis and test, by ideas and data, and it also proceeds by obtaining good descriptions of ecological events. Parasitology like ecology is an empirical science that cannot be done solely on the blackboard or on the computer; it requires data from the real world. However, ecological data on parasites do not say everything about ecology of parasites.

Data represent only one half of this science; ecoparasitological hypotheses are the other half. Some evolutionary parasitologists even feel that hypotheses are more important than data themselves, while others argue the contrary. The central tenet of modern empirical science is that both are necessary. Hypotheses without data are not very useful, and data without hypotheses are wasted (*e.g.*, Krebs 1999; Henderson 2003). One problem that all research fields face is: what to measure? So selection of good, relevant and correct data is essential for the study and understanding of ecological or parasitological systems.

Host fish as habitat and sampling unit

Selection of a suitable and proper habitat unit is among the key questions in sampling design in the ecology of free living animals. In the case of parasites, a host organism represents the environment colonised and inhabited by parasites and due to that host organism, infrapopulation and infracommunity or local host population, metapopulation and component community can be conceptually identical to the concept of habitat and sampling units for free-living animals, respectively (see Fig. 3.1.3).

At the outset, a scientist must be sure about the problem he/she is proposing to investigate. As it is normally impossible to count and identify all the animals in a habitat, it is necessary to estimate data on the population or community by sampling. Naturally, these estimates should have the highest possible accuracy in relation to the effort spent. This requires a plan that includes a sampling program stipulating the number of samples, their distribution and their size. For example, the number of hosts is typically seen as sufficient to characterise a population at a given point in time. The importance of careful formulation of hypotheses to be tested cannot be overstressed (*e.g.*, Southwood & Henderson 2000; Sutherland 2006).

Sampling design and field work

In community studies, preliminary work should explore species richness and potential problems with species identification. The appropriate degree of taxonomic discrimination must be decided as it is important to maintain a consistent taxonomy. Sample sorting and species identification are often the most labour-intensive parts of a study and it may be useful to carry out a pilot trial to assess the effort required. Planning of the timing requires knowledge of life cycles. Preliminary work will be necessary to gain some knowledge of the occurrence of parasites to be studied.

The first decision concerns the scale of the environment to be sampled. A correct definition of the target population or community is essential: if too small, it may not produce results representative of the structure as a whole; if too large, it will waste resources. The second decision must be to define the accuracy or precision of the population estimates required. These decisions must be taken by considering both the objectives of the study and the variability of the system under study.

According to Henderson (2003), the following elements should be considered in any preliminary sampling design for populations of a host fish and for populations and communities of its parasite species.

- The need for sampling
- The scale of the study
- Safety
- Care for the environment and animal welfare
- Taxonomy
- Recording, labelling and noting down observations
- Data security and processing
- Effect of the time of year on sampling
- Effect of the time of day on sampling
- Size of population and community estimate
- Definition of the habitat unit
- Proper selection of unit area for sampling
- Subdivision of the habitat unit
- Statistical considerations

The selection of habitat and sampling unit for parasite ecology research

In general, the criteria for sample unit selection are, for parasites, broadly those of Morris (1955), where the term ‘habitat unit’ is identical with the term metapopulation of the parasites on a local metapopulation of host fish and the term ‘sample unit’ is identical with infrapopulation/infracommunity of fish parasites infecting the above mentioned metapopulation of host fish (*e.g.*, Krebs 1999; Southwood & Henderson 2000; Henderson 2003).

- All units of the environment must have an equal chance of sampling.
- It must have environmental stability.
- The proportion of the population using the sample unit as a habitat must remain constant.
- The sampling unit must lend itself to conversion to unit areas.
- The sampling unit must be easily delineated in the field.
- The sampling unit should be of such a size as to provide a reasonable balance between the variance and the cost.
- The sampling unit must not be too small in relation to the animal's size, as this would have edge-effect errors.
- The sampling unit for mobile animals should approximate the average ambit of an individual.

Conclusions – Top 10 golden rules

- Not everything that can be measured should be.
- Find a problem and state your objective clearly.
- Collect data that will help achieve your objective and make a statistician happy.
- Some ecological questions are impossible to answer at the present time.
- With continuous data, save time and money by deciding on the number of significant Figures in the data before you start field work/an experiment.
- Never report an ecological estimate without some measure of its possible error.
- Be sceptical about the results of statistical tests of significance.
- Never confuse statistical significance with biological significance.
- Code all your ecological data and enter it on a computer in some machine-readable format.
- Garbage in, garbage out.

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Chapter 3.2.

PARASITOLOGICAL EXAMINATION OF FISH (DISSECTION)

Tomáš SCHOLZ, Eva ŘEHULKOVÁ & Roman KUCHTA

Introduction

Parasitological examination, *i.e*., dissection or necropsy, is the basic method necessary to obtain parasites, especially for endoparasites (some macroscopical ectoparasites can be taken from live fish without their euthanasia). The extent of the examination depends on the purpose of a given study and the group of parasites studied because different methods are used to study eukaryotic microorganisms (parasitic protists and myxozoans), ectohelminths (Monogenea), endohelminths (Trematoda, Cestoda, Acanthocephala and Nematoda), and mostly ectoparasitic crustaceans. Therefore, the methods used in studies of these four principal groups of parasites are described separately in the following chapters (3.3.1-3.3.4). The present text provides only basic information about the most important requirements.

Basic requirements and rules

Equipment and facilities

Examination of fish usually does not require extremely sophisticated equipment and facilities, especially if focused only on those groups of parasites that do not need to be handled with special techniques. Overall, inspecting fish for eukaryotic microorganisms (see chapter 3.3.1) and monogeneans (see chapter 3.3.3) is more complicated; good optics including a light microscope and special chemicals are needed. In contrast, dissection of fish for some large-sized endohelminths can be done even without the use of a dissecting microscope (or just with a simple magnifying glass), but this does not enable the researcher to find all endoparasitic helminths, especially if they are tiny (< 1 mm). Therefore, the best recovery technique for any parasite group is observation of organs with a dissecting (helminths and parasitic crustaceans) and compound (eukaryotic microorganisms) light microscope. Since some helminths, especially monogeneans, are very tiny and translucent, a dissecting microscope equipped with bottom light (transmitted illumination) is preferred to effectively shed light on these parasites.

For dissection of fish in the field, a table is needed on which fish are examined, dissecting tools, several Petri dishes of different sizes, plastic pipettes, sample storage and transport equipment (vials, tubes, microscopic slides, coverslips and boxes) for fixed parasites, nail varnish to fix coverslips, a burner, water and/or

saline, fixatives and a camera. Headlights or torches may help find parasites in the organs examined if electricity is unavailable. Containers with aeration to keep living fish should also be available because fish euthanised just before dissection should be used (see below).

Catching fish for examination

Since ectoparasites can be lost during capture and transport of live fish to the place of examination, catch methods that do not damage the external surface, *e.g.*, electrofishing, sport fishing, scap net, small trawl or seine (see chapter 2.2), should be used. Methods that damage the fish (*e.g.*, gill nets) cause substantial injury and fish captured by such a method may suffer high mortality. Care has to be taken not to disturb the outer surface of fish. In particular, the fish surface should not dry up because this would incur the loss of ectoparasitic protists, crustaceans and monogeneans from the skin and fins. To become familiar with the general situation in the fauna of fish parasites in a locality, the fish sample should include at least 10-15 specimens of each fish species.

Condition of fish

The freshness of the hosts examined is a key factor that considerably influences the quality of parasites found, because decomposition and autolysis of their tissue and surface is very fast following the host's death. This negatively affects subsequent processing such as staining and light or scanning electron microscopic (SEM) observations. If fresh hosts cannot be examined, fish should be placed on ice to slow down autolysis of their tissues including their parasites, and examined as soon as possible (within several hours). Examination of dead fish in the field using a provisional laboratory is recommended rather than loosing time by transporting the fish for several hours to the laboratory. However, hosts should not be frozen, because parasites from frozen hosts may be deformed (contracted or artificially relaxed) and their tissues will have disintegrated, making them unsuitable for reliable morphological characterisation and correct species identification. In the case of protists, they can be completely lost. Hosts from fish markets may be suitable for parasitological examination provided they are alive or fresh (the gills should be red and without much mucus), and have not been kept in captivity for a long time or were not previously frozen.

If the number of hosts to be examined is too high for quick processing, the best option is to keep them alive. They can be maintained for some time in large tanks or wide plastic buckets with aerated water from the place of origin (or with dechlorinated water). However, the interval between the capture of hosts and their dissection should not be too long, because parasites may disappear from living hosts within a couple of days, mainly ectoparasites, but also intestinal helminths due to their starvation, stress and different water conditions. In addition, their community composition may change considerably, thus impeding reliable ecological study (changes in infection intensity and hence relative abundance, etc.).

Humane killing of fish

Before parasitological examination/dissection, the fish must be killed humanely in a dissecting dish with local water. Collecting and killing fish always need ethical approval and permits from a relevant authority. It is most important that researchers make sure that they follow the regulations and ethical procedures as prescribed by the country where the research is undertaken. For killing fish, pithing or stunning followed by interruption of the spinal cord should be used. Pithing (also spiking, coring, ikejime) is usually applied to smaller fish. A spike is quickly inserted into the brain of the fish (diagonally through the upper part of the eye or slightly behind and above the eye) and this is immediately followed by physical disruption of brain tissue by rotary movement of the spike. Bigger fish should first be stunned with a stroke on the head and then killed by interruption of the spinal cord immediately beyond the head using scissors or a sharp knife.

As an alternative to killing the fish, the fish can be sedated, anaesthetised or euthanised with chemicals such as tricaine (MS-222), clove oil, quinaldine sulfate, 2-phenoxyethanol, sodium bicarbonate and benzocaine. However, only MS-222, which does not seem to have an effect on parasites, is currently approved for use with fish that are destined for human consumption. More details about sedation, anaesthesia and euthanasia of fish are provided in the monograph by Ross and Ross (2008).

Host identification and labelling

Correct identification of the host is crucial for any parasitological survey or ecological study. Relevant data for the host such as its size (total and standard length), weight and sex should be recorded. Photographs of the host should be taken from a vertical position (not at an angle) with its snout directed to the left. The photos should include a ruler for size estimation and a unique host code (Fig. 3.2.1A). Morphological characters important for identification in individual fish groups such as details of the mouth, the fins and their rays, the number of scales on the lateral line, etc., should also be documented in these photographs. It is highly recommended to take samples of the host's tissues (around 5 mm in diameter, samples of muscles, fins or liver) and fix them with molecular grades 99% ethanol to enable later DNA-based identification. This is important especially in taxonomically complicated groups of fishes.

A unified system of hosts numbering with country codes and consecutive numbers (see Chapter 3.3.3) is strongly recommended because it avoids possible confusion if the same numbers are given to different fish hosts. Widely used abbreviations of fish names as codes may be helpful in some cases, but generally are not recommended because scientific names including genus of fish may change. In addition, this system of host coding is inapplicable when fish cannot be properly identified, which may happen with African fish, *e.g.*, cichlids or species of *Synodontis*.

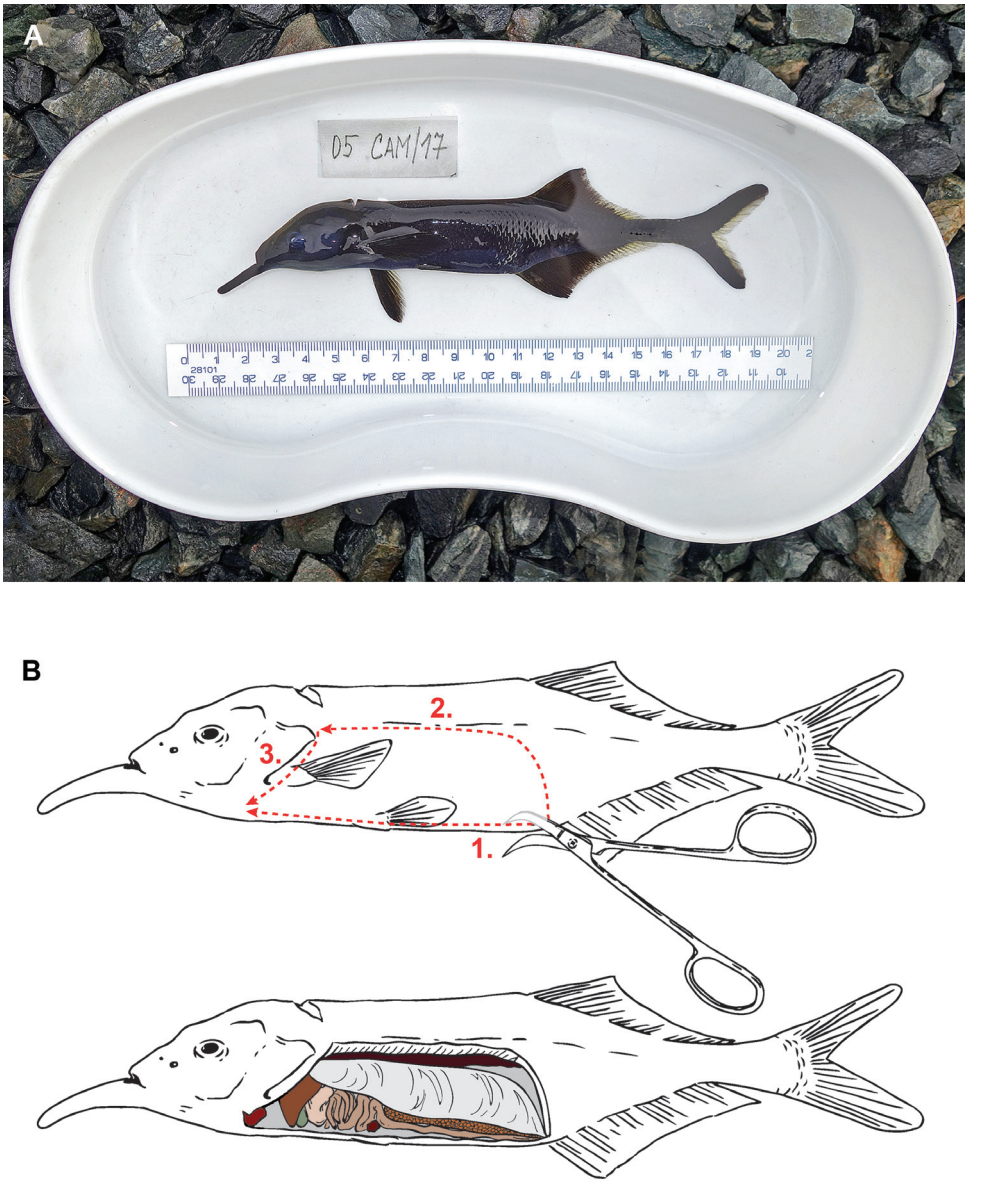


Fig. 3.2.1. A. Labelling fish hosts. Note that the fish snout is positioned to the left side and a ruler is added for estimation of fish size. The surface of the fish should be kept wet during any manipulation and handling of the fish; **B.** Illustration of how to open the body cavity of a fish to reveal the internal organs. (Photograph by E. Řehulková; illustration by M. Luo.)

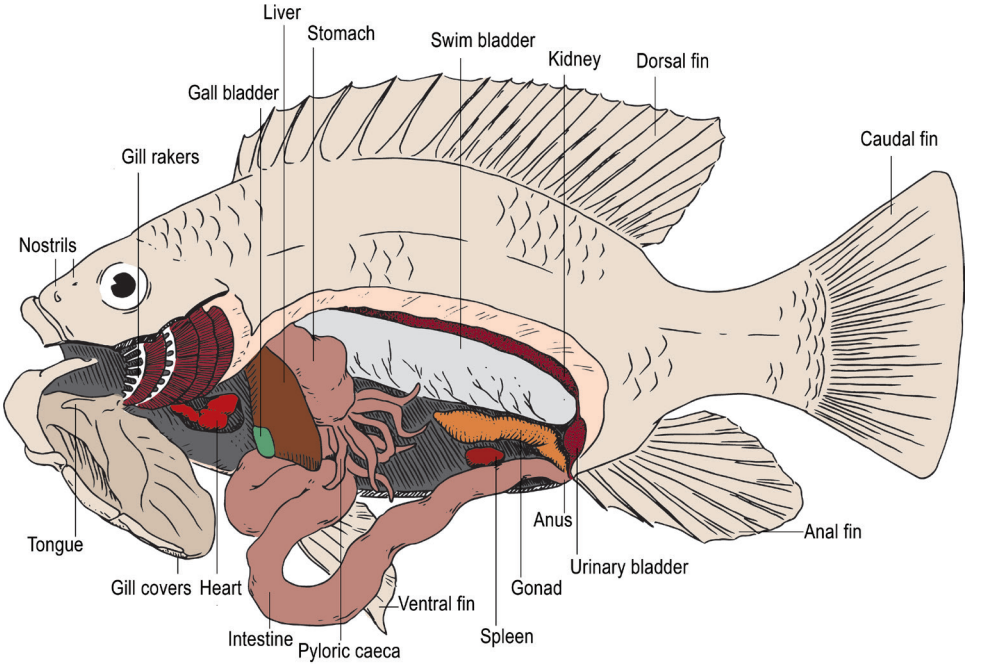


Fig. 3.2.2. External and internal anatomy of a bony fish. (Modified by M. Luo from Hile, R. 1960, U. S. Fish and Wildlife Service, Fishery Leaflet, no. 132, 6 pp.)

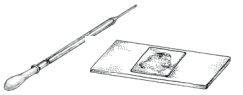
Information on the sampling date and locality (GPS coordinates, water temperature, etc.) should be recorded. The scientific name of the host, the infection site, the number of specimens found and fixed, the fixative used, the date of dissection and the name of the collector should be written in a field notebook for all parasites found. Recording of vernacular names (in addition to scientific ones, though) can be useful in interviewing fishermen or people in the market to find a particular species, to learn about its ecology, occurrence, etc. Thereafter, all the data can be transferred to spreadsheets, best as Excel files.

Fish anatomy and handling

Basic knowledge of fish anatomy is necessary before fish examination starts, especially the appearance and location of individual organs (Fig. 3.2.2). For the examination of head organs, the fish should be decapitated (see chapter 3.3.3). Access to the organs of the body cavity can be facilitated by removing one side of the body wall (Fig. 3.2.1B). The organs should be properly excised (avoid cutting them and releasing their contents) and should not be confused. For example, the excretory bladder can be difficult to find in some fish and the examination of kidneys requires scraping them from their location alongside the spinal cord. Superficial organs such as gills and fins, and scrapings from the surface should be placed in water. Internal organs and eyes should be treated in saline.

Reference

Ross, L.G. & Ross, B. (Eds) 2008. *Anaesthetic and Sedative Techniques for Aquatic Animals*. Blackwell Publishing, Oxford: 222 pp.



Chapter 3.3.

METHODS TO STUDY THE PRINCIPAL GROUPS OF FISH PARASITES

3.3.1. FISH-INFECTING EUKARYOTIC MICROORGANISMS (EMs)

Iva DYKOVÁ, Tomáš TYML & Astrid HOLZER

Introduction

EMs belong to several taxonomically divergent groups (Kabata 1985; Paperna 1991; Lom & Dyková 1992; Noga 2011; Adl *et al.* 2012). Their identification is traditionally carried out using a series of classical keys (see references to individual groups of parasites below) based upon the morphology of the whole organism, with confirmation or additional classification by DNA sequencing (predominantly 18S rDNA). Fresh smears are of special importance as many taxonomic features are not visible in fixed and stained EMs. However, tissue sections are important to determine the exact location of the parasite in the host and histopathological changes. Ideally, infected tissues are fixed for and studied by all possible methods. Often, light microscopical morphology allows assignment to a group or even genus but species identification requires molecular analyses or detailed ultrastructural studies (Aldrich & Todd 2012).

Groups of EMs

The following EMs are commonly found on freshwater fish:

- **Ciliates** (Alveolata, SAR) – ciliated protists with nuclear dimorphism (micro- and macronuclei). Motile. On external epithelia or inside the host, ranging from harmless to extremely pathogenic. See Figs 3.3.1.1A-I, 3.3.1.2A-G (for further reading, see Lynn 2008; Foissner 2014).
- **Blood flagellates** (Kinetoplastida, Excavata) – highly motile protists with one or two flagella, often forming an undulating membrane, characteristic kinetoplast (single large mitochondrion), associated with flagellar kinetosome. See Fig. 3.3.1.3A-C (Lom 1979; Davies 1995).
- **Amoeboid organisms** (Amoebozoa, Excavata, Opisthokonta, Rhizaria) – protists with amoeboid movement and pseudopodia. Most common are amphizoic amoebae (free living but able to colonise fish) on external epithelia, some other representatives in intestine or internal organs. See Fig. 3.3.1.3F,G (Page 1988; Dyková & Lom 2004; Dyková & Kostka 2013).
- **Coccidia** (Apicomplexa, SAR) – obligate intracellular protists, unsporulated/sporulated oocysts predominantly in enterocytes and faeces,

some other species in parenchymatous organs (*e.g.*, liver, spleen). See Fig. 3.3.1.4A-G (Dyková & Lom 1981, 1983).

- **Microsporidia** (Opisthokonta) – obligate intracellular protists with small, refractile spores with polar tube, which is used for injecting the sporoplasm (infective germ) into the host. Formation of large xenomas (infected and distended host cells) in different organs. See Fig. 3.3.1.5A-F. (Lom 2002; Lom & Dyková 2005).

- **Myxozoa** (Cnidaria) – multicellular (metazoan) parasites forming characteristic spores that contain 1-7 polar capsules, containing a polar filament for attachment to the host. Extremely diverse endoparasites. See Fig. 4.3.2A-M (Lom & Arthur 1989; Lom & Dyková 2006; Okamura *et al.* 2015).

Practical key for preliminary determination of fish-infecting EMs in fresh material

1 (2) Infection detectable as macroscopic whitish aggregations, from tiny dots to cyst-like structures of several mm or even cm in size; on the skin, gills, in or on the internal organs……3

2 (1) No macroscopic changes visible. EMs only detectable by light microscopy……9

3 (4) Microorganisms visible as tiny dots on the body surface and gills. Under the microscope the dot proves to be large (up to 1 mm) slowly rotating cells, uniformly covered with synchronously beating cilia; next to large cells, there may be small ones of different sizes; their cytoplasm is full of granules and contains a large horseshoe-shaped macronucleus. (Fig. 3.3.1.1G-I)…… ……..…***Ichthyophthirius multifiliis*** (Ciliata, Alveolata, SAR)

4 (3) Dot-, nodule-, or cyst-like structures composed of a mass of small, uniform, refractile bodies (spores or oocysts)…… 5

5 (6) The spores, typically 7-20 µm in size, most commonly have 2 (1-7) capsules containing a coiled filament, at one or both poles (Fig. 4.3.2A-M)… ……..……**Myxozoa** (Cnidaria)

6 (5) Spores without polar capsules……7

7 (8) Spores very small, typically 3-10 µm in size, usually ovoid and often showing a prominent vacuole in the posterior part (Fig. 3.3.1.5A-F)…… ……..……..**Microsporidia** (Opisthokonta)

8 (7) Organisms are spherical or ellipsoidal bodies of about 10-20 µm in size, each containing four ellipsoidal bodies, each of which contains two slender cells. Whitish nodules within the body organs are not sharply delimited (Fig. 3.3.1.4A-G)…… ……**coccidian oocysts** (Apicomplexa, Alveolata, SAR)

9 (10) EMs infecting the surface (skin, fins, nasal pits or gills)……11

10 (9) EMs infecting the intestine, other internal organs or blood……24

11 (12) Organisms that move…….. 13

12 (11) Sessile or motionless organisms attached to the surface……17

13 (14) EMs with flagella or cilia on the cell surface……15

14 (13) Cells with amoeboid movement and changes of body shape (Fig. 3.3.1.3F,G) ……..…….. **Amoebae**

15 (16) Cells up to 15 µm in size, possessing two flagella, moving with jerky, creeping motion or swimming spirally forward……....……flagellates, *e.g.*, ***Cryptobia*** (Kinetoplastida, Excavata) and ***Ichthyobodo***

16 (15) Cells 20 µm and larger, either covered uniformly with cilia or with several ciliary belts or circular ciliary wreath; they move directly forward, glide over the surface, or roll on the spot (Fig. 3.3.1.1A,B)…… ……..**ciliates,** *e.g.*, scuticociliates (Alveolata, SAR)

17 (18) Pyriform or sac-like cells, attached to the skin or gills of fish……19

18 (17) EMs attached to surface of host via stalks……21

19 (20) Transparent, attached pyriform cells not exceeding 15 µm in size ……..…***Ichthyobodo*** (Kinetoplastida, Excavata)

20 (19) Pyriform or sac-like cells, 30-300 µm in size, their cytoplasm yellowish or greenish and containing many refractile granules ....**Dinoflagellata** (Alveolata, SAR)

21 (22) Cells 40-100 µm in size, with cytoplasm dark due to refractile granules, and with bundles of tubules with knob-like ends protruding from their surface……..**suctorian ciliates** (Ciliata, Alveolata, SAR)

22 (21) Goblet-like or cylindrical cells about 40-90 µm in length, each with a wide free end encircled by wreaths of beating cilia; the cells may contract a little (Fig. 3.3.1.1E,F)……..…..**sessiline peritriches** (Ciliata, Alveolata, SAR)

23 (24) EMs in internal organs, urinary tract or bile……25

24 (23) EMs in blood…….. 31

25 (26) Myxozoa (see 5; in any organ, urinary tract or bile), microsporidia (see 7; in any organ), coccidian oocysts (see 8; in intestine); or amoebae (see 14)

26 (25) EMs with surface showing flagella or cilia……27

27 (28) Cells up to 15 µm in size, with up to 8 flagella, moving about with a jerky motion or swimming directly forward.....……**flagellates – Diplomonadida** (Excavata)

28 (27) Cells ciliated…….. 29

29 (30) Spindle-shaped cells, of about 30-140 µm in size, uniformly covered with cilia, with both ends pointed and with sluggish movement……...**Protoopalina** (Stramenopiles, SAR)

30 (29) Ciliated cells of another shape, up to about 120 µm in length…….**other ciliates** (Alveolata, SAR)

31 (32) Motile EMs…….33

32 (31) Non-motile EMs only visible in stained blood smears……35

33 (34) Slender cells, typically 10-15 µm long, moving with a wriggling or undulating motion, with 1 or 2 flagella (Fig. 3.3.1.3A-C)…… **flagellates – *Trypanosoma* and *Trypanoplasma*** (Kinetoplastida, Excavata)

34 (33) Cells of about 3-15 µm in size, of amoeboid shape, displaying a twitching motion on the spot (Fig. 3.3.1.1E)…… ……**developmental stages of some myxosporeans** (Myxozoa, Cnidaria)

35 EMs inside red blood cells (Fig. 3.3.1.3D)…… ……...**Haemogregarina** (Apicomplexa, Alveolata, SAR)

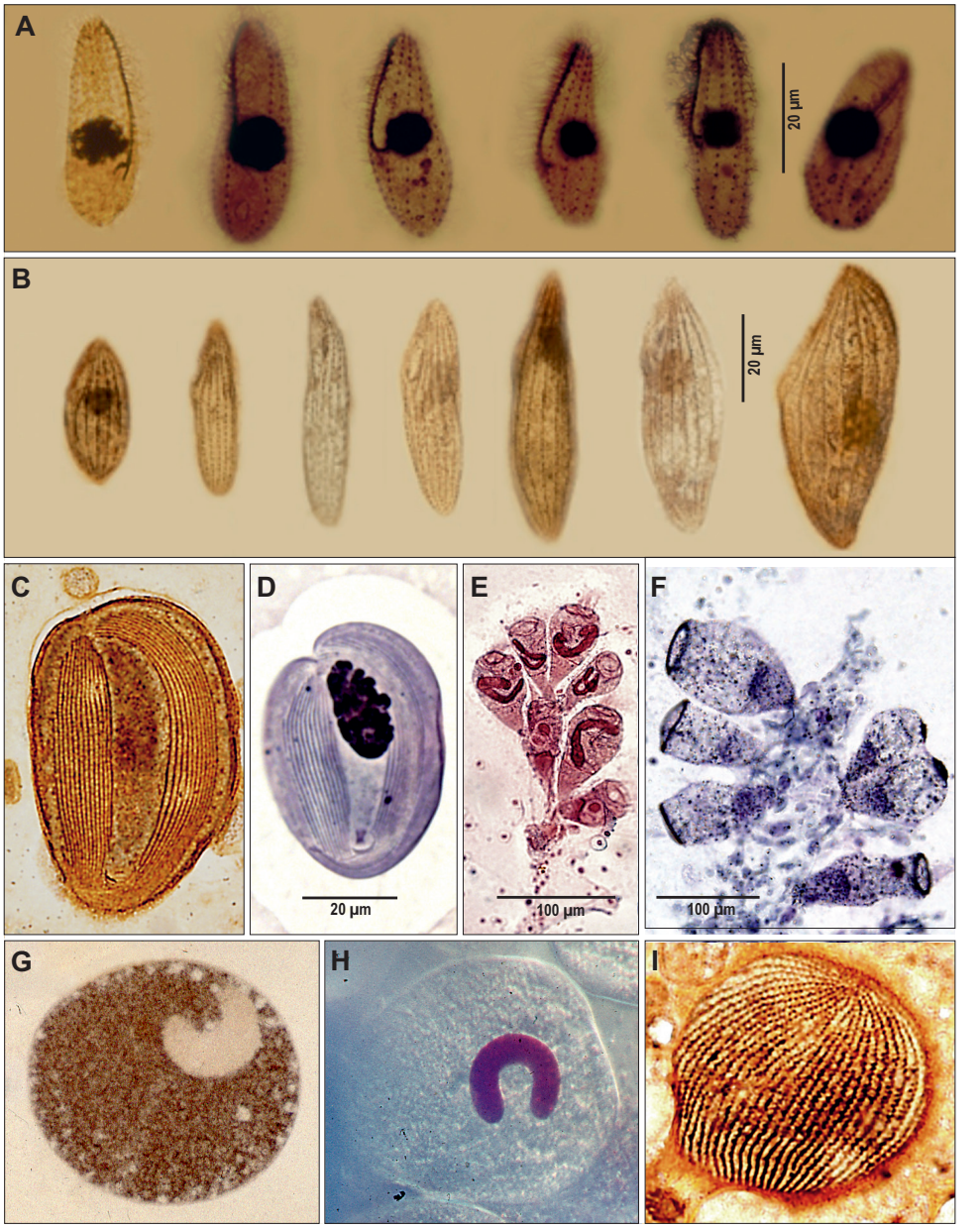
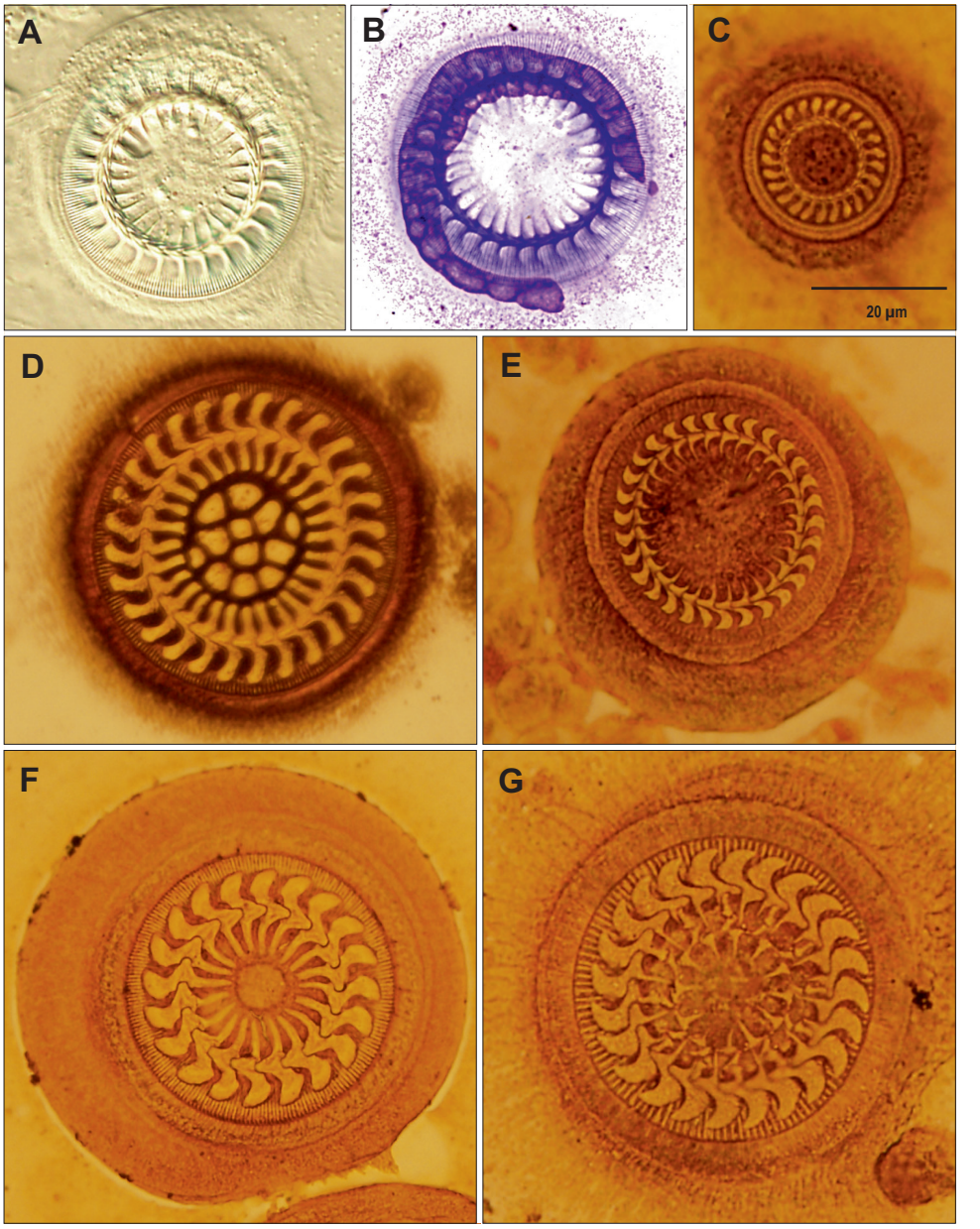


Fig. 3.3.1.1. Fish-infecting ciliates. **A, B.** Scuticociliates. **C, D.** *Chilodonella piscicola* (Zacharias, 1894). **E, F.** *Sessiline peritrichs*. **G-I.** *Ichthyophthirius multifiliis* Fouquet, 1876. Staining: protargol (A, D), 'dry' silver nitrate (B, C), Feulgen (nucleus stain; E,H), Klein's method (I). (All microphotographs by I. Dyková.)



3.3.1.2. Diagnostic features of trichodinid ciliates: skeletal parts of adhesive disc (AD) and shape of nucleus. **A.** AD seen in fresh (Nomarski differential interference contrast); **B.** Horse-shoe shaped macronucleus stained with haematoxylin; **C-G.** ADs stained with Klein's silver impregnation method: (C) *Trichodinella* sp., (D-F) ADs of various species of *Trichodina*. **G.** Dividing *Trichodina* with an outer well developed denticulate ring and a newly formed one indicated by concentrically arranged thorns. Scale bar C applies to all images.

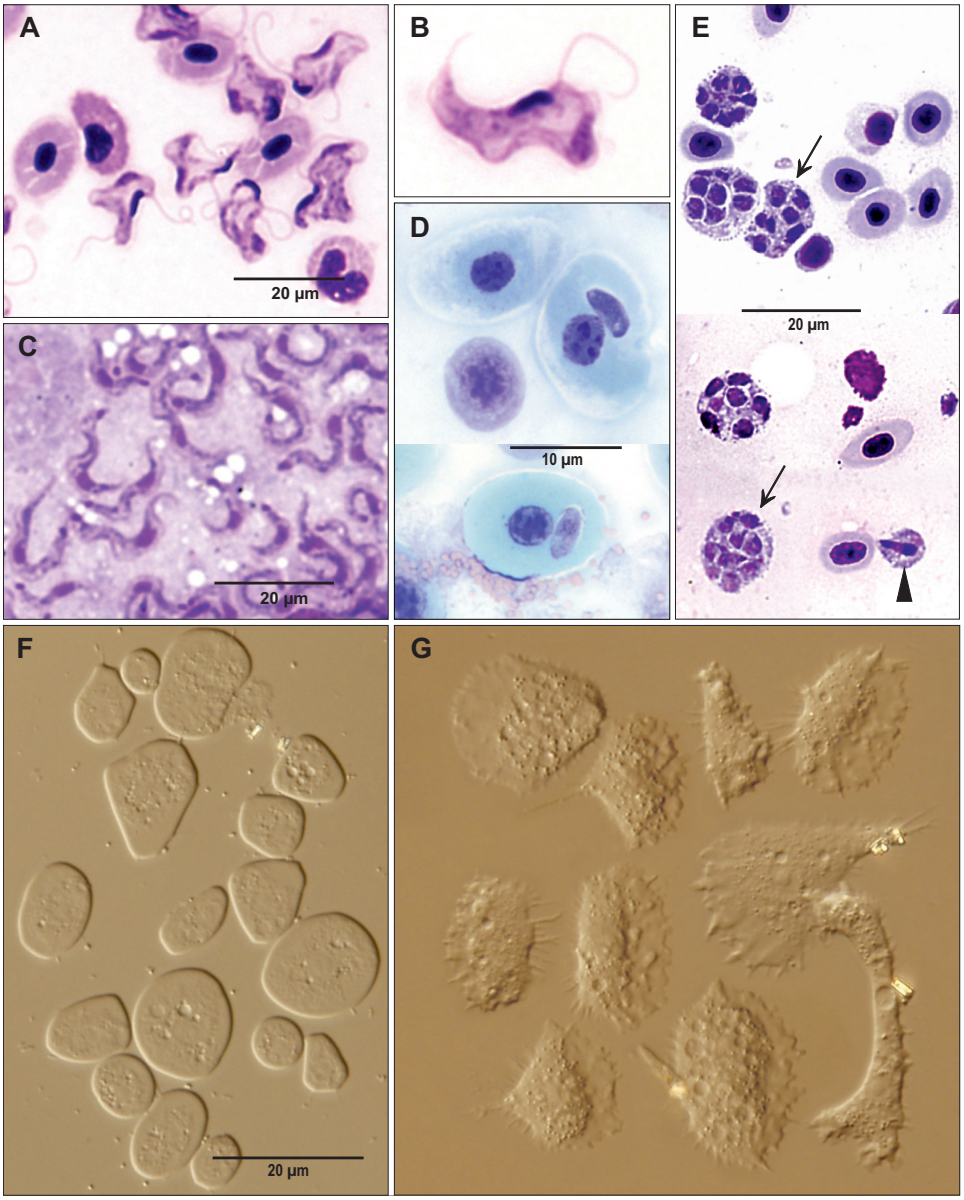


Fig. 3.3.1.3. A, B. *Trypanoplasma borelli* Laveran et Mesnil, 1901 stained with Giemsa. **C.** *Trypanosoma carassii* Mitrofanov, 1883 in Giemsa-stained blood smear. **D.** Intraerythrocytic stages of *Haemogregarina* sp. fixed and stained with Diff Quick. **E.** Proliferative stages of myxosporeans 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.

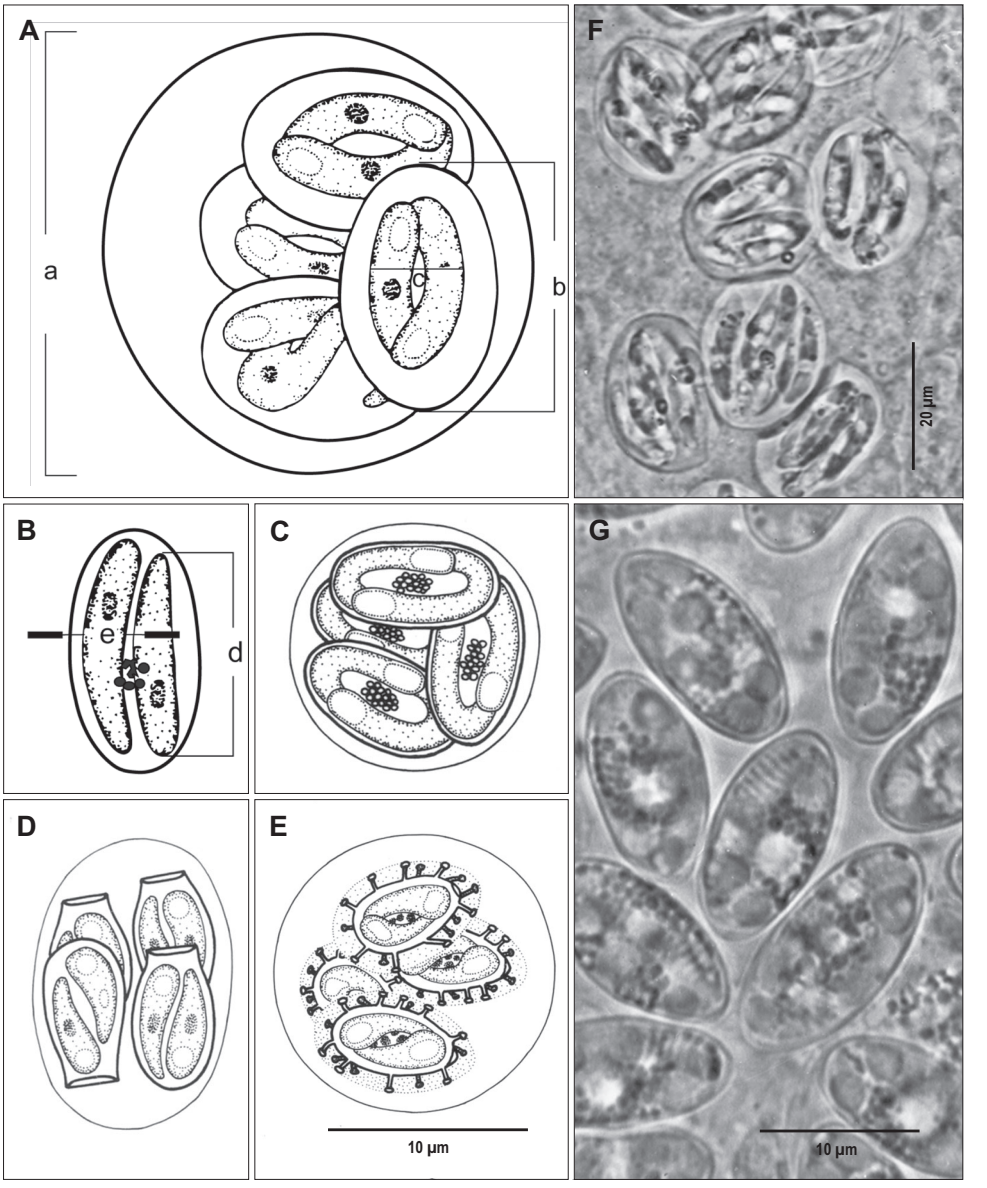


Fig. 3.3.1.4. Coccidia. Spherical oocysts (**A**), diameter (a), (length and width are measured in ellipsoidal oocysts), length and width of sporocyst (b and c, respectively). **B.** Sporozoite measurements (d, e). **C.** Oocyst of *Goussia carpelli* (Léger et Stankovich, 1921) contains sporocysts with 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).

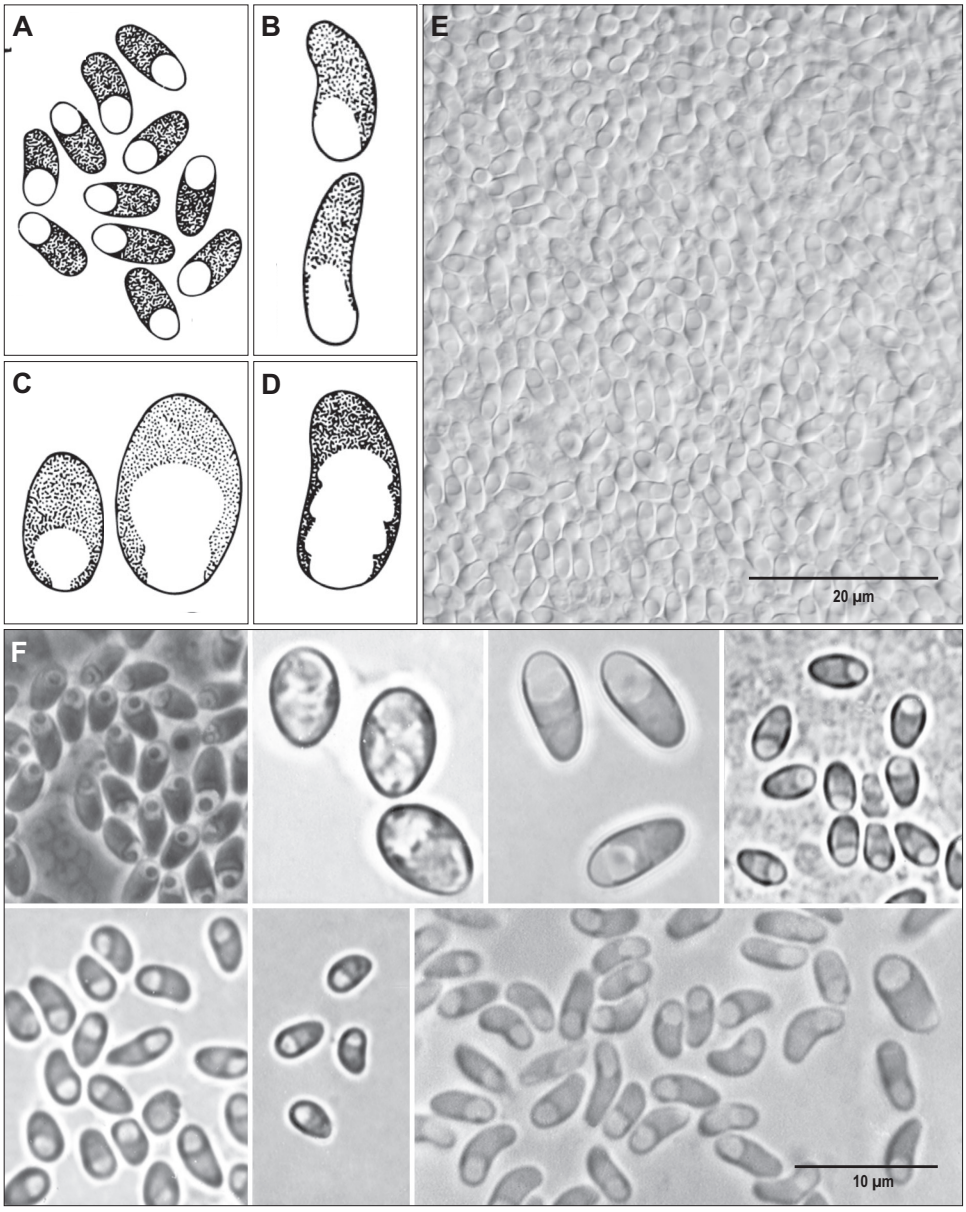


Fig. 3.3.1.5. Microsporidian spores observed in light microscope and documented in fresh state. In spores of fish-infecting species often contain conspicuous vacuole. **A-D.** Line drawings of *Microsporidium* sp., *Glugea* sp., *Pleistophora* sp. and *Heterosporis* sp., respectively. **E.** Fresh spores 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

Pavla BARTOŠOVÁ-SOJKOVÁ & Ivan FIALA

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

period (Heil 2009). Alternatively, 10% formalin can be used to fix samples for 24-48 hours, followed by replacement of the fixative by 70%, 80%, 90% and 96% ethanol (each concentration for one hour). Samples can be kept in 96% ethanol in the freezer for a longer period. Afterwards, the samples are embedded in paraffin and cut into slides that can later be stained by haematoxylin-eosin or Giemsa. When fixing the samples, do not forget to label each vial with a tissue sample on the outside and by inserting a label with the code of the host written in pencil.

12. Fix another small part of the infected organ in 96-99% ethanol or, for longer sample storage, in TNES urea buffers (Asahida *et al.* 1996) for subsequent DNA extraction and molecular characterisation of the parasite.

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3.3.3. ECTOPARASITIC HELMINTHS (MONOGENEA)

Eva ŘEHULKOVÁ

Introduction

Monogeneans are common, almost exclusively, ectoparasitic flatworms of freshwater, brackish water and marine fishes. Most monogeneans are tiny, which makes their sampling and further processing more difficult compared with larger-sized endohelminths such as most tapeworms, acanthocephalans or nematodes. Species identification of monogeneans may be difficult and its accuracy depends, to a large extent, on the quality of the material available. Therefore, adequate methods of sampling and processing monogeneans are required. If monogeneans are not collected and fixed correctly, it may affect the reliability of the morphometric data on taxonomically important structures.

Examination of fish for monogeneans

Fish should be examined immediately following their death while the monogeneans are still alive because living monogeneans are more easily detected by their movements. In addition, observations of living parasites may yield valuable information on internal structures (*e.g.*, digestive and excretory system) and the natural configuration of sclerotised hard parts. *Post-mortem* changes of monogeneans, which usually disintegrate quickly after they die, might make taxonomical evaluation of the specimens collected difficult or even impossible. The only disadvantage of collecting the living monogeneans is that they are sometimes harder to isolate because they are difficult to mount and orientate on a slide.

Fixed or preserved fish should be studied in a similar way as described below, but the quality of the specimens obtained is always much worse compared with fresh material; in some cases, a reliable identification of the worms cannot be made. It is important to point out that the surface of the fish should be kept wet during any manipulation and handling of the fish (taking photos, measurements, tissue samples, etc.), because drying up results in the damage or loss of monogeneans on the skin and fins. Therefore, the surface organs (skin, fins, nostrils, mouth and gill cavity) must be examined first after all the necessary data are recorded (see Chapter 3.3.1).

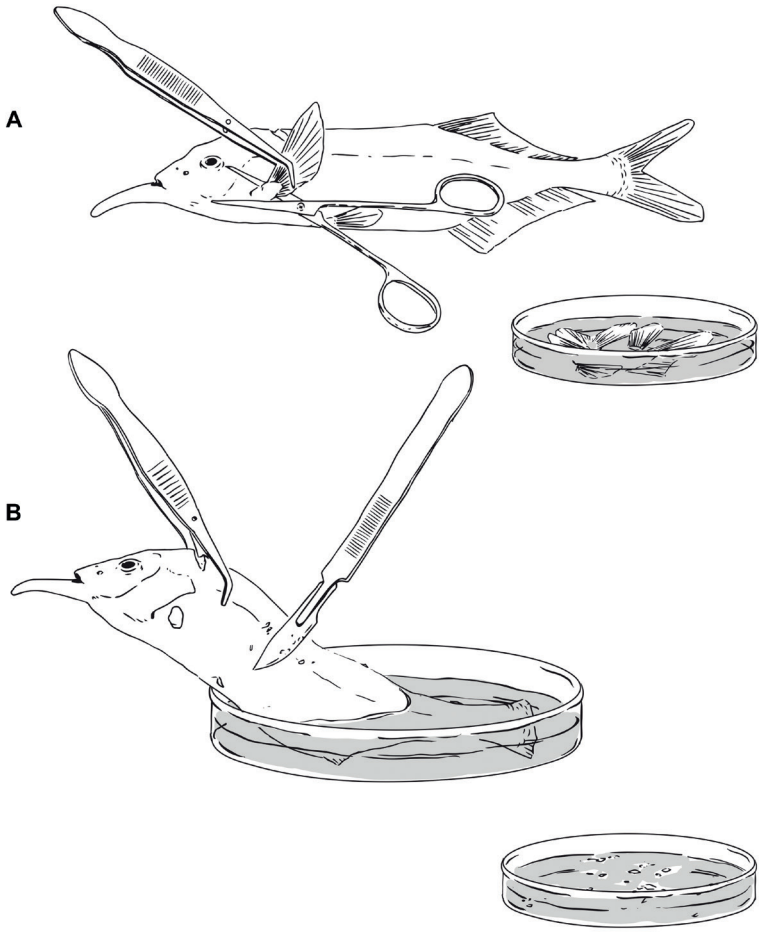

Fig. 3.3.3.1. Examination of fish for monogeneans. **A.** Cutting off fins; **B.** Scraping off mucus. (Illustration by M. Luo and E. Řehulková.)

PROCEDURE

1. Kill the fish using approved methods of euthanasia if it is not dead (*e.g.*, bought at the market or dead after capture).

2. Holding the fish with forceps, cut off the fins using scissors and place them in a Petri dish with water (preferably site water, *i.e.*, from the same source as the fish) (Fig. 3.3.3.1A).

3. Using a scalpel or slide, gently scrape mucus from the whole surface of the fish into a Petri dish with site water (Fig. 3.3.3.1B). If the fish is small (less than 10 cm), examine the whole fish directly under a dissecting microscope (magnification 20×). In this case, an upper illuminator for incident light viewing is required.

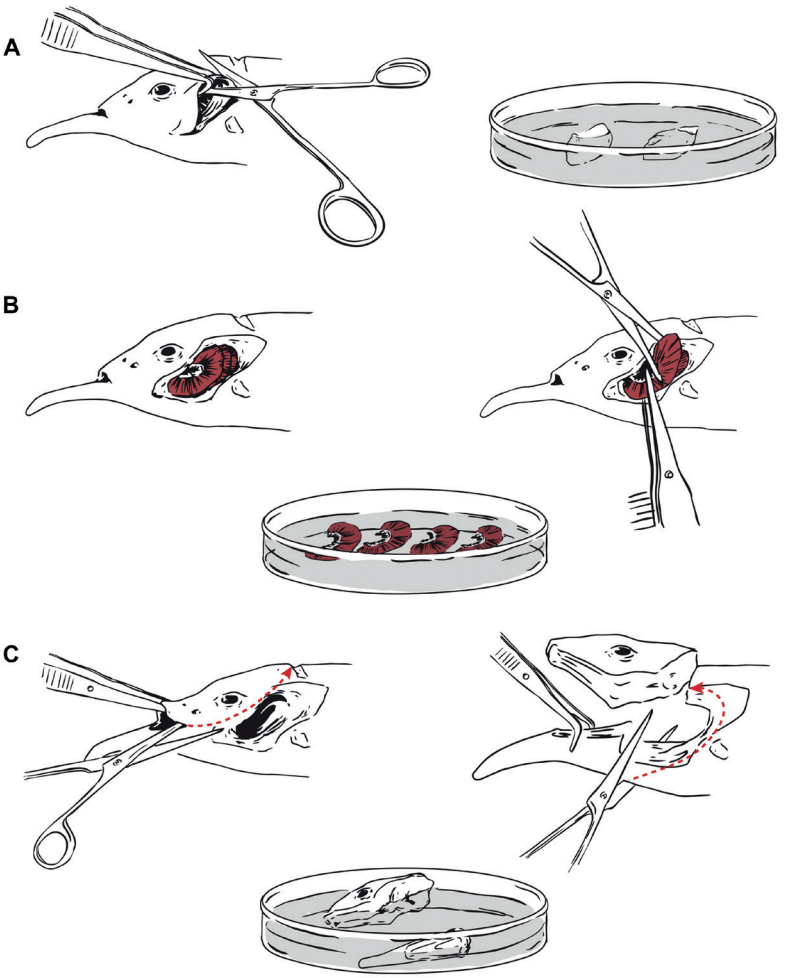

Fig. 3.3.3.2. Examination of fish for monogeneans. **A.** Removing of operculum; **B.** Extraction of gill arches; **C.** Separation of the upper part of the head from the lower part. (Illustration by M. Luo and E. Řehulková.)

4. Remove the operculum of the fish with scissors (Fig. 3.3.3.2A), cut off the gill arches (one by one) from the gill cavity and transfer them to a separate Petri dish with site water (Fig. 3.3.3.2B). If microhabitat preference is studied, each Petri dish should be labelled with the side/number of the gill arch (ideally 1 to 4 from external to internal).

5. Separate the upper part (nostrils, mouth) of the head from the lower part (mouth, pharynx, gill cavity); cut the mouth on both sides of the head towards the oesophagus (scissors following the dorsal side of the pharynx), decapitate the fish just behind the opercula, and place both parts directly in a separate dish with site water (Fig. 3.3.3.2C).

6. Carefully examine the mucus and all organs in Petri dishes with the aid of fine needles under a dissecting microscope at about 20× magnification. Check also the water in each Petri dish for detached monogeneans.

7. Carefully remove each worm from host tissues and place it in a drop of water on a slide, where it is can be fixed immediately (see below) or observed *in vivo* and photographed if the microscope is equipped with a digital camera.

8. After monogeneans from surface organs including gills are collected and fixed, the internal organs should also be examined for endoparasitic monogeneans (*e.g.*, species of *Enterogyrus* in the stomach of cichlids).

Fixation of monogeneans

A variety of methods are used to preserve monogeneans on slides, but some of them do not provide permanent preparations suitable for a deposition in museum collections as types (if a new species is described) or vouchers (faunal surveys and ecological studies). Basically, there are two methodological approaches to processing these parasites. The first one is focused on a study of sclerotised structures, the second one on observations of soft internal structures. To obtain the best results from both these approaches, two different preparation techniques should be used.

For a study of sclerotised structures the method of 'completely flattening' specimens is applied, where monogeneans are flattened under coverslip pressure until their body wall ruptures (see Fig. 3.3.3.3). Using this method, the vitelline follicles disintegrate after the rupture of the body and do not hamper observation of the male copulatory organ and vagina. If monogenean specimens are not sufficiently flattened, the shape of sclerotised structures may not be properly interpreted and their measurements tend to be shorter because of their twisted position. In contrast, coverslip pressure may affect the actual orientation of sclerotised structures with respect to the body axis. For that reason, the orientation of taxonomically important structures should be taken from non-flattened stained specimens.

Fixation to study sclerotised structures

To study the sclerotised structures of the haptor and the distal parts of the reproductive system (*i.e.*, male copulatory organ and vagina), the methods (formalin-glycerine fixative), proposed by R. Ergens in 1956 (in a Czech-written unpublished technical report) and later corroborated by Malmberg (1957; glycerine-ammonium picrate fixative or GAP), should be used. Formalin-glycerine fixative is prepared by mixing five parts of 4% formaldehyde solution and one part of glycerine/glycerol. GAP is prepared by mixing one part of saturated ammonium picrate solution and one part of glycerine.

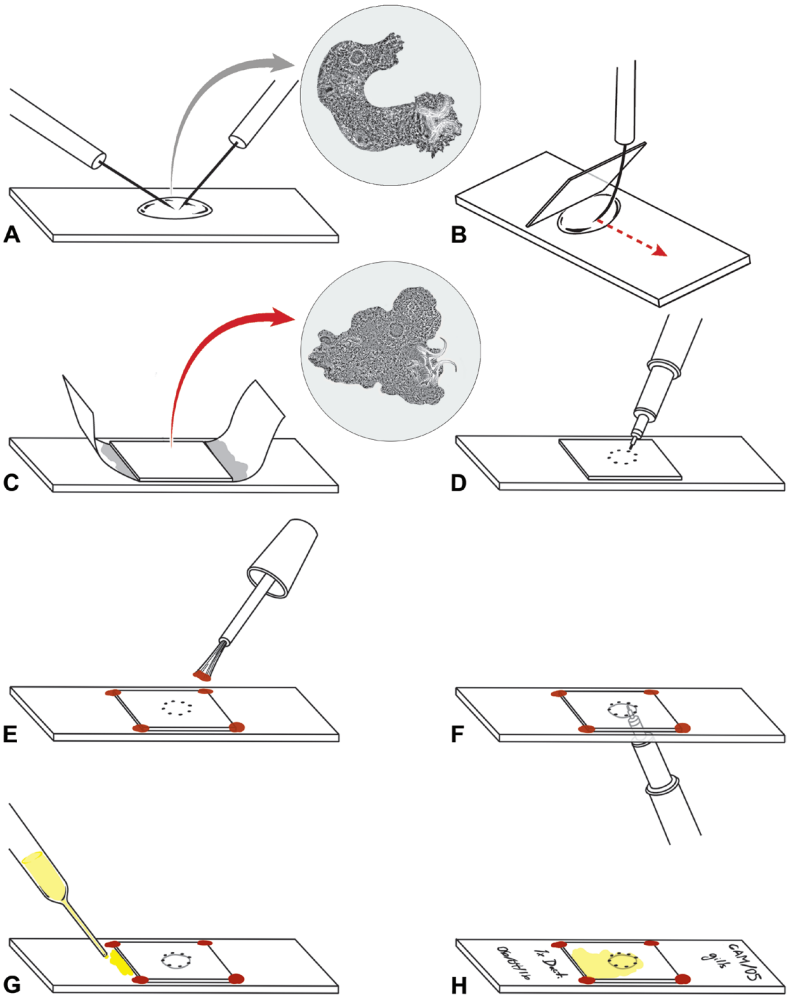

Fig. 3.3.3.3. Slide preparation of monogeneans mounted in GAP (glycerine-ammonium picrate) for subsequent morphological examination of the sclerotised structures. (Illustration by M. Luo and E. Řehulková.)

PROCEDURE (Fig. 3.3.3.3)

1. Place at maximum five clean worms, *i.e.*, worms without host tissue, mucus or any debris, which should be removed using fine needles, in a water drop on a slide using fine needles.
2. Lay a coverslip on the worm(s) while observing its/their position under a dissecting microscope to avoid the loss of the worm(s).
3. Remove excess water from under the coverslip by placing a piece of filter paper at the edge of the coverslip (best from both sides), thus further flattening the specimen(s) until the body wall ruptures.

4. Under the dissecting microscope, gently mark the position of the worm(s) by a dotted circle on the upper side of the coverslip.
5. Seal all four corners of the coverslip with Noyer's lacquer or nail varnish.
6. Trace the dotted circle (using an ethanol-resistant pen) around the worm on the reverse (lower) side of the slide.
7. Add a small drop of formalin-glycerol fixative (or GAP) on the edge of one side of the coverslip. Avoid adding a large volume of fixative as it can lift the coverslip and the flattened/ruptured worm(s) will disintegrate.
8. Label the slide (using an ethanol-resistant pen) with a field number (unique code) of the fish examined, date of collection, infection site (on the host), higher-rank taxon name (usually family) to which the specimen belongs (if known), or unique code of the worm (if part of it was fixed separately for subsequent DNA analysis).
9. Leave the slide on the table in a horizontal position to saturate the worm with formalin-glycerol (or GAP) for a couple of hours (overnight) before storage.
10. Seal the coverslip with enamel paint (nail varnish or Canada balsam) to prevent the mount from drying out.

Since both formalin-glycerine fixative and GAP are semi-permanent mediums, it is necessary for long-term storage, including deposition in museum collections, to remount these preparations using the method of Ergens (1969). This method produces permanent mounts, but some worms may be lost during the remounting procedure, especially if they are broken when the coverslip is detached. It is therefore strongly recommended to make drawings and take measurements from formalin-glycerine or GAP-fixed specimens before remounting them; another option is to take a photo as a photo-voucher.

Fixation to study the soft structures and further processing

To observe the soft internal structures, monogeneans should be relaxed during fixation and then stained with appropriate stains. Fixation with a fixative at ambient temperature (4% formalin or 70% ethanol) is useful only when monogeneans are being (moderately) flattened under a light coverslip pressure. This is best accomplished by placing the worms in a drop of water in a small Petri dish and covering them with a coverslip with a small weight on top (*e.g.*, a metal bolt or nut of approximately 2 g). It is important to note that too much pressure will distort the arrangement/size of the internal organs. Fixation with a hot fixative can avoid this disadvantage. Using hot 4% formalin is the best option (similarly as for trematodes, tapeworms and nematodes – see Chapter 3.3.4), because it penetrates fast into tissues and makes them well-preserved and more suitable for staining compared with samples fixed using hot water. If heating formalin is a practical problem, hot water can be used as described by Justine *et al.* (2012). Hot-water fixation makes it possible to use the worms for both morphological observation (after fixing with 4% formalin or 70% ethanol and subsequent staining) and DNA sequencing (fixed worms are immediately placed in molecular grade 96-99% ethanol).

Fixation of monogeneans for molecular studies

Even though the identification of monogeneans is based mainly on morphological characteristics, molecular data are important for taxonomic, phylogenetic and ecological studies. Therefore, it is strongly recommended always to fix some (parts of) specimens (see below) for genetic analyses (DNA sequencing). Simultaneous infections of fish with several, morphologically similar species represent a serious obstacle in molecular studies because the identity of sequenced worms cannot be ascertained without the availability of a corresponding morphological voucher, *i.e.*, hologenophore (see Pleijel *et al.* 2008 for terminology). In this case, the worms should be divided into three parts; the anterior body part comprising the male copulatory organ and the posterior part with the haptor are prepared for morphological observation as described above (*i.e.*, fixed with formalin-glycerine or GAP), whereas the middle part of the body is fixed in molecular-grade ethanol. However, this procedure is often inapplicable due to the small size of most monogeneans (*i.e.*, species of the families Dactylogyridae and Gyrodactylidae). Therefore, worms are cut just into two parts; that part which enables species-level identification (the posterior part with the haptor in gyrodactylids and diplozoids, the anterior part with the male copulatory organ in dactylogyrids) is fixed for morphological study and the remaining half of the body is fixed for molecular work.

It is important to note that only live or ethanol-fixed monogeneans are suitable for molecular studies. Formalin-fixed worms should not be used because their DNA is fragmented or considerably damaged. The procedure for dividing worms for both morphological and molecular studies is briefly described below.

PROCEDURE (Fig. 3.3.3.4)

1. Place the living or ethanol-preserved worm in a drop of water on a slide.
2. Under a dissecting microscope, divide the body of the worm into two parts using fine needles.
3. Transfer half of the body which does not contain the most important diagnostic structures, to an Eppendorf tube with molecular grades, *i.e.*, non-denaturated 96-99% ethanol and, if possible, store the sample in a refrigerator or freezer.
4. Fix the rest of the body in formalin-glycerine or GAP under coverslip pressure (if the worm is alive) or with Hoyer's medium, as described below.
5. Use identical labelling for the tube and slide to match the morphological voucher (hologenophore and paragenophores) with the sample to be sequenced.
6. After morphological evaluation, deposit the hologenophore in an internationally accessible collection, ideally together with type (holotype, paratypes) or voucher specimens from the same host.

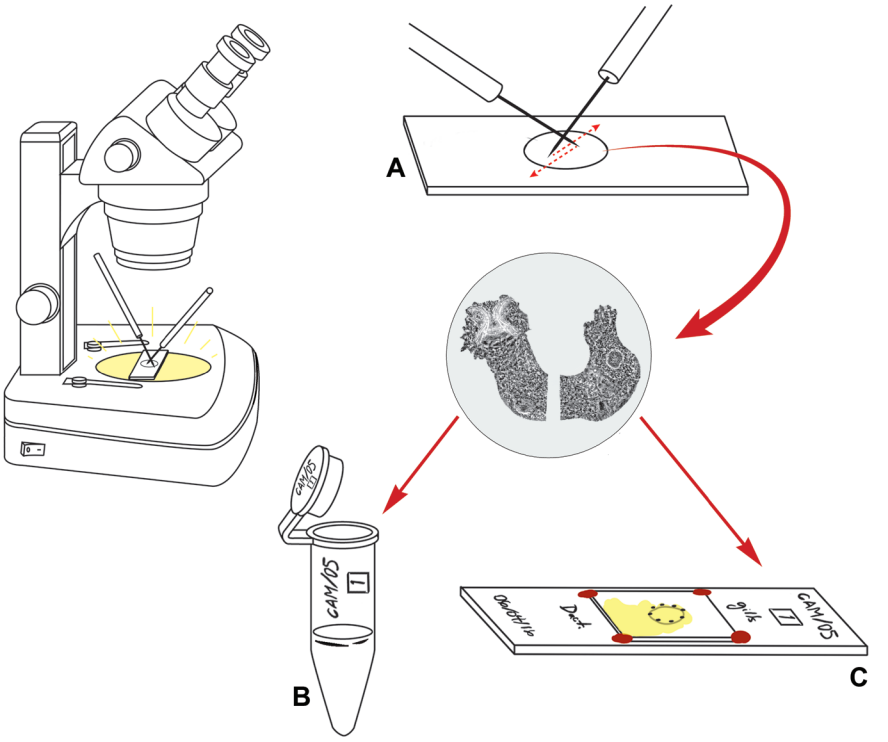

Fig. 3.3.3.4. Collection and identification of specimens for DNA analyses: specimen bisection using fine needles. (Illustration by M. Luo and E. Řehulková.)

Processing of fixed/preserved monogeneans

Study of sclerotised structures

Fixed/preserved monogeneans in vials are observed after being placed into a drop of water on a slide, removing excessive water and mounting them in Hoyer's medium. The slides should be kept in a horizontal position until the medium had solidified. As the worms are cleared rapidly, sclerotised structures and internal organs can be readily observed. Since this is a semi-permanent medium, it is best to ring the coverslip with enamel paint (or Canada balsam) after the medium has solidified. Hoyer's medium is prepared by mixing 30 g Arabic gum, 50 ml distilled water, 20 ml glycerol and 200 g chloral hydrate, followed by filtering the solution through 8-10 layers of cheesecloth or fine gauze before use (Ash & Orihel 1991).

Study of soft internal structures

To study soft parts, the monogeneans should be studied following staining. Different stains, mostly carmine-based, are used to visualise the internal structures and organs of monogeneans, *e.g.*, iron acetocarmine (Georgiev *et al.* 1986; see the procedure below), Schneider's acetocarmine, Mayer's acid carmalum, Gomori's trichrome, etc. (see also Humason 1979; Ash & Orihel 1991 for more details on several staining techniques). After staining, the worms are dehydrated in ascending series (increasing concentration) of ethanol, cleared (with clove oil or xylene), and finally mounted in Canada balsam as permanent preparations, which are suitable for long-term storage in museum collections.

PROCEDURE

1. Prior to staining, rinse the fixed worms in distilled water (30-60 min); worms fixed/preserved in 70% ethanol can be stained directly, without previous rinsing.
2. Transfer the worms to iron acetocarmine in a small Petri dish and keep them in the staining solution until they acquire a deep red colour (1-10 hours).
3. Rinse the worms by placing them into 70% ethanol.
4. Destain the worms in a weak solution of acid ethanol (1 ml or 4 drops of concentrated hydrochloric acid in 100 ml of 70% ethanol); leach the colour from the worms until they turn into a pale pink whereas the internal organs remain red-coloured. Destaining may take from several minutes to several hours, but it must be observed carefully to avoid excessive destaining. If too much stain is removed, rinse the specimens in 70% ethanol and return them to the stain (*i.e.*, start again with step 2), otherwise continue with step 5.
5. Rinse the worms by placing them into tap water until they turn into a deep red colour.
6. Dehydrate the worms through 70% (5 min), 96% (10 min) and 100% ethanol (5 min).
7. Clear the worms in clove oil (eugenol) for 5 min.
8. Mount the worms in Canada balsam as permanent slides.

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3.3.4. ENDOPARASITIC HELMINTHS

Tomáš SCHOLZ, Roman KUCHTA, Šárka MAŠOVÁ & Aneta KOSTADINOVA

Introduction

In the present text, general study methods are briefly described for different groups of endoparasitic helminths, *i.e.*, adults and larvae of flukes (Trematoda, *i.e.*, Aspidogastrea and Digenea), tapeworms (Cestoda), parasitic nematodes (Nematoda) and spiny- or thorny-headed worms (Acanthocephala). The main focus of this section is on the most important steps in searching for endohelminths and on their fixation and processing to ensure adequate quality of the material collected for subsequent evaluation. More detailed information can be found in specialised papers or books on individual groups of endoparasitic helminths.

Examination of fishes for endoparasitic helminths

Parasitological (helminthological) dissection is the basic method to obtain parasites. The extent of the examination depends on the objectives of a given study. The present text is focused on endoparasitic helminths and thus only the examination of internal organs will be described. As mentioned in the introduction to this methodological section (see 3.2), it is necessary to examine fresh hosts because worms, especially tapeworms and tiny trematodes, die quickly following the host's death. As a result, endoparasitic worms from long-time dead or frozen hosts are decomposed and unsuitable for subsequent studies including their reliable identification. Data on the host identity, site of infection, number of specimens found and fixed, fixative used, the date of dissection and the name of the collector should be written in a field notebook. It is highly recommended to record this information digitally on spreadsheets (such as Excel files) following fieldwork.

PROCEDURE (Fig. 3.3.4.1)

1. Take (a) photograph(s) of the host to be examined (the head of the fish should be on the left side) with its unique code (see below) and measurements (usually total and standard lengths). It is strongly recommended to excise a small piece of fish tissue (*e.g.*, muscle, fin – 'finclip', or liver) and fix it in molecular-grade ethanol to allow DNA-based identification of the host or other genetic work on the hosts, *e.g.*, barcoding, co-phylogenetic work, etc.

2. Place the complete digestive tract and other internal organs either in a suitable Petri dish or on a glass plate and add a small volume of saline (0.8-0.9% physiological solution, *i.e.*, 8-9 g of NaCl in 1 l of water). Under no circumstances should the organs dry out. In the tropics, you can add small pieces of ice to the Petri dish with the organs to cool the saline and thus slow down the decomposition of organs and parasites. Add labels with a unique host

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

Iva DYKOVÁ

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).

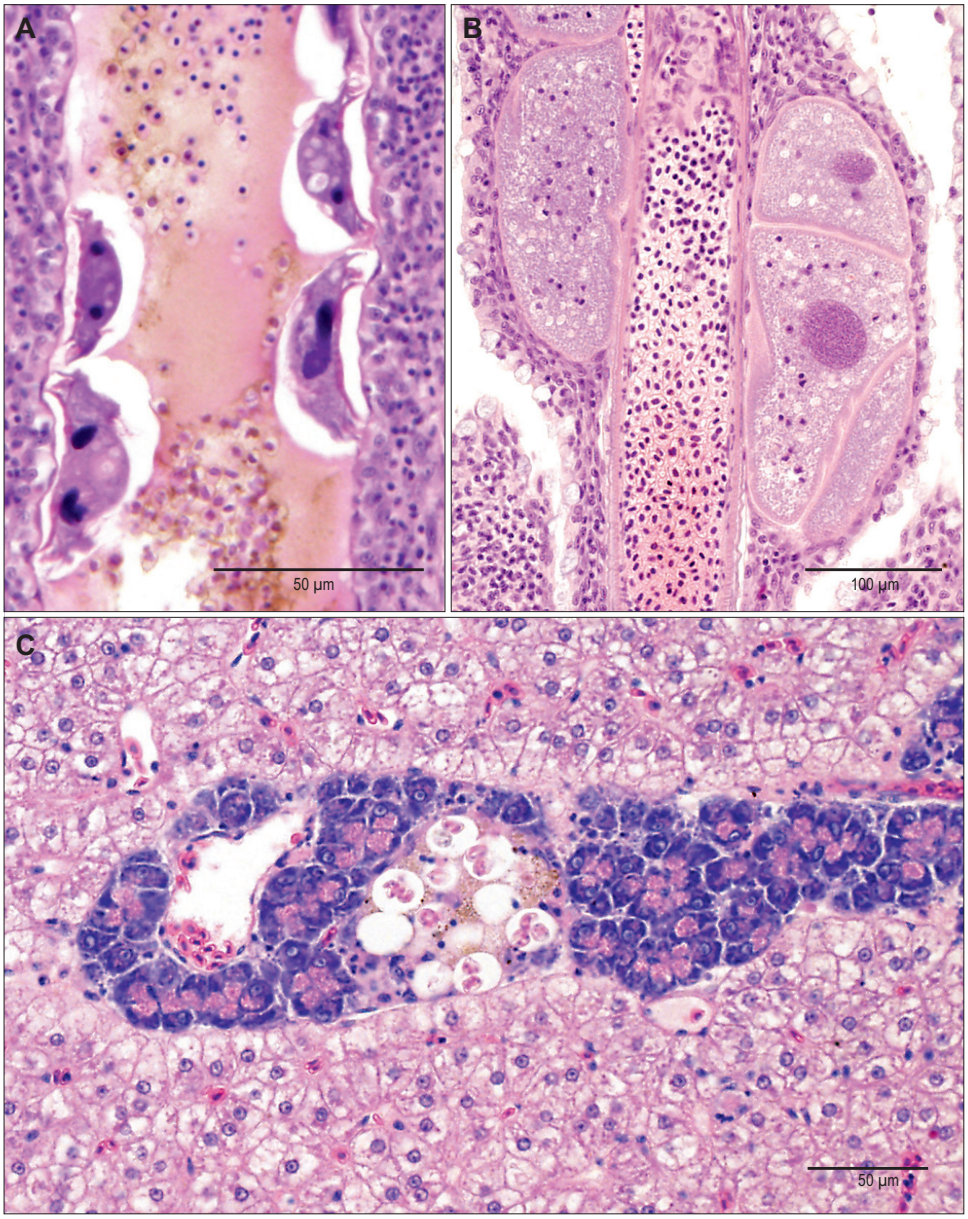

Fig. 3.4.1. A. Trichodinid ciliates seen on the surface of gill filaments of cichlid fish; **B.** Histophagous ciliate *Ichthyophthirius multifiliis* Fouquet, 1876 in the gill filament tissue of *Pseudotropheus* sp.; trophozoites with prominent macronuclei and host cells in the cytoplasm; **C.** Thin-walled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

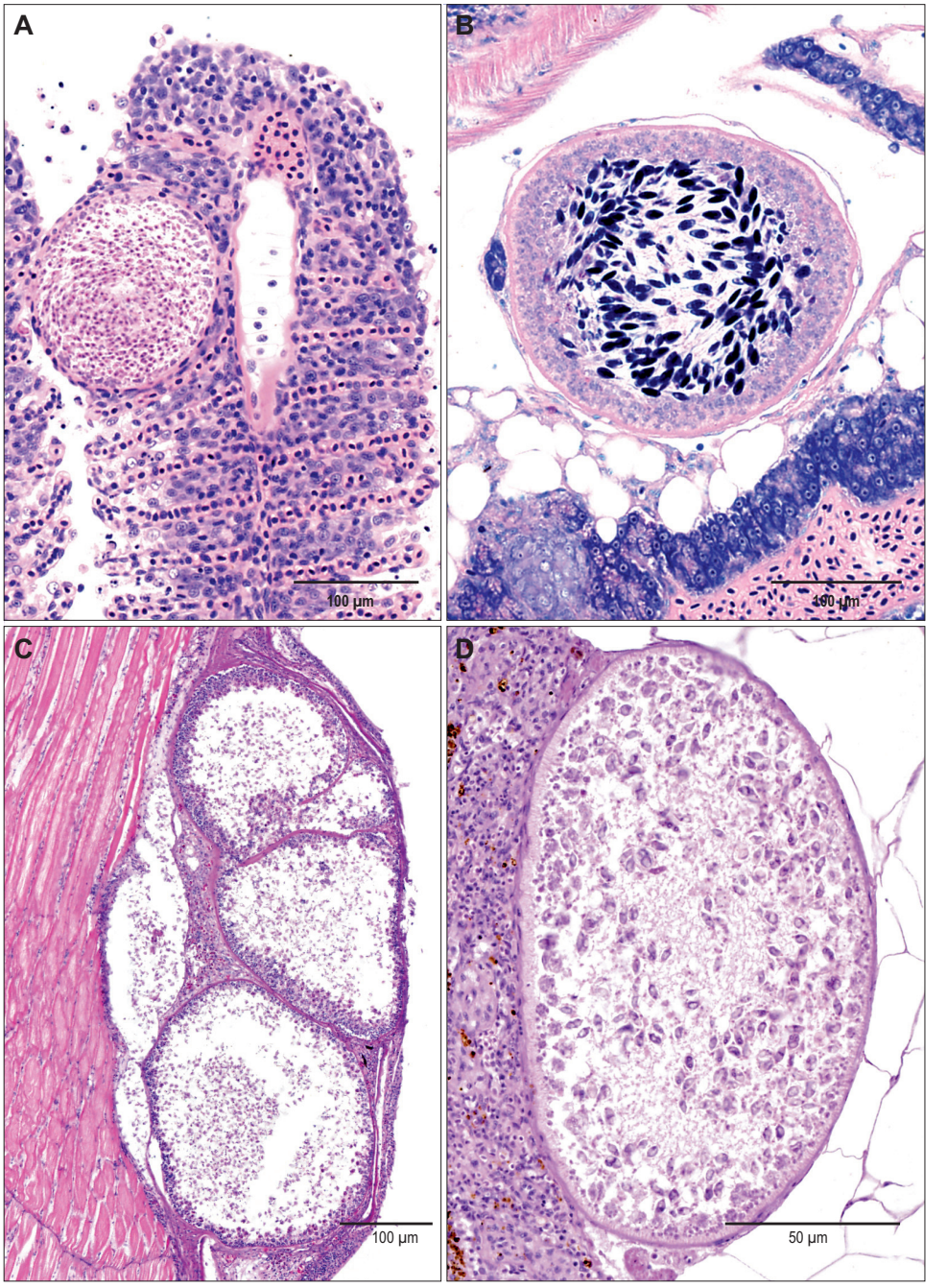

Fig. 3.4.2. A. Polysporic plasmodium of a myxosporean in the gill tissue of *Cichlasoma* sp. H & E; **B.** The plasmodial stage of a myxosporean species localised in the body cavity of *Leporinus* sp. contains intensely stained myxospores. Giemsa stain; **C.** Four myxosporean plasmodia localised subcutaneously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

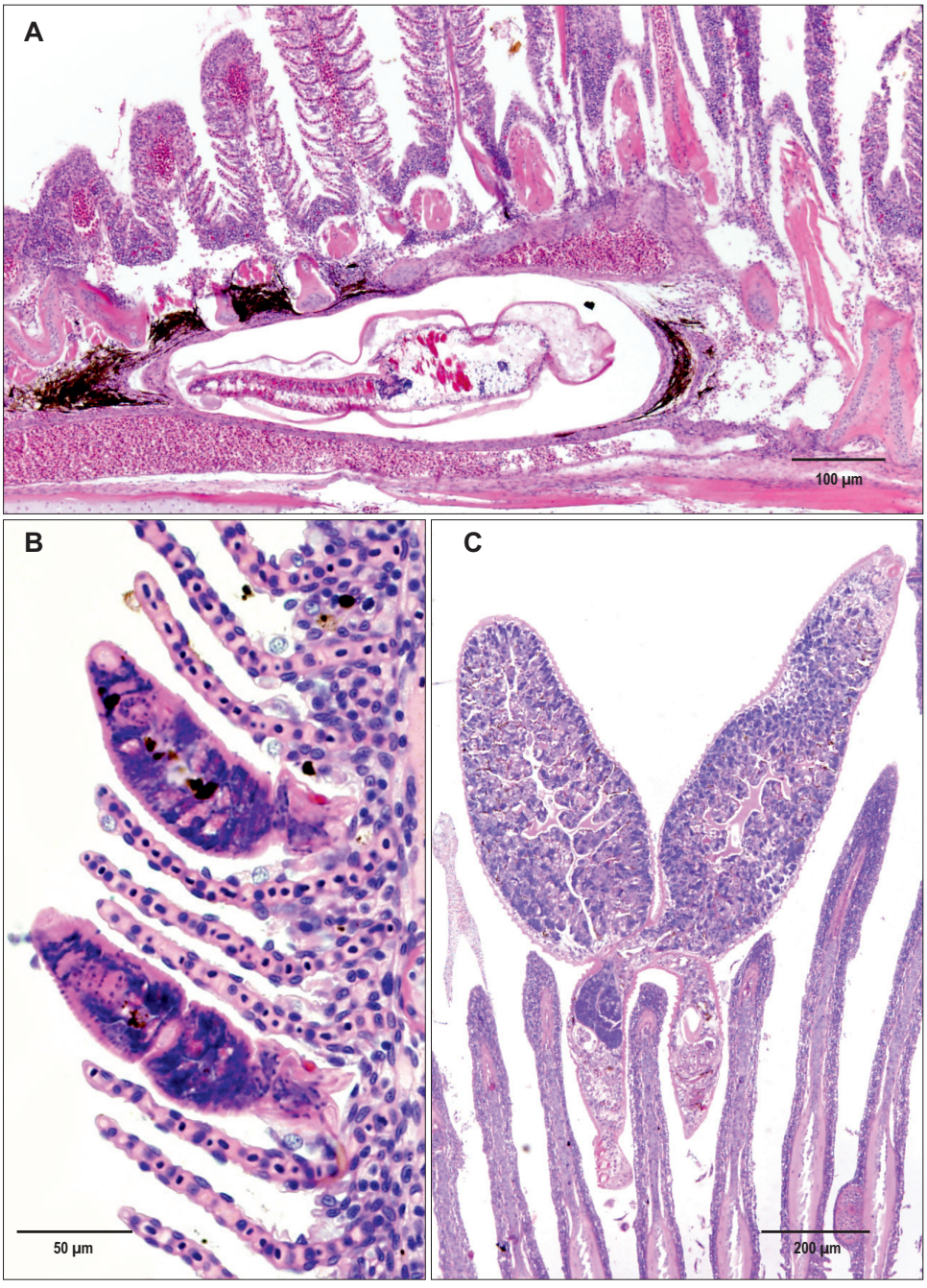

Fig. 3.4.3. A. Metacercarial stage of a digenean (Trematoda) in the gill arch of *Cichlasoma* sp.; **B.** Longitudinal sections of dactylogyrid monogeneans among secondary gill lamellae of *Haplochromis* sp., attached to epithelial tissue of gill filament; **C.** Complete section of a polyopisthocotylid monogenean *Diplozoon* sp. among gill filaments exemplifies the potential of histology in parasite identification. All H & E.

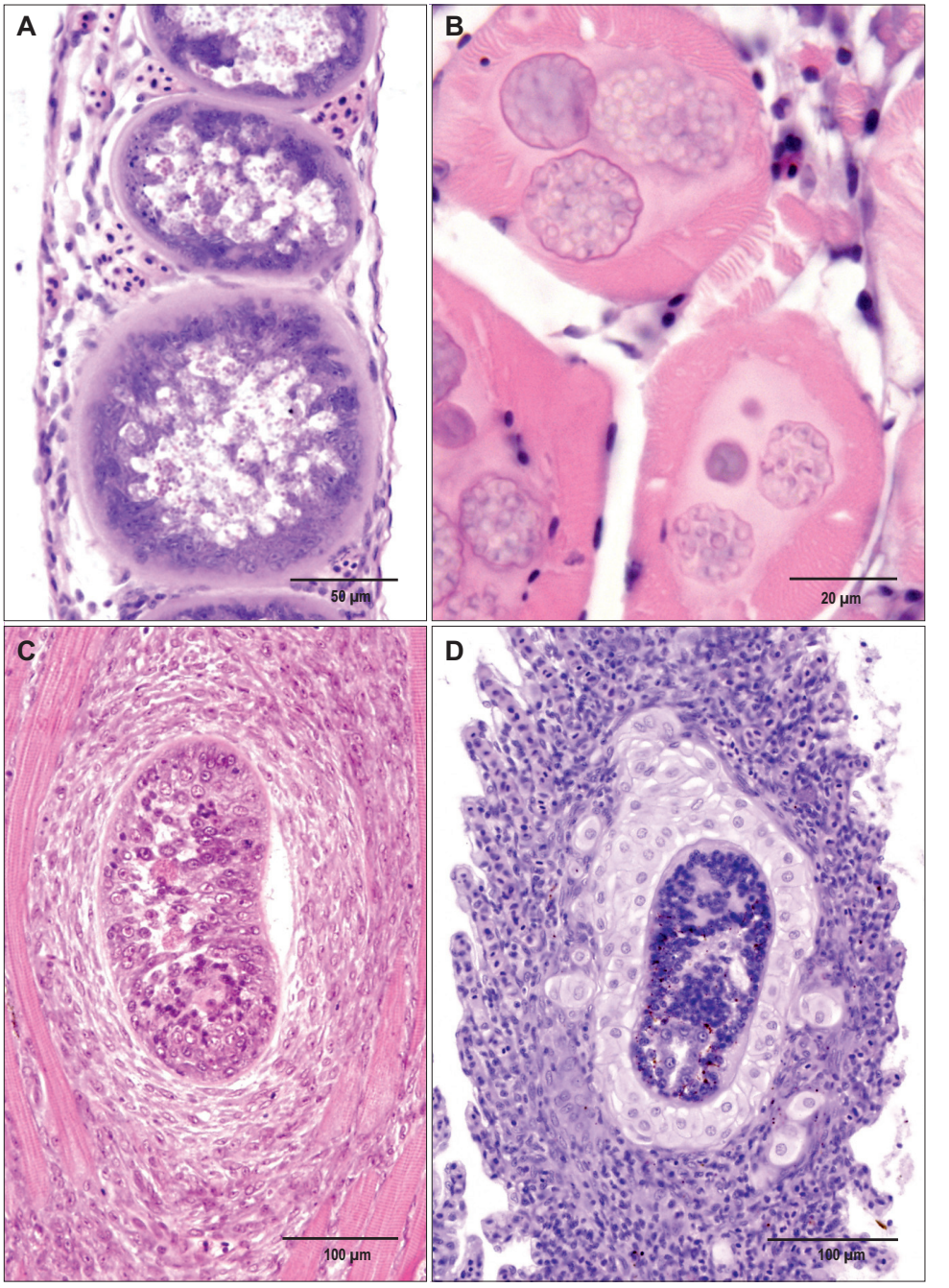

Fig. 3.4.4. A. Xenoma formations induced by microsporidia in connective tissue of *Nothobranchius* sp. Spores concentrated in the centre are almost indistinguishable whereas the whole formation can reach macroscopically visible size; **B.** Developmental stages of microsporidia in muscle fibres of *Paracheirodon innesi*; **C.** Metacercaria of a digenean trematode in muscle tissue of *Haplochromis* sp.; **D.** Metacercaria in cartilage of gill filament. All H & E.

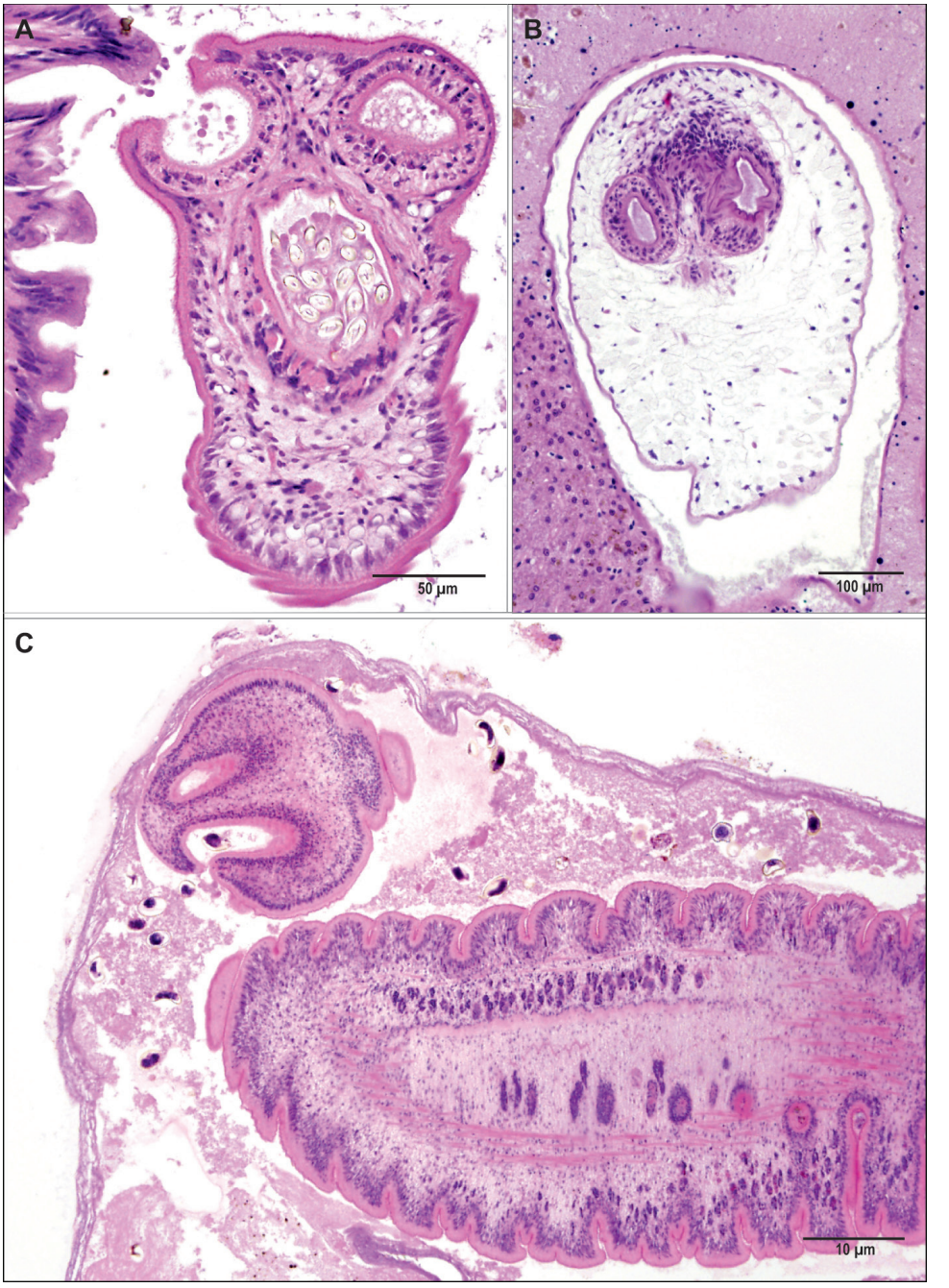

Fig. 3.4.5. A. A sucker-bearing gryporhynchid cestode with hooks on the rostellum in the intestine of *Sandelia* sp.; **B.** Larval stage (plerocercus) of a gryporhynchid cestode in the liver of a cichlid fish; **C.** Cestode *Schyzocotyle acheilognathi* (Yamaguti, 1934) with a pair of dorsoventral grooves (bothria), part of neck and a short part of the strobila in the intestine of *Symphysodon* sp. The fish tissue is autolytic whereas the structures of cestode are well maintained. All H & E.

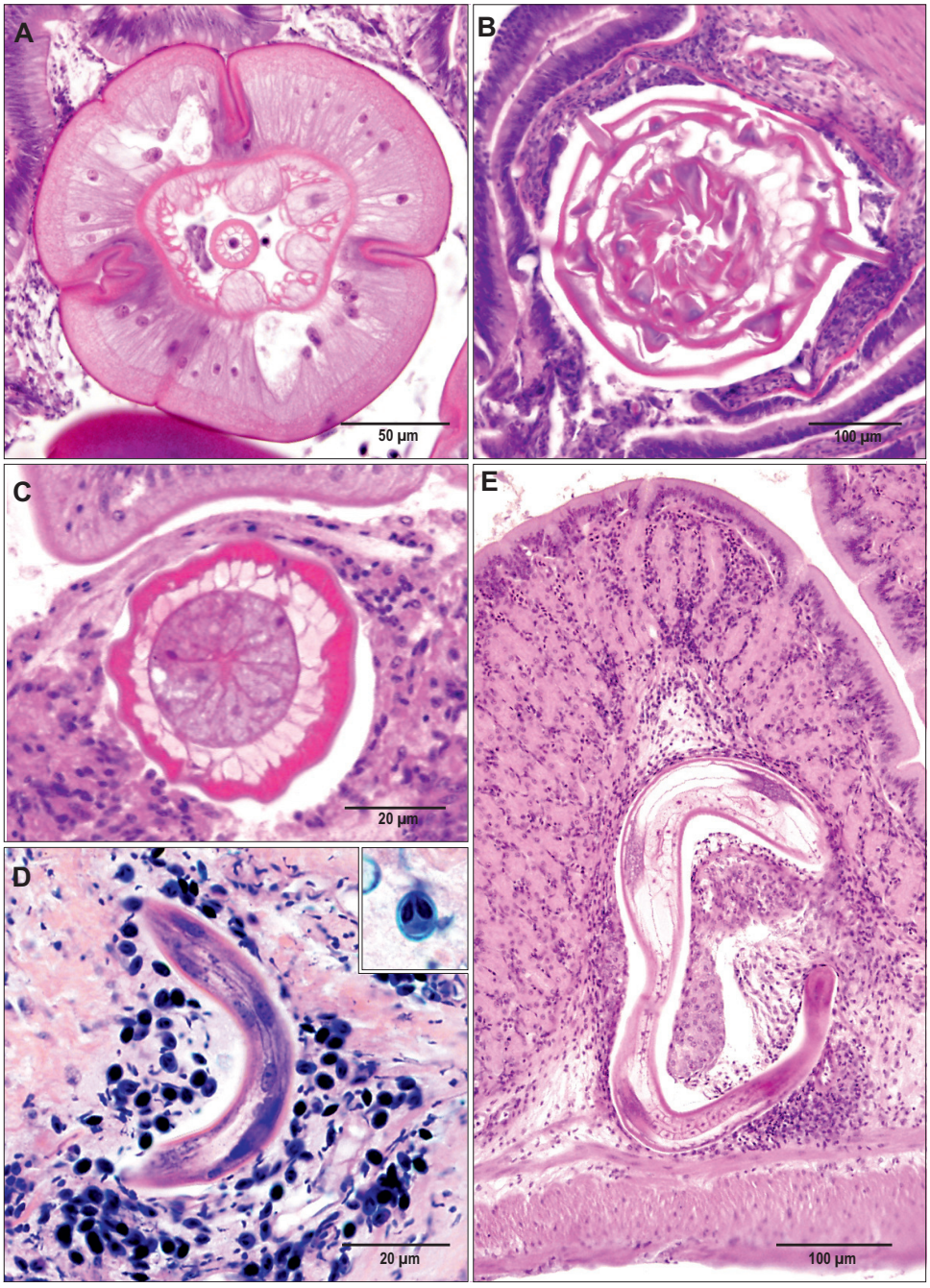

Fig. 3.4.6. A. Transverse section of the anterior part of an acanthocephalan. H & E; **B.** Transverse section of everted acanthocephalan proboscis armed with hooks. H & E; **C.** Transverse section through the oesophagus of a nematode. H & E; **D.** Larval stage of a nematode in host connective tissue. Also note the darkly stained myxospores (inset). Giemsa stain; **E.** Larval stage of a nematode in connective tissue of the stomach. H & E.

Fixation

The objectives of fixation are to preserve tissue samples, preventing autolysis and putrefaction. Fixation has to be adequate and complete. These requirements determine the type of fixative and the method of fixation applied. There are routinely used fixatives which fix tissue samples relatively slowly (*e.g.*, neutral buffered formalin solution), aggressive, rapidly penetrating fixatives (*e.g.*, mixtures of formol, acetic acid and ethanol), and fixatives which preserve specific cell components for specific staining procedures (*e.g.*, non-aqueous fixatives for glycogen). If a fixative causes tissue distortions and deformities, it is recommended to trim tissue samples before the next step (dehydration) starts.

Dehydration

To avoid excessive shrinkage of tissue samples, which ultimately causes difficulties in the evaluation of lesions, water should be eliminated from the samples almost completely by using ascending grades of ethanol before being transferred into an organic solvent. The best results are obtained with adequate concentrations of ethanol and adequate exposure times to ethanol and organic solvent.

Embedding

Embedding following dehydration consists of gradual impregnation of tissue samples with a firm medium (paraffin with a melting point of 56.6°C, mixtures of paraffin with other components, etc.) and blocking out in appropriate moulds. Of the considerations that should be kept in mind in the three-step impregnation procedure (three baths of paraffin), the most important ones are to follow the impregnation schedules given for each paraffin to eliminate remnants of organic solvents (xylene, toluene, etc.) completely. Too long exposures in paraffin and/or the presence of solvents in the last paraffin bath impair the quality of blocks and sections. The paraffin-impregnated tissue should be oriented with the side of interest facing the bottom of the mould.

Sectioning

The essential equipment required for sectioning properly prepared tissue blocks includes a microtome adjusted for the type of knife used, a water bath, slides and a hot plate (or a safe place to dry sections). To ensure good results from the sectioning, several adjustments may prove necessary. Of those, the crucial one is an appropriate knife angle as specified by the manufacturer. Also important are the temperatures of the water bath, hot plate and oven (with paraffin, with its melting point 56.6°C, neither of these temperatures should exceed 45°C) and drying the sections completely.

Staining of histological sections

The staining procedure completes the preparation of tissue material for histological examination. It includes deparaffinisation of sections with xylene or another organic solvent, their hydration to water (through descending concentrations of ethanol), staining proper and counterstaining, followed by dehydration (through an ascending series of ethanol), clearing (with xylene) and mounting in a medium of choice. It is advisable to have a sufficient number of consecutive sections in order to avoid missing important details which might require special staining.

Haematoxylin and eosin are universally accepted basic dyes used to demonstrate tissue morphology. Haematoxylin stains the nuclear material whereas eosin stains the cell cytoplasm. Some special methods also deserve to be mentioned here, *e.g.*, the Periodic Acid Schiff reaction for demonstration of mucopolysacharides, Trichrom methods with various counterstaining agents for connective tissue, Van Gieson's method for collagen, Giemsa staining for protozoans, Gram's stain for Gram positive and Gram negative bacteria, Ziehl-Nielsen for acid alcohol fast organisms, Von Kóssas for demonstration of calcium salts, Perl's method for iron, Gomori for fungi, etc. There are many methods elaborated by specialists and many modifications of individual techniques. One can find dozens of recipes or modifications of basic staining methods but hardly ever a clear explanation of the chemical processes taking place during the staining. More than 50 staining procedures have been modified for microwave technology, saving time and liquids. For more detailed information, see list of references below.

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Chapter 3.5.

MOLECULAR TAXONOMY

Ivan FIALA & Jan BRABEC

Introduction

Deoxyribonucleic acid (DNA) sequences are a valuable source of information that stores the elementary instructions for how individual parts of an organism should be assembled and operate. DNA-encoded information can also be used to gain insights into the evolutionary history of an organism. Recovering this information has become an essential strategy to study and compare organisms. The field of downstream computational molecular evolution approaches has grown into a complex and rapidly evolving scientific discipline.

Analyses of DNA sequences have become an important part of various studies on the parasites of fish globally, including alpha taxonomy, diagnostics of disease agents, phylogeographical distribution and various studies on the biology of parasites including ecological, life cycle or host specificity-oriented surveys, to name a few. In strong contrast to that, the use of molecular data in studies on African fish parasites remains limited. Studies of Pouyaud *et al.* (2006), de Chambrier *et al.* (2008), Kuchta *et al.* (2012) and Přikrylová *et al.* (2013) are among the few available examples, where analyses of gene sequences assisted substantially in resolving the phylogenetic position of various fish helminths from the African continent. Schaeffner *et al.* (2011) and Chibwana *et al.* (2013) used molecular data to study phylogenetic relationships within individual genera of fish tapeworms and trematodes, respectively.

Co-phylogenetic analyses allowed Mendlová *et al.* (2012) and Vanhove *et al.* (2015) to propose speciation mechanisms in monogeneans infecting African cichlid fishes. Bouzid *et al.* (2013) studied genetic divergence within populations of the diphyllobothriidean cestode *Ligula intestinalis* (Linnaeus, 1758) using highly variable sequences of non-coding regions of DNA, whereas Kmentová *et al.* (2016) used sequence data from the nuclear ribosomal DNA region and the cytochrome *c* oxidase subunit I gene to look at, respectively, host range and intraspecific diversity in the dactylogyrid monogenean *Cichlidogyrus casuarinus* Pariselle, Muterezi Bukinga et Vanhove, 2015. Brabec *et al.* (2016) used next generation sequencing to study intraspecific differences within isolates of the invasive Asian fish tapeworm *Schyzocotyle acheilognathi* (Yamaguti, 1934) parasitising African fishes. Additionally, sequence data are frequently used in species descriptions to support the identification or discovery of parasite species, or to get an idea of their phylogenetic position.

Among the molecular markers most frequently used to study phylogenetic relationships and life history characteristics of fish parasites are ribosomal RNA (rRNA) encoding genetic loci, which include three rRNA encoding genes called 18S, 5.8S and 28S rDNA, according to their molecular weight, as well as the noncoding sequences of so-called internal transcribed spacers 1 and 2 (ITS-1, ITS-2) that are situated between 18S and 5.8S, and 5.8S and 28S rDNAs, respectively. Individual coding and noncoding regions from this genetic locus together form a unit called the nuclear rRNA operon, which is typically found in several hundreds to thousands of copies that are tandemly repeated one unit after another on certain chromosomes, depending on the parasite group. A wealth of information on molecular characteristics of rDNA has been summarised by Blair (2006) for parasitic flatworms and by Fiala *et al.* (2015) for myxozoan parasites.

Given the heterogenetic nature of individual parts of the nuclear rRNA operon, individual regions differ by their relative mutation rate and thus their speed of evolution. Therefore, they can be used across a range of taxonomic levels, spanning from populations of a single parasite species to orders and classes of parasites. Typically, the noncoding regions (such as ITS regions) are used at lower taxonomic levels (*i.e.*, populations and species), whereas the gene sequences (18S, 28S rDNA) are useful at higher levels, typically from genera to orders. However, for many parasitic groups, *e.g.*, Myxozoa or ciliates of the family Trichodinidae, 18S rDNA is a standard universal marker from species to order levels (Tang *et al.* 2013; Fiala *et al.* 2015). Combination of 18S and 28S rDNA or both noncoding and coding regions can be used in studying the phylogenetic relationships of parasites (*e.g.*, Bartošová *et al.* 2009; Přikrylová *et al.* 2017). Thanks to the presence of relatively conserved regions, rRNA loci can be characterised using a universal set of short strands of nucleotides called primers that are necessary to amplify a given region of DNA during polymerase chain reaction (PCR).

Sequences of mitochondrial protein-coding and rRNA-encoding genes (mitochondria are remnants of a primary endosymbiotic event and thus carry their own pair of rRNA genes originally belonging to an alpha proteobacterium) are further examples of commonly used molecular tools. Contrary to the nuclear rDNA, their overall speed of evolution tends to be higher (in some cases roughly comparable to ITS regions of the nuclear rRNA operon). This makes mitochondrial genes useful candidates for lower-level taxonomical studies. However, they may also be used on higher taxonomic ranks, when the protein-coding nucleotide sequences are translated into the corresponding sequence of amino acids. However, the increased mutation rate also means that universal primers are difficult to design. Moreover, flatworms substantially differ from other metazoans in amino acid content over cytochrome c oxidase I, *i.e.*, the sequence homology of flatworm and other metazoans' *cox*1 sequences is generally lower than sequence homology within metazoan *cox*1 (Vanhove *et al.* 2013) and researchers are thus left with no other option than to design a specific set of primers for their parasitic group of interests.

Recently, next generation sequencing techniques have been developed and gained popularity to bulk-characterise sequence data on large scales (*i.e.*, from thousands of loci to entire genomes) without previous knowledge of primer sequences. However, these sequencing approaches remain expensive and require the use of sophisticated technologies and highly trained laboratory staff and bioinformaticians, and are thus not suitable for routine taxonomy.

Fixation of fish parasites for molecular studies

The most critical step that allows successful isolation of DNA and generation of sequence data is quick and correct processing of the dissected parasite tissue and its immediate preservation in a suitable preservative. As a rule of thumb, parasites should be processed after their isolation from the host without any time delays, preferably immediately after the host's death. Extracted parasites (or infected tissues) should either be immediately preserved or kept in conditions that allow parasite survival (*i.e.*, in cool temperatures, appropriate pH and salt concentration). Before being completely submerged in the appropriate preservative (see Chapter 3.3), cells of parasitic protists or tissues of metazoan parasites have to be carefully cleaned of any remnants of the host cells and tissues, eliminating carry-over and subsequent simultaneous extraction of host DNA. Nearly absolute (96-99%) molecular-grade ethanol is used as a preservative of choice, notably in hot weather climate conditions.

DNA sequencing

Sequencing of selected molecular markers includes several steps (principally DNA isolation, PCR amplification and electrophoresis), which require adequate equipment and laboratory experience. A number of essential laboratory skills need to be acquired first to ensure successful and safe work in the laboratory. A good start for those not familiar with basic laboratory practice is to get familiar with individual chapters of the Current Protocols Essential Laboratory Techniques (http://onlinelibrary.wiley.com/book/10.1002/9780470089941) and preferably to obtain practical skills personally in an established laboratory under the supervision of a technician experienced in all relevant methods. Most of these complex issues can be eased through collaboration with an expert parasitologist with a publication record that includes the use of molecular taxonomy and phylogenetic approaches.

DNA isolation

The first step in the entire process of characterising novel sequences is isolation and purification of the DNA from the cells, the basal building blocks of any parasite's body. Within the cells, the DNA is located in membrane-bound organelles, where it is part of high-molecular complexes that consist of DNA itself together with a number of associated proteins. The goal of the DNA extraction step is to get the DNA out of these cells, into a protein- and other contaminant-free water solution called a buffer. It is essential to obtain well-purified DNA in this step, otherwise the following step (*i.e.*, PCR amplification) is likely to fail.

Generally, there are two basic, frequently used ways of isolating DNA. The first involves the use of a commercial DNA extraction kit (basically a box that includes all the chemicals and silica membrane spin columns necessary for DNA isolation, commercially available from many biotech companies). The second option is to go through a more traditional procedure called phenol-chloroform extraction. Both of these methods can vary slightly from one another according to the company that manufactures the kit and the authority that originally established the actual phenol-chloroform protocol. General principles and practical descriptions of sample protocols can be found in Dowhan (2012). As an oversimplification, both DNA extraction strategies are based on the digestion of the cells or tissue and the separation of the DNA from its associated proteins in a clean, water-based buffer.

Independent of the extraction protocol, all workflows start with transferring a certain volume of parasite cells or a small piece of tissue from the ethanol preservative into a new, clean 1.5 ml Eppendorf tube. The transferred material needs to be ground into as small pieces as possible (in the case of tissue) without risking the actual loss of the tissue, especially when you possess only small snippets of, for example a tapeworm strobila (often barely seen with the naked eye). In the case of larger parasites, you should cut up to 5 mm^3 of the tissues with clean, sterilised stainless steel dissecting scissors or a blade, and after a transfer into the new tube, cut it into as small pieces as possible immediately, before the ethanol evaporates and the tissue becomes solid, hard to cut and starts moving because of static electricity. Between processing individual specimens, make sure to thoroughly clean the forceps and scissors/blade used for transferring and cutting the tissue, to avoid cross-contamination of the sample by exogenous DNA that would be impossible to discover in later steps. A recommended method of cleaning is rubbing the forceps/scissors/blade well with a sterile piece of tissue soaked with absolute ethanol, and sterilising the steel tools over a laboratory burner. The tools should be cooled down before processing the next tissue sample.

PCR (polymerase chain reaction) amplification

Polymerase chain reaction is a method to amplify, starting from the solution of parasite DNA, a selected molecular marker that will be used, *e.g.*, to reconstruct the phylogeny of the studied parasite taxon. To amplify the chosen marker (*e.g.*, 18S rDNA), the following chemicals and tools are needed: Taq DNA Polymerase, Taq Reaction Buffer, dNTPs, forward and reverse primers, PCR-grade water and DNA template; thermocycler, pipets, tubes, tips and gloves. PCR is a routine method in many molecular laboratories and detailed protocols can be found elsewhere (*e.g.*, Sambrook *et al.* 1989). For successful amplification, good quality DNA and well-designed primers are crucial.

Electrophoresis

Agarose gel electrophoresis is the most effective way of separating DNA fragments of varying sizes ranging from 25 kb to 100 bp. The phosphate backbone of the DNA (and RNA) molecule is negatively charged. Therefore, DNA fragments will migrate to the positively charged anode when placed in an electric field. Since

DNA has a uniform mass/charge ratio, DNA molecules are separated by size within an agarose gel in a pattern such that the distance travelled is proportional to their molecular weight.

Sequencing

The PCR product of the proper size must be cleaned from unused nucleotides and primers. The product is directly sequenced using a DNA sequencer if available or making use of the services of commercial DNA sequencing companies. The result is a chromatogram file with the desired sequence of nucleotides of the genetic marker.

Phylogenetic analysis

BLAST analysis

The chromatogram sequence file should be checked to confirm that the sequences obtained actually belong to the studied organism. PCR may accidentally amplify the host gene instead of the desired gene of the parasite species. This usually happens when the primers are not specific enough for the studied parasitic group. The easiest way to clarify the sequence origin is to perform a BLAST (Basic Local Alignment Search Tool) search at the web page: https://blast.ncbi.nlm.nih.gov/Blast.cgi. BLAST search of the nucleotide sequences will find the closest match with the sequences stored in GenBank.

Aligning and tree reconstruction

The phylogenetic relationships of the studied organism can be revealed by aligning the sequence obtained with a selected number of sequences downloaded from GenBank at www.ncbi.nlm.nih.gov. Such a dataset of sequences is subjected to tree reconstruction analyses using several methods of choice. The most commonly used methods are maximum likelihood, maximum parsimony and Bayesian inference.

There are plenty of phylogenetic programs that can be used for phylogenetic analysis. One of the best programs including all methods is Geneious, which is a very user-friendly programme. A trial version can be downloaded and used for a limited time (https://www.geneious.com/). Another option is to use MEGA – a free programme with very good user-friendly interface (http://www.megasoftware.net). A very useful manual for beginners called “Introduction to Walk through MEGA” can be obtained at: http://www.megasoftware.net/web_help_7/hc_introduction_to_walk_through_mega.htm

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Chapter 3.6.
ECOLOGICAL STUDIES

Andrea VETEŠNÍKOVÁ ŠIMKOVÁ & Jiří JARKOVSKÝ

Introduction

Basic ecological studies in fish parasitology focus on parasite distribution in host populations, the structure of parasite communities and host-parasite interactions. The effects of some abiotic or biotic factors on parasite distribution (usually measured by parasite prevalence, abundance or aggregation) or parasite diversity have been analysed. The most commonly studied abiotic factors are season, time, water temperature, habitat types and environmental pollution. The most commonly studied biotic factors associated with hosts are species, body size, age, sex, food spectrum, stress, reproduction, immunity, or genetic diversity of hosts. The presence and abundance of a given parasite species in the parasite community may also be strongly affected by other parasite species currently occurring (or coexisting) in the parasite community.

General challenges in ecological studies on fish parasites

The protocol of an ecological study basically depends on the hypothesis to be tested, *i.e.*, predictions and objectives should be set prior to any ecological study. As parasite abundance and diversity can be affected by multiple abiotic and biotic factors, the ecological study should be designed to eliminate these effects. Before starting to investigate ecological patterns in fish parasites, the correct identification of host specimens should be confirmed by a specialist. In case of doubt over host identification or if hybridisation between phylogenetically related host species seems to play a role, molecular markers should also be applied to confirm morphology-based identification.

Sample size is important when investigating parasite diversity (for example, when studying the structure of parasite communities or in the case of comparative analyses of determinants of parasite diversity), investigating parasite distribution in host populations or delimiting host specificity of parasites. However, there are mathematical methods that allow correction for unequal sampling (rarefaction method or simulated random sampling of given sample size). Another confounding effect may be the host body size as parasite diversity (and parasite abundance) generally increases with increased host body size due to allometric relationships. Larger hosts represent a larger and more stable habitat for parasite colonisation. Therefore, when comparing the parasite communities of a given host species between different sites, hosts of similar body size should be selected.

For parasites with a complex life cycle, the presence and abundance of intermediate hosts is another biotic factor influencing the composition of parasite communities and should be taken into account even if the study includes sites with equal sample size and fish hosts of similar body size. Furthermore, parasite diversity and parasite abundance may vary in time and space. In addition, the biotic variables linked to hosts also exhibit temporal and spatial variability (*e.g.*, seasonal changes in water temperature induce changes in fish immunity which affect the level of parasite infection). Therefore, when investigating spatial variability in parasite diversity, the ecological study should be performed under similar environmental conditions (*e.g.*, when comparing the parasite diversity of a given host species among different sites, the fish from all sites should be sampled in the same season, with similar water temperature or water flow).

A very important part of ecological studies on parasite diversity is fish storage following sampling and the time between the collection and processing of fish specimens (*i.e.*, fish dissection and parasite collection). Fish should be quickly transported to the laboratory and placed into containers with the original water and aeration. All fish should be dissected and parasites should be collected and fixed within 48 hours after capture. Alternatively, fish may be frozen and dissected later, but in that case most parasites found are useless for a detailed morphological study. In addition, parasites cannot be detected based on their movement. Finally, host phylogenetic relationships should also be considered. Two congeneric hosts may share parasite species due to common ancestry.

Fish in the life cycle of parasites

Parasites exhibit direct or complex life cycles. In the case of a direct life cycle, parasites require only one host species to complete their ontogenetic development. All monogeneans, some nematodes and most arthropods have a direct life cycle. Parasites with a complex (or indirect) life cycle have one or more obligatory intermediate host species in different stages of their life cycle in which the parasites undergo some developmental and morphological changes (*i.e.*, multiplication of infective stages in intermediate hosts) and definitive hosts (parasites reach sexual maturity in definitive hosts). For many endoparasites with a complex life cycle (*e.g.*, trematodes and nematodes maturing in fish-eating birds), fish act as intermediate hosts. Some endoparasites (*e.g.*, heterophyid metacercariae in the brain of fish and plerocercoids of diphyllobothriidean cestodes in the body cavity) are able to manipulate the behaviour of their intermediate host (here, a fish) to successfully reach the definitive host (PITT – Parasite Increased Trophic Transmission).

Population ecology of parasites – basic terminology

> **Population:** a group of individuals belonging to the same species living at a given time and in a given space; each individual host is parasitised by one or more parasite infrapopulations. The following types of parasite populations have been defined (Margolis *et al.* 1982; Bush *et al.* 1997, 2001; Morand & Šimková 2005).

Infrapopulation: the group of all individuals of a given parasite species infecting a single host specimen; each individual host is parasitised by one parasite population of a single parasite species or more parasite infrapopulations of different parasite species; an infrapopulation is short-living, *i.e.*, its maximal life span is equal to (but usually shorter than) the life of the individual host harbouring this infrapopulation. Parasite infrapopulations are subunits of a metapopulation.

Metapopulation (sometimes termed component population): consists of all infrapopulations of a given parasite species in all host individuals of the same host species in an ecosystem.

Suprapopulation: consists of all parasites of a given species including all developmental stages of this parasite in all hosts in a given ecosystem.

Population ecology of host-parasite interactions is analogous to metapopulation theory. The principal idea of metapopulation theory is that the local populations are interconnected, *i.e.*, there is migration of specimens among local populations. Each individual host represents the equivalent of a habitat patch, which usually includes the infrapopulations of more metapopulations of different parasite species infecting a given host population.

To describe the size and distribution of a parasite population in a given host population, Margolis *et al.* (1982) and Bush *et al.* (1997) proposed the **basic epidemiological parameters** describing the level of parasite infection in a host population:

Prevalence: the proportion of hosts infected by a given parasite species (*i.e.*, the proportion of hosts infected in the whole sample of host specimens examined).

Intensity of infection: the number of parasite specimens found in/on a given host specimen infected.

Mean intensity of infection: the mean number of parasites of a given parasite species over all infected hosts in the sample.

Mean parasite abundance: the mean number of parasites per host specimen in a given host population, *i.e.*, the mean number of parasite specimens calculated when considering both infected and uninfected hosts in the sample.

Parasites are typically aggregated within a host population, which means that many hosts are parasitised by one or very few parasites or are uninfected, and a few hosts are infected with many parasite specimens. The simplest way for the description of this parasite distribution is to calculate the variance/mean ratio. A ratio equalling 1 indicates random distribution, a ratio below 1 indicates a uniform distribution and a ratio higher than 1 indicates an aggregated distribution.

Parasite communities – basic terms

Several types of parasite communities have been defined (Bush *et al.* 1997, 2001; Poulin 2007):

Infracommunity: all populations of different species of parasites in the same host individual.

Component community (or metacommunity): all parasite species exploiting a host population.

Compound community: all parasite communities in an ecosystem.

As infracommunities are subsets of the component community, the maximum number of species in an infracommunity is equal to the number of species in the component community (however, this maximum number of parasite species in an infracommunity is typically not reached and usually no single infracommunity contains all species that are locally available). Infracommunities are short-lived, their maximum life span is equal to that of the host. As component communities are subsets of the parasite fauna, the maximum number of parasite species in a component community is equal to the number of species in the parasite fauna (however, this maximum number of parasite species in a component community is typically not reached). Component communities are longer-lived assemblages than infracommunities as the host population persists in time (Poulin 2007). Component communities are often saturated (expressed by a curvilinear function) by parasite species (the saturation by species is below the number of species in the parasite fauna).

Parasite species are not randomly distributed among infracommunities due to species interactions or other structuring forces. Parasite infracommunities may exhibit so-called **nested patterns** of parasite species distribution when a common parasite species (*i.e.,* usually a parasite with high prevalence and abundance) is distributed in all infracommunities, but rare parasite species occur only in species-rich infracommunities (Patterson & Atmar 1986). This nested pattern is usually explained by different colonisation and extinction rates of species.

Parasite interactions: competition versus coexistence in parasite communities

There are two types of parasite communities:

(1) non-interactive (isolationist) communities, in which niche space is not saturated with parasite individuals and thus interspecific interactions do not play a role (parasites may coexist in the communities);

(2) interactive communities, in which niche space is saturated and interspecific competition plays an important role (Rohde 1977, 1991).

The ecological niche of a given parasite species is the multidimensional habitat volume occupied by specimens of this parasite species. It is defined by physical and biotic variables (Hutchinson 1957 and modified for parasites by Poulin 2007). The comparison of basic niche (measured for a single species infection) and real ecological niche (measured for a multispecies infection) under experimental conditions is the basic way to reveal ongoing competition. The ecological niche of a parasite species is determined by host specificity, microhabitat, macrohabitat (*i.e.*, the habitat of the host), geographical distribution, host age, host food and rarely by host sex (Rohde 1979).

Host specificity

The most widely used descriptor of parasites in their communities is the host specificity. According to the most widely accepted definition, host specificity is the extent to which a parasite taxon is restricted in the number of host species used at a given stage in the life cycle (Poulin 2007). Using a basic measure of host specificity (*i.e.*, host specificity measured by the number of host species), a specialist (or strictly host-specific parasite) is restricted to a single host species, while a generalist (*i.e.*, parasite species with low host specificity) is able to infect at least two host species. Host specificity decreases with an increasing number of host species (*i.e.*, with increasing host range).

Special attention should by paid to parasite species with a complex life cycle. A parasite species with a complex life cycle is often restricted to a single intermediate host species (*i.e.*, it is a specialist at the intermediate host level), but is able to infect a wide range of definitive hosts (*i.e.*, it is a generalist at the final host level). Host specificity may also be expressed by including quantitative ecological data (like abundance), phylogenetic relatedness of hosts or the geographical distribution range of parasite species (Poulin *et al.* 2011). When evaluating host specificity, the scale of the study should be taken into account. Some parasites may exhibit strict host specificity at the local level, but are recorded on a wide range of host species at the regional level.

Analyses of parasite communities – biodiversity indices

Diversity of parasite communities is expressed by species richness or by the relative abundance of species. Species richness is a simple count of the number of species in the community. Relative abundance specifies the number of individuals per species. Biodiversity indices are frequently used to express the diversity in parasite communities (see Maguran 2003). The Shannon index and its evenness have been widely applied for parasite component communities. In contrast, the Brillouin index is useful at the level of the infracommunity. Species dominance in parasite communities can be evaluated using the Simpson index or the Berger-Parker index (see Table 3.6.1 for equations).

Table 3.6.1 Overview of biodiversity indices (S – number of species, N – number of individuals, n_i – number of individuals of the i-th species).

Index	Equation
Margalef index	$D_{Mg} = \frac{(S-1)}{\ln N}$
Menhinick index	$D_{Mn} = \frac{S}{\sqrt{N}}$
Shannon index	$H' = -\sum p_i \ln p_i$, where $p_i = \frac{n_i}{N}$
Brillouin index	$HB = \frac{\ln N! - \sum \ln n_i!}{N}$
Simpson index	$D = \sum \left(\frac{n_i(n_i-1)}{N(N-1)} \right)$
Berger-Parker index	$d = \frac{N_{\max}}{N}$ where N_{max} – abundance of the most abundant species

Parasite communities are compared by calculating the similarity between parasite communities (*e.g.*, similarity between two parasite component communities of the same host species collected from two different sites). The coefficient of associations is calculated with or without taking into account the problem of double zero values (asymmetrical and symmetrical coefficient, respectively). Binary or quantitative data are used to evaluate the similarity between parasite communities. The most often applied asymmetrical indices are the Jaccard index of similarity for binary data and the Sørensen index for quantitative data (see Table 3.6.2 for equations).

Table 3.6.2 Basic similarity indices.

Index	Equation
Jaccard similarity coefficient	$S = \frac{a}{a+b+c}$, where *a* is the number of species occurring at both sites and *b*, *c* is the number of species occurring only at one of the sites
Sørensen quantitative coefficient	$C_N = \frac{2jN}{aN+bN}$, where *aN* and *bN* are the abundance of species at sites A and B, and *jN* is the sum of abundances of species occurring at both sites

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