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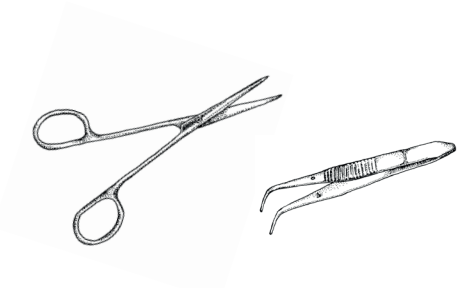
PART 3

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).

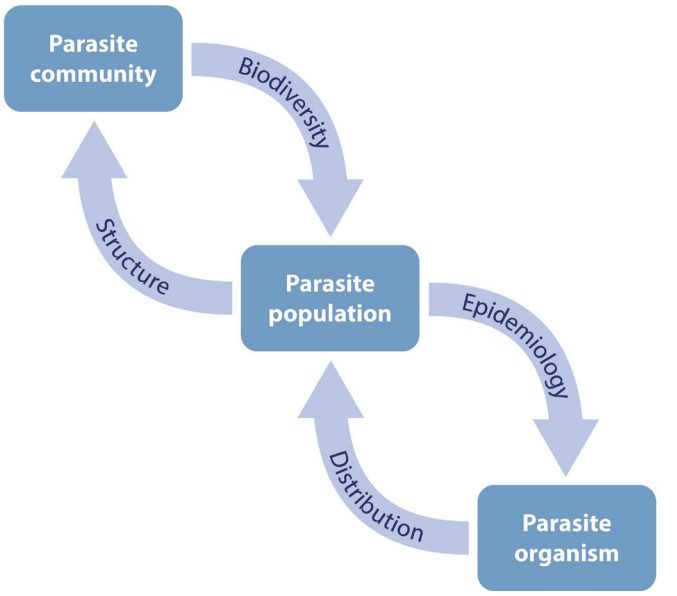

Fig. 3.1.1. A schematic representation for the three hierarchical levels of organisation of parasite-host associations. (Illustration by M. Luo and M. Gelnar.)

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*).

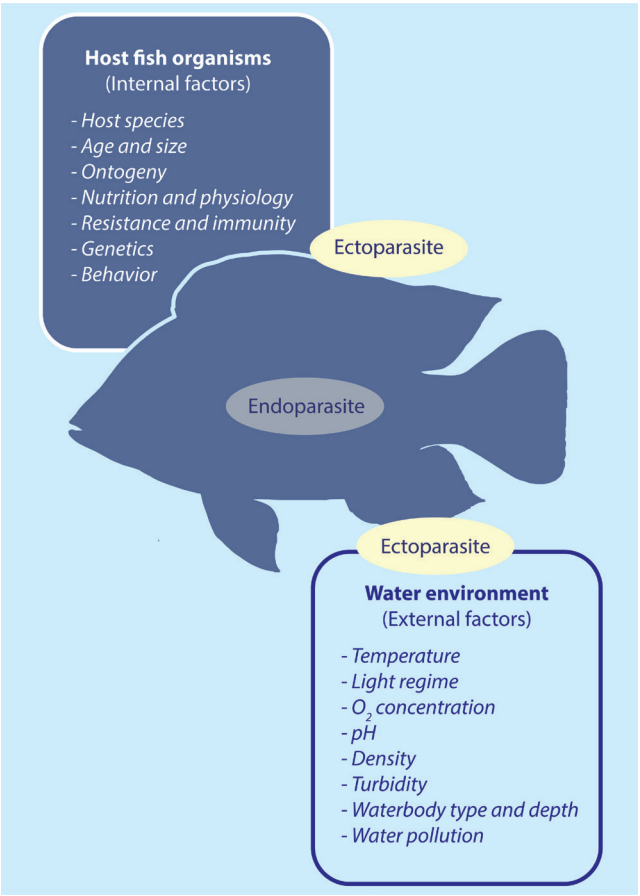

Fig. 3.1.2. A schematic representation of parasite-host interactions in an aquatic environment. (Illustration by M. Luo and M. Gelnar.)

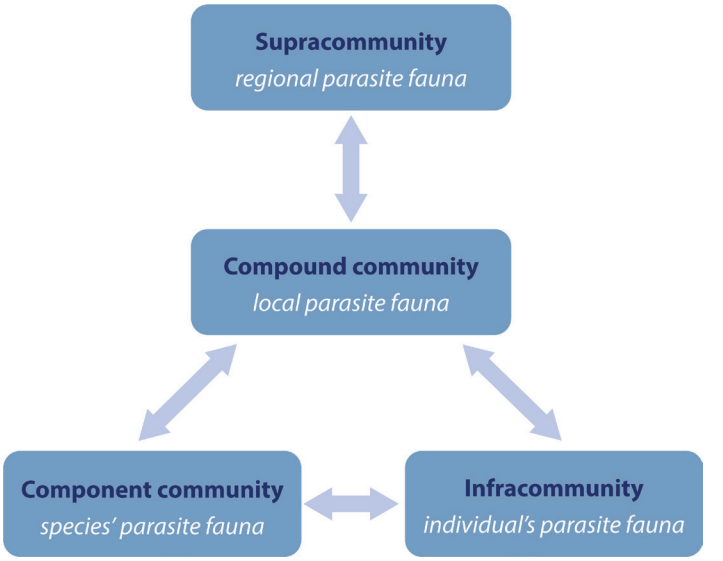

Fig. 3.1.3. A schematic representation of the hierarchical organisation of parasite supracommunity, compound community, component community and infracommunity. (Illustration by M. Luo and M. Gelnar.)

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*.

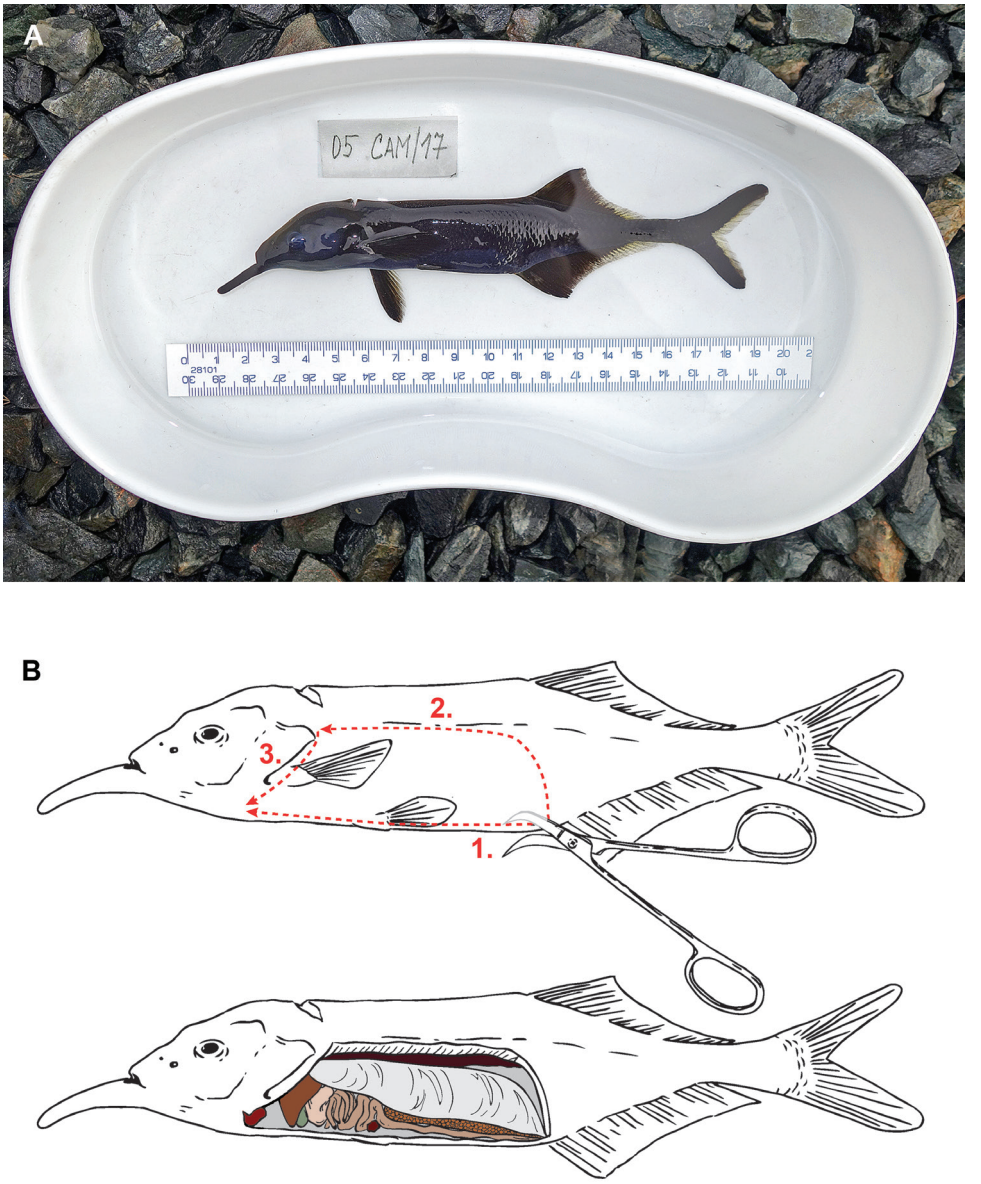


Fig. 3.2.1. A. Labelling fish hosts. Note that the fish snout is positioned to the left side and a ruler is added for estimation of fish size. The surface of the fish should be kept wet during any manipulation and handling of the fish; **B.** Illustration of how to open the body cavity of a fish to reveal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

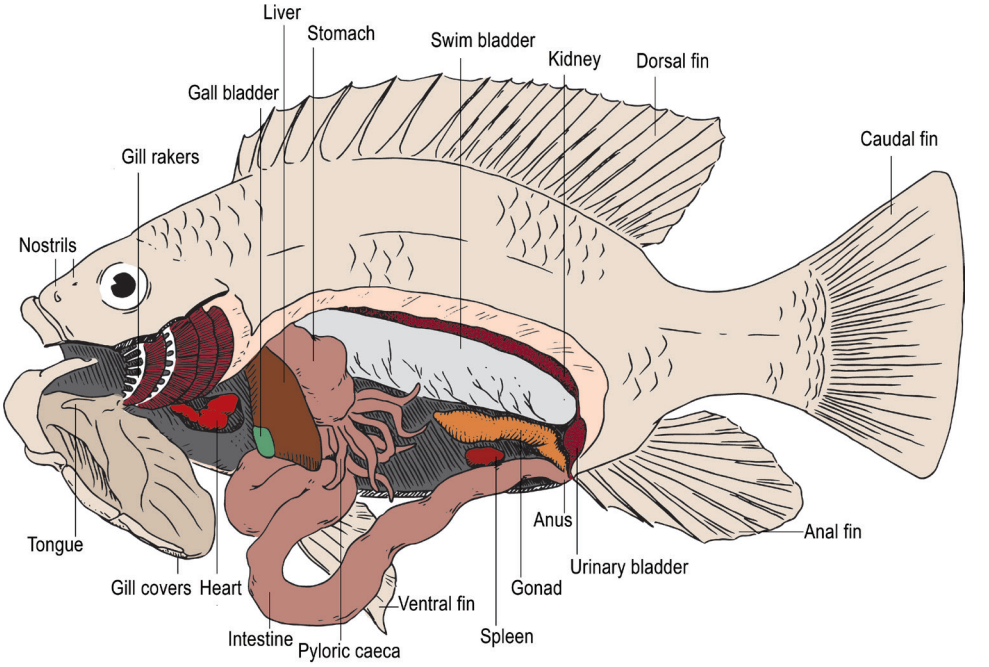


Fig. 3.2.2. External and internal anatomy of a bony fish. (Modified by M. Luo from Hile, R. 1960, U. S. Fish and Wildlife Service, Fishery Leaflet, no. 132, 6 pp.)

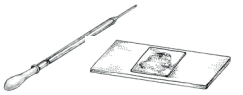
Information on the sampling date and locality (GPS coordinates, water temperature, etc.) should be recorded. The scientific name of the host, the infection site, the number of specimens found and fixed, the fixative used, the date of dissection and the name of the collector should be written in a field 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)