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Corresponding author:
S.C. Cutmore;
Email: s.cutmore@uq.edu.au

Twenty thousand fishes under the seas: Insights into the collection and storage of trematodes from the examination of 20,000 fishes in the tropical Indo west-Pacific

S.C. Cutmore[1,2], R.A. Bray[3], D.C. Huston[4], S.B. Martin[5], T.L. Miller[1,2], N.Q-X. Wee[1], R.Q-Y. Yong[6] and T.H. Cribb[1]

[1]Queensland Museum, Biodiversity and Geosciences Program, South Brisbane, Queensland, 4101, Australia; [2]The University of Queensland, School of the Environment, St Lucia, Queensland, 4072, Australia; [3]Department of Life Sciences, Natural History Museum, Cromwell Road, London SW7 5BD, UK; [4]Australian National Insect Collection, National Research Collections Australia, CSIRO, PO Box 1700, Canberra, ACT 2601, Australia; [5]Centre for Sustainable Aquatic Ecosystems, Harry Butler Institute, Murdoch University, Murdoch, Western Australia 6150, Australia and [6]Water Research Group, Unit of Environmental Sciences & Management, North-West University, Potchefstroom, North-West Province, South Africa

Abstract

The techniques employed to collect and store trematodes vary between research groups, and although these differences are sometimes necessitated by distinctions in the hosts examined, they are more commonly an artefact of instruction. As a general rule, we tend to follow what we were taught rather than explore new techniques. A major reason for this is that there are few technique papers in the published literature. Inspired by a collaborative workshop at the *Trematodes 2024* symposium, we outline our techniques and processes for collecting adult trematodes from fishes and discuss the improvements we have made over 40 years of dissections of 20,000+ individual marine fishes. We present these techniques for two reasons: first, to encourage unified methods across the globe, with an aim to produce optimally comparable specimens across temporal periods, across geographic localities, and between research groups; and second, as a resource for inexperienced researchers. We stress the importance of understanding differences in host biology and the expected trematode fauna, which ultimately enables organised and productive dissections. We outline our dissection method for each key organ separately, discuss handling, fixation, and storage methods to generate the most uniform and comparable samples, and explore ethical considerations, issues of accurate host identification, and the importance and potential of clear record keeping.

Introduction

The methods used to find trematodes in fishes have been in continual evolution since the collection and description of *Hirudinella ventricosa* (Pallas, 1774) Baird, 1853 by Pallas (1774). It is unsurprising that this was the first trematode described from a fish; this giant hemiuroid, found in the stomach of large and commercially important pelagic scombriform fishes, is one of the most conspicuous trematodes encountered in marine fishes. As work on the Trematoda of fishes progressed over the next 250 years, those species that were large, obvious, or morphologically or ecologically distinctive were generally, consciously, and unconsciously prioritised (Cribb *et al.* 2021). Naturally, larger trematodes found in important food fishes or that infect commonly opened sites in their host (e.g., the stomach) were encountered most readily. Trematodes that are smaller, are less abundant, are found in rarely encountered fishes, or infect cryptic sites within their hosts were, and are still, frequently overlooked (Bennett *et al.* 2022). Such is the nature of species discovery and taxonomy for almost all organismal groups: the more obvious species are described before those that are obscure (McClain *et al.* 2024). For the Trematoda, fishes provide a wide range of sites for infection in which common and sometimes morphologically and ecologically distinct species can remain overlooked.

The conference *Trematodes 2024*, held in Brisbane, Australia, in September 2024, included a series of workshops focused on incorporating attendee participation and knowledge transfer between research groups (see Martin *et al.* 2024). One of these workshops was dedicated to discussing the best practices in the collection, processing and description, and long-term management of trematodes. This workshop stimulated the sharing of our experiences, and here we detail our methods for finding, collecting, fixing, and preserving trematodes of fishes, and the improvements that we have made over 40 years of dissections. Like many before us, our initial dissections, starting in 1981, focused primarily on the gastrointestinal system. This focus was for

good reason; the majority of trematodes infect this organ system. Although this is still the most important element of almost all our dissections, we have constantly modified how we examine the gut, often on the fly, tailoring different methods for different groups of fishes and different parts of the intestinal tract. Over time and with each dissection, we have also gained a more nuanced understanding of the groups of trematodes that infect non-intestinal sites.

We are not under the illusion that our methods are perfect, and it is not our intention to be prescriptive; we acknowledge that there are doubtless further improvements to be made and that there is a range of other tried-and-tested methodologies that work (e.g., Gibson 1984; Lutz *et al.* 2017; Pritchard and Kruse 1982). We do, however, think that the knowledge gained from 20,000 dissections is worth sharing, with the goal of a global equality of trematode collections that leads to optimally comparable data for species delimitation and identification, especially over geographical range. The following is a description of the process of fish dissection in search for adult trematodes; the search for metacercariae can incorporate a range of methods not covered here, described in a range of previous papers (e.g., Cribb and Bray 2010; Duflot *et al.* 2021; LaFonte *et al.* 2015; Scholz and Aguirre-Macedo 2000). Most of our work has involved the dissection of teleosts. The dissection of elasmobranchs is a rather different study, as their endoparasite fauna is dominated by cestodes, but we do refer here to these fishes where they are relevant to trematodes.

Preface

Before delving into detailed dissection techniques, it is important to emphasise a few fundamentals. It is our opinion that a dissection should be started as we mean to go on: efficiently and with purpose, but also carefully, systematically, and with respect for the host. More than being just a matter of ethical principle, treating a dissection with rigorous care lays the groundwork for quality data and specimens, and informative scientific inference. By the end of this article, it will be clear that trematodes 1) variously occupy distinct niches within their hosts; 2) are not always easy to find; and 3) require care in handling to maximise scientific value. The methods detailed below describe our ideal dissection process. Effective characterisation of the global fish trematode fauna requires the examination of multiple individuals of many thousands of species. Our 'ideal process' therefore incorporates appreciation for efficiency. Although the circumstances under which a dissection is performed may not always be ideal, sensible management can ameliorate circumstantial challenges. Conversely, crude handling of hosts, organs, and parasites leads to crude data.

Preparation

Priorities and order of operations

Regardless of how fishes are procured, it is often the case that there are multiple fishes to be examined. If the fishes are fresh, having multiple fishes creates time pressure and necessitates prioritisation, both within each fish dissection and collectively across all fishes to be dissected. Only experience can reliably inform the appropriate number of fishes examinable per person per day, as many factors need to be considered, including the size and species of the fishes, the proficiency and efficiency of individuals and teams, the objectives of the dissection, and circumstantial constraints. For those with limited experience, our advice is to start by doing a better job of fewer fishes – perhaps aim for three to five per day.

Focus and defined objectives are important. Our process described in this article assumes the focus is the search and collection of adult trematodes. It is often tempting to aspire to examine the host comprehensively and collect all parasites. Our experience is that this is worthwhile only where a team of parasitologists with diverse taxonomic interests is available to divide the labour of dissection and, importantly, also to divide collected material and subsequent work translating that material to outputs. Otherwise, we typically do not extend our dissections to search for other parasite fauna; doing so significantly extends the dissection with little prospect of additional output.

Even where the focus is constrained to the search for trematodes, it needs to be decided whether that focus will include or exclude the search for trematodes other than in the gastrointestinal system, particularly the blood flukes in the circulatory system. Searching for blood flukes is the most difficult, time-consuming, and frustrating aspect of the comprehensive search for adult fish trematodes in fishes. Discovery is still often serendipitous, depending on encounters while dissecting other organs. Ideally, a specialist should be deputised for specifically undertaking dissections of the circulatory system to look for them, as it is usually onerous and time-consuming, often taking as long as inspecting the rest of the organs combined. We think it is always quick and worthwhile to check the heart, urinary bladder, and gall bladder, and our routine procedure involves a quick diagnostic check for blood flukes before deciding whether to undertake a thorough search. If the circulatory system is to be examined, it should be prioritised; the coagulation of host blood and the delicate nature of blood flukes make finding adult worms suitable for morphological characterisation difficult shortly after the death of the fish.

Once the fish is dead and requisite information recorded, dissection should commence promptly to minimise parasite degradation. Given our emphasis on the gastrointestinal tract, these are usually the organs that are removed first, but trematodes can be found in almost every organ, so a systematic dissection requires isolating other key organs from the carcass.

Equipment

The effective collection of adult trematodes is more difficult than for most other fish parasite groups, yet the necessary laboratory set-up and equipment remains simple and inexpensive. Nevertheless, a few particular considerations improve the dissection process and outcome.

First, a basic stereo microscope and light source are essential; although larger trematodes are detectable with the naked eye, most will be overlooked without a microscope. Because many trematodes, especially smaller species, are semi-transparent, we recommend reflected light and an opaque black stage (a bright desk lamp and a sheet of black card suffices). A basic compound microscope is required if gills are to be screened for blood flukes (technique below) and is useful in the search and study of metacercariae, but otherwise is unnecessary for the search of adult trematodes.

Standard and assorted dissection tools are mostly sufficient. However, we have two specific recommendations. First, and most importantly, fine dissection tools, specifically needle-point forceps and microscissors, dramatically improve the fine dissection of the organs, especially the neat opening of the entire length of the gut, including pyloric caeca. Second, a pair of stronger cutters, such as straight, compound-action (aviation) snips (sheet-metal cutters) or

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similar, greatly expediates the gross dissection of medium and larger fishes. Performing the gross dissection on a tray allows for any liquid to easily be added to the soak of the carcass.

Assorted and ample buckets, containers, and jars are needed for preparing saline and soaking carcasses and organs. Jars to be used for washes should be selected more carefully: we recommend screw-top and a volume of 500–1,000 ml. Avoid jars that have an inner wall indented from the thread for the cap or have a neck or narrow aperture like a bottle, as these can cause a vortex during pouring that will agitate the sediment and risk losing parasite specimens. Similarly, provision of ample petri dishes for isolating organs and examining washes and smaller receptacles for isolating specimens will improve flexibility and efficiency. We use 14-cm diameter plastic petri dishes with deep sides (2 cm, to hold ample saline); glass petri dishes offer no advantages. Small receptacles are necessary to isolate and temporarily hold specimens. We prefer excavated glass cavity blocks because they have a small volume and concave sides that encourage worms to the centre. However, these are expensive; small petri dishes (the smaller, the better; < 6 cm diameter) are sufficient. To handle trematode specimens (and other parasites), it is best to use a pipette rather than forceps to avoid damage. For this purpose, glass Pasteur pipettes are superior to plastic transfer pipettes because some flatter trematodes species, such as aporocotylids and transversotrematids, will often become stuck to the side of plastic pipettes. Nevertheless, plastic transfer pipettes are useful for handling larger trematodes.

Finally, but perhaps most importantly, sufficient saline is paramount when dissecting fresh fishes. Insufficient saline is among the most common and most easily rectifiable flaws in novice dissections. Live trematodes should always be collected and kept alive in saline (salt concentration of roughly 8,000 ppm); water too salty or too fresh will have a catastrophic osmotic effect on trematodes, causing them to die prematurely and shrivel or explode. Live trematodes are more easily detectable and will survive longer in ample saline, and the best quality specimens are achieved by fixation of live, active worms. This is easy for us to recommend, as most of our examinations have been performed at marine research stations with seawater and fresh water available on tap. At such locations, one can simply dilute one part seawater with three parts fresh water by the bucket; such large quantities are needed when doing multiple washes for multiple fishes. In other locations, we have filled a 20-L drum or similar with seawater while procuring and transporting marine fishes to the laboratory. We understand that unlimited saline is not always available and needs to made with purchased salt. Nevertheless, our advice is to not be sparing with saline; a small investment in salt will result in considerable increase in the quality of the trematodes collected. A means of boiling saline and small heatproof beakers are required for fixation.

Fresh is best

The number and quality of adult trematodes collected from fishes is usually in a sharp decline from the time of host death. Indeed, of the various metazoan parasite groups that can be found on and in fishes, adult trematodes appear to deteriorate most rapidly and substantially – undoubtedly a consequence of being both soft-bodied and (typically) located within the gastrointestinal tract. Live, active trematodes are more easily detectable, whereas trematodes from long-dead or frozen fishes may have disintegrated entirely, and those that are collected are dead or dying. Although such worms are still valuable, their worth is certainly not equal to that of well-fixed, fresh worms. In our experience, the time by which trematodes die and become unfixable varies. Some groups, such as hemiurids, opecoelids, and didymozoids, are more resilient than others, lasting many hours, or up to a day, after host death. Others, such as some aporocotylids and monorchiids, begin to lose structural integrity and are moribund within an hour or two of host death. Notably, trematodes infecting herbivorous fishes generally deteriorate more rapidly than those infecting predators or omnivores. An understanding of these differences, and the expected trematode fauna of the fish to be examined, should influence the order in which hosts are examined once collected. In general, if the goal of the work is taxonomy, then it is best to examine fishes as soon after death as possible. However, we understand that obtaining fresh samples is not always possible. Fishes from the deep sea and remote locations cannot necessarily be examined immediately after host death and are sometimes only accessed opportunistically. The examination of hard-to-obtain hosts is certainly valuable even if they are dead or frozen, as the number of trematodes previously from them are generally limited. However, we continue to stress the importance of studying fresh hosts when this is possible.

Ethics and euthanasia

All parasitological surveys need to be conducted with consideration for the death of the host. This includes minimising the suffering of the individual fish and also consideration for the impact of the removal of hosts and parasites from ecosystems, maximising output, and minimising waste. Techniques for euthanasia are largely dictated by local ethics regulations and evolving best practices. In our collective experience, cranial pithing (ikejime) is the most universally accepted method of fish euthanasia, and the most effective. Although there may be debate surrounding the ethics and effectivity of other methods (e.g., use of anaesthetic overdoses, spinal severance, ice baths, etc), the consensus is that none are more effective than traumatic destruction of the brain. Even those countries that require the use of a combination of two euthanasia methods effectively predicate their protocols on cranial pithing being one of those methods. Pithing, in principle (for all but the largest of fishes), is simple: the fish is firmly held down on a fixed, flat surface and a robust, sharp implement is driven through the braincase, severing nervous connections. When well-practiced, it can be done in a single movement and will not damage any other organs or structures. We find purpose-made fish spikes and acute-ended surgical scissors to be most effective for these purposes. *Post mortem* nervous twitching may occur, occasionally including violent trembling of the tail; these can be mitigated (though not always avoided) by directing the blade (through the same initial incision) posterior-wards to ensure that spinal connections to the brain are severed, or performing a separate spinal severance further along the back, behind the fish's skull. It must be noted that spinal severance, by itself, is not an effective euthanasia technique, as it does not destroy the brain and therefore does not compromise cerebral or cephalic sensory functions.

Techniques

Wash and soak

The 'gut-wash' method has evidently been developed independently by multiple research groups. Perhaps first outlined by Nagaty (1937), the method was most clearly detailed by Cribb and Bray (2010). The method effectively dislodges trematodes from their

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infection site without causing damage to the worms and simultaneously cleans them from debris. The method is quick, expediating dissection, and improves confidence that most worms present a found; the gut-wash frequently yields numerous worms overlooked in the initial inspection, whereas inspection of the gut wall following the wash typically yields no further specimens. Indeed, it is so effective that we now routinely extend the method for washes of the body cavity, body surface, gills, head, and sometimes, for large fishes, the heart (e.g., Cutmore and Cribb 2021; Power *et al.* 2019; Widdicombe *et al.* 2020). We think the gut wash is among the most important techniques for the effective and efficient search of trematodes and should be included routinely in essentially all parasitological dissections.

After the initial fine dissection and visual examination, the part of the fish being examined (and the saline in which it was initially examined) should be poured into a large jar, half filled with fresh saline, sealed, and then shaken vigorously for 15–20 seconds. It is our experience that this never damages trematodes, although it is likely to damage adult cestodes, which can break and become entangled. The jar should then be filled with saline, the host tissue removed from the jar for a second visual examination and the contents of jar left to settle for 1–2 min. The top three-quarters of the supernatant should then be smoothly poured off in one motion to avoid agitating the sediment layer; if the sediment layer is excessively agitated, it must be allowed to resettle. Once most of the supernatant has been discarded, the jar may then be refilled with clean saline and the settling process repeated until the saline is clear enough to facilitate effective inspection. Finally, the sediment is examined in an appropriate petri dish using a stereo microscope.

Although the wash method is effective, it is not infallible. Some trematodes with larger ventral suckers, such as hemiurids and some opecoelids (e.g., Martin *et al.* 2018), may remain attached to the gut wall despite vigorous shaking and thus need to be picked off individually. Some other trematodes embed themselves within folds or pockets of the gut (e.g., monorchiids and zoogonids in the rectum), such that the vigorous shaking might not detach them. Some species, even when dislodged, may reattach themselves to the gut if it is left in the jar during settling. It should therefore be standard to remove and inspect the washed gut or other tissue while waiting for the wash to settle. Doing this picking after the gut has been washed and cleaned of mucus and gut contents renders any remaining trematodes easier to find.

We recommend removing especially large, clumpy, and hard gut contents like shell and coral fragments, partially digested fish, large faecal clumps, and vegetable matter before putting the guts to wash. In our experience, relatively few worms are in or on these contents (though they should always be separately and carefully inspected) and removing them before shaking will prevent potential damage to worms and make for cleaner, faster washes (see our section on the gastrointestinal tract for more details). Third, if the gut is very large, or very dirty, washing should be performed across multiple jars, either by cutting the gut into sections and individually washing each or, after shaking and the gut is removed, splitting the unsettled wash into multiple jars and allowing to settle. The multiple washes can be recombined when clear or separately examined; trying to pour off the liquid from such a wash when undivided, even from quite a large jar, will risk losing some of the worms. Finally, for gastrointestinal sites, there will often be a dense froth layer produced by the washing. This layer can trap one or two worms, so when the jar is first topped up after shaking, it should be stirred down with a dissection tool like forceps to sink any trapped worms and then skimmed off.

Hot fixation of the gut

We find that a hot fixation of the cleaned intestinal tract (after it has been removed from the wash) may dislodge some trematodes that were not detected visually or dislodged in the wash. For a hot wash, the intestinal tract is carefully lowered into saline just off the boil in a heatproof beaker. The gut should then either be gently dunked in and out of the hot saline a couple of times or gently washed to remove the worms. Hot saline should *never* be sealed and shaken as in the gut wash technique above, as the pressure may cause the container to explode, along with the boiling contents. By this process, any trematodes still attached to the gut will be heat-fixed instantly and release from the gut wall. The sediment from the hot wash should be diluted with cool saline to avoid cooking the worms and then left to settle and examined under a stereo microscope. We use this technique occasionally rather than routinely when we suspect (or hope!) further specimens of rare species, or in rare hosts, might be found.

Bulk fixation

Justine *et al.* (2012) proposed a method for the expeditious collecting of all gastrointestinal parasites by bulk fixation. The method is implemented without the use of a microscope or any direct search for parasites. In brief, they recommended gut-washing followed by bulk fixation in hot saline and preservation in ethanol. This method has the advantages of speed and simplicity and is thus useful in field situations such as working at sea, especially in limited space or in rough conditions, or when many fresh fishes need to be processed quickly. However, the unavoidable disadvantage is that the nature of the parasites collected (which otherwise influences immediate collecting strategy) becomes clear only following future work away from the collection location. Our collection strategies are modified daily throughout a field trip based on findings from the previous days' collections; such improvements are impossible if all processing is done in the home laboratory. This method also has the disadvantage of requiring searching for fixed worms that blend in with the surrounding sediment. When searching for live worms in a fresh dissection, the movement and slight transparency help to clearly distinguish trematodes. Nonetheless, this is still an excellent method for when complete dissections are impossible or there are too many fishes to dissect fresh.

Dissection

Accessing the visceral mass

For most fishes, we find that the best way to access the viscera is to remove one side of the abdomen; simply opening the fish along the ventral margin and prying the cavity open is quicker but does not allow the same level of observation of the cavity. We generally cut along the ventral margin from the anal vent to level with the pericardial cavity and do a second, diagonal cut from the anal vent, along the dorsal edge of the cavity to just posterior to the operculum. For dorsoventrally flattened fishes (e.g., pleuronectiforms and platycephalids), the entire ventral portion of the body cavity wall is removed, and for the hard-plated boxfishes (e.g., Ostraciidae and Aracanidae), we have found it simplest to remove the entire ventral surface. For some fishes, such as the tough- and saggy-skinned, soft-bodied pufferfishes (Tetraodontidae) and porcupinefishes (Diodontidae), removing one side of the abdomen is difficult and

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time-consuming; for such fishes, opening the ventral margin from the vent to the pericardial cavity suffices.

Before the viscera is removed from the fish, we attempt to locate and remove the urinary bladder; this organ is often ruptured when the rectum is cut at the vent, so it is best to remove it first. Once the viscera are removed from the fish, we attempt to isolate the gastrointestinal tract, the gall bladder, the liver, the spleen, and the gonads. The gastrointestinal tract, our key focus, is isolated in a petri dish and immersed in fresh saline. We start by cutting all the connective tissue that holds the tract in its characteristic folds (if one is confident that there are no blood flukes living in the mesenteric vessels; otherwise, careful removal of the mesenteric connective tissue along with all its vessels is necessary). We then remove the organs not relevant to the wash (the spleen and gonads). We have never found adult trematodes in the spleen or gonads, and as such, we examine them only briefly, but the former can be an important infection site for metacercariae and the latter an important infection site for philometrid nematodes.

Gastrointestinal tract

The most important organ system in the search for trematodes is the gastrointestinal tract. The great majority of trematode species are found somewhere along the length of this organ. On the basis of communication with colleagues, the process of examination of the gastrointestinal tract varies between research groups, including combinations of visual examination, scraping, and rudimentary washes in a petri dish. Although we are open to learning from each method, we consider the washing approach the optimal method for finding the greatest number of quality specimens for the least time invested.

The isolated gastrointestinal tract can be divided into constituent parts (stomach, intestine, pyloric caeca, rectum) for detailed individual washes or washed as a whole (with the loss of precise infection site information). This choice usually comes down to the time available and the size and the type of fish to be examined. Multiple organ washes take longer than just one wash; even if the actual washes can be done simultaneously, at the minimum, much more time is needed to examine the sediment. If there are many dead fish that need to be dissected, doing a single wash of the entire tract certainly expedites the process. We find that the smaller the fish, the less chance there is of missing worms. Small fish usually have less digesting food in the gut, and the worms are likely to be seen immediately on first inspection, so the entire tract can be done as one wash. Larger fish have longer guts, often with more food in them, and deeper villi that may hide small trematodes; for these, dividing the gastrointestinal tract into its component organs enables less cluttered sediment for examination and a clearer understanding of infection location. The structure of the intestinal tracts of different fishes varies, and different taxonomic groups require different approaches. Pelagic fishes such as carangids and scombrids, and some herbivorous fishes such as kyphosids, often have an enormous number (up to thousands) of tiny pyloric caeca; for these fish, it is best to process the pyloric caeca separately, opening them roughly with a stick blender or mincing with scissors. Such blending and mincing is likely to release some specimens effectively as well as destroying some; we find the balance useful when it is simply impossible to manually open every pyloric caecum individually. In contrast, other fishes such as belonids and labrids have short, almost straight guts with no stomach and no pyloric caeca and demarcation only of the rectum from the intestine; for these fishes, the gastrointestinal tract can be examined in just one or two sections. Herbivorous fishes have exceptionally long intestines, usually full of partly digested algae; despite an enormous number of wash iterations, the sediment from gut washes of herbivorous fishes will often be overwhelmed by partly digested plant material and sand, making finding the worms difficult. For such species, the stomach and intestine are best examined separately, with the intestine divided into smaller sections, to be split across several jars for washing and clearing. Washing the intestinal tissue and intestinal contents separately, through gentle scraping of the intestinal contents into a separate wash container, can be helpful for detecting trematodes that might have otherwise been missed when examining a potentially dirty wash. For fishes with an invertebrate diet (particularly crabs), such as some lethrinids, the hard sediment from the stomach will overwhelm the wash sediment (like the intestine contents of herbivores), making finding worms more difficult. For both omnivores and herbivores, the judicious use of wide-gauge sieves may help remove obscuring gut contents without removing trematodes. For tiny fishes (e.g., those under 3 cm), it can be efficient to use a compound microscope to examine the entire gut lightly pressed between microscope slides; trematodes are relatively uncommon in such small fishes but easily seen in such preparations, and the tiny gut need not be opened at all. These are just a few examples of how the nature of the fish can affect the wash of the gut, and thus, it is useful to approach each examination slightly differently.

Whether the gut sections are split or washed together, the process is essentially the same. The gastrointestinal tract should be opened along its length in saline and visually inspected for obvious and large worms under a stereo microscope. Lumps of undigested food in the gut and intestine should be immediately removed. As discussed above for herbivores, this material will cloud the wash and make it harder to find the worms in the final sediment. There are rarely any trematodes within this material because most trematodes are preferentially associated with the gut wall. Microscaphidiids, a family lacking oral and ventral suckers, are exceptional in this respect, but they are generally large and conspicuous. Once the obvious worms have been found and removed (into a cavity block of fresh saline), the intestinal tract and the saline it is in should be poured into large jar and washed as described above.

Circulatory system

Our understanding of the ecology of blood flukes (Schistosomatoidea excluding Clinostomidae), and thus the manner in which we examine the circulatory system of fishes for them, has improved over 20 years. In the early years of our dissection for blood flukes, only the heart was considered worth the time for detailed examination; this organ seemingly had the greatest amount of space in which blood flukes could live, and most of the species known to that point were found from it. We now know that blood flukes infect a diverse range of sites beyond the heart, including the body cavity, the ocular, cranial, hepatic, renal, mesenteric and branchial vessels, and even the tiny gill filament vessels themselves (Cutmore and Cribb 2021; Cutmore *et al.* 2018; Ogawa *et al.* 2015; Palacios-Abella *et al.* 2017; Yong *et al.* 2018). A minority of species (e.g., some species of *Paradeontacylix* McIntosh, 1934 and *Skoulekia* Alama-Bermejo, Montero, Raga & Holzer, 2011) are not associated with any vessels at all, instead occupying sites like the braincase, eyes, and the interstitial spaces between pectoral girdle musculature. We also know that there is variation in site specificity. For example, the threadlike species of *Ankistromeces* Nolan & Cribb, 2004 and *Phthinomita* Nolan & Cribb, 2006 live exclusively intertwined

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in the ventricular and atrial tissue (Cutmore *et al.* 2021; Nolan and Cribb 2006). In contrast, some species exhibit little site specificity, seemingly freely dispersing throughout the circulatory system. This is exemplified by *Holocentricola rufus* Cutmore & Cribb, 2021, which, in a single species of squirrelfish (Holocentridae), has been found infecting the cardiac ventricle, the branchial arteries, and the hepatic vessels, and in washes of the head and body once split open (Cutmore and Cribb 2021). Thus, we now routinely examine a range of likely infection sites once we have found evidence of infection in a fish, even if adult worms are found immediately.

The first step in examination of the circulatory system is removing and opening the heart; it takes under a minute to complete and can be highly productive. The incision for opening the body cavity along the ventral ridge of the fish should be extended to the jaw, and the pericardial cavity gently opened. For teleost fishes, the bulbus should be clamped by forceps as far anteriorly as possible and then cut just anterior to the clamp. For elasmobranchs, the conus arteriosus can be anteriorly clamped and the ventral aorta cut at the level of the branchial arteries. The sinus venosus should then be cut as far posteriorly as possible and the removed heart submerged in a petri dish of saline. The ventricle and atrium are then carefully ripped opened (we find ripping rather than cutting leads to fewer worms being found in small pieces), and the ventricle is then slowly teased apart. If large worms are present (e.g., species of *Cardicola* Short, 1953; *Psettarium* Goto & Ozaki, 1929; and *Spirocaecum* Yong, Cribb & Cutmore, 2021), they will simply fall or wriggle out once the heart is opened; threadlike species such as those of *Ankistromeces* and *Phthinomita* will be intertwined among the trabeculae and will need to be teased out with fine forceps or a needle point. Heart dissections should always be performed in generous volumes of saline and the heart regularly flushed with clean saline, as blood clouding the water can easily obscure blood flukes, which are often translucent and difficult to spot in murky water. The use of citrated saline solution has shown to be very effective in alleviating blood coagulation, which will ultimately result in blood flukes that are in better condition and more easily found (Platt 1988; Platt and Blair 1996; Snyder and Clopton 2005).

Absence of adult worms in the heart certainly does not mean that the fish is not infected, but a detailed examination for blood flukes can be time consuming, and, as such, we prefer to look for signs of infection before investing this time. Current infections are often, but not infallibly, identified by the presence of live eggs in the gill filaments and heart tissue (see Power *et al.* 2020; Yong *et al.* 2013). The process involves clipping several individual gill filaments (a few for large fishes and up to 30 for small fishes) to examine under a compound microscope; filaments are usually taken from at least two gill arches to account for variability in egg laying behaviour. The filaments are fanned out on a microscope slide with a small amount of saline and lightly squashed under a large coverslip. Notably, however, eggs are sometimes heavily concentrated in just one or two filaments (e.g., for species of *Holocentricola*). On such occasions, the egg aggregations may even be externally visible as arrays of tiny silvery specks embedded in the lamellar tissue, so an overall scan of the gill arches should always precede the filaments being cut off. A small amount of heart tissue from inside the ventricle should also be removed, roughly torn apart into small pieces on a microscope slide with a small amount of saline, and squashed under a coverslip with significant pressure. Eggs in the gill tissue are almost always viable and often contain active miracidia; these are almost always a sign of current infection. Eggs in the gills are continually hatching and thus being shed, and there is little opportunity for them to be lodged long enough to be nonviable. In contrast, eggs lodged in the heart can be a sign of previous infection if they are all old and degraded and in varying states of being removed from the tissue by the host immune functions (e.g., McElroy *et al.* 2020; Shirakashi *et al.* 2012; Yong *et al.* 2013). When searching for eggs in the gills and the heart, one needs be familiar with the difference between aporocotylid and didymozoid eggs (typically elongate and spined vs. round and unarmed) and between aporocotylid eggs and very small metacercariae (the two being clearly distinct when examined in detail but confusable when initially located).

If the fish is found to be infected on the basis of the presence of viable eggs, then the hunt is on to find the adult worms! The first place to look, after the heart, is the gills. The gills should be removed from the fish and the separated arches placed in clean saline. We use two approaches to find worms in the gills. The arches may be cut into smaller pieces along their length (not too small to avoid cutting any worms present) and examined using the washing method; the worms may emerge from the fragments spontaneously while cutting the arches. The other approach is to 'unzip' the gills by tearing the gill filaments from the gill arches and splitting the arch along its length, attempting to thereby open the associated blood vessels in which the blood flukes live. Just occasionally, we have found adult blood flukes in the gill filaments when these are examined for eggs; for some species (e.g., *Psettarium hustoni* Yong, Cutmore, Jones, Gauthier & Cribb, 2018), this was their only infection site (Yong *et al.* 2018).

The liver can be removed from the viscera, shredded, and examined with the washing method. The kidneys can be roughly shredded, and the body cavity soaked. Blood vessels of the viscera can be carefully removed from the visceral mass and shredded, dissected, and washed, separate to the rest of the viscera. Suspicious aggregations of mesenteric granulomas and melanocytes, especially if accompanied by some deformation of the adjacent vessel walls (visible lumpiness or haemorrhage), should be investigated under a high-powered microscope for eggs. The entire fish head may be removed from the body, split in half along the midline (powerful cutters are needed for this), and examined with the washing method. This will help dislodge worms living in the cranial vessels or in association with the eyes, although determining the precise site of infection is often difficult. Finally, the rest of the body can be filleted (large fishes) or cut down the middle (small fishes) and examined by the washing method. This last method is the least productive for blood flukes in our work, but it has discovered some new species that likely only infect vessels around the spine.

Body cavity

We have encountered trematodes from a range of families in the body cavity, a couple that genuinely occur there and others that are dislodged from their actual site of infection while cutting the body cavity open or removing the viscera; at worst, a thorough examination of the body cavity has proven an effective failsafe against messy or rushed dissecting. Those trematodes that do genuinely infect the body cavity are some genera of aporocotylids (e.g., *Plethorchis* Martin, 1975 and *Deontacylix* Linton, 1910) in teleosts (e.g., Lester *et al.* 2009; Linton 1910; Martin 1975) and gorgoderids in elasmobranchs (e.g., Caballero 1945; Curran *et al.* 2003; Cutmore *et al.* 2010; Tandon 1969). Trematodes likely to be found from messy dissections include zoogonids dislodged from the rectum, gall bladder, and urinary bladder; gorgoderids from the urinary bladder; some hemiuroids from the swim bladder; and some

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aporocotylids (e.g., species of *Cardicola* spp. and *Holocentricola* Cutmore & Cribb, 2021) from vessels in the liver and the kidney. After the cavity is initially inspected for large and obvious worms, the body of the fish should be placed in a large jar of saline and examined using the washing method. In this wash, it is likely that copepods and monogeneans will be dislodged from the gills and body surface.

Urinary bladder

Digeneans of two families, the Gorgoderidae and Zoogonidae, may be found infecting the urinary bladder of some fishes (e.g., Ho *et al.* 2014; Machida *et al.* 2006; Manter 1972; Yamaguti 1934). The urinary bladder is often remarkably small and inconspicuous, especially relative to the size of the worms it may contain, and removing it requires a careful dissection when first opening the body cavity, particularly regarding the initial cuts made around the anus. The urinary bladder always lies immediately dorsal to the rectum, and if it is still intact before the viscera are removed, it can be carefully pinched with forceps at the base and cut from the vent posteriorly. It is then placed in a small receptacle filled with saline and opened with microscissors. If the urinary bladder is not found, trematodes inside it may either be completely overlooked or discovered by its accidental opening, most likely via examining the washes of the gut and the body cavity.

Gall bladder

Like the urinary bladder, the gall bladder is not a rich site for trematodes, but species of Fellodistomidae, Lepocreadiidae, Opisthorchiidae, and Zoogonidae can be found infecting it in certain teleost fishes (e.g., Cribb *et al.* 1999; Duong *et al.* 2022; Krupenko *et al.* 2020), and some Aspidogastrea in certain elasmobranchs (e.g., Bray 1984; Méndez and Vidal-Martínez 2017). The gall bladder is best isolated under a stereo microscope after the viscera has been removed from the body cavity. The gall bladder varies in shape, colour, and size and can be long and narrow to almost spherical; sometimes it is partly imbedded in the liver. Like the urinary bladder, the gall bladder can be cut free by pinching the base with forceps and cutting from the rest of the viscera. It should then be isolated in saline and opened with microscissors. Worms may occasionally be found in the base of the bile duct (Bray 1987).

External body surface

The external surfaces of fishes are little occupied by trematodes. However, species of one family, the Transversotrematidae, are exclusively found externally, under the scales of their hosts (e.g., Cutmore *et al.* 2023; Hunter and Cribb 2010; Manter 1970). A small fauna of other trematode families (e.g., syncoeliids and didymozoids) can be found in the gill chamber, and other didymozoids encyst in fin membranes.

To find transversotrematids, the entire body of the fish is soaked orientated with the head up in saline for at least 30 min. It is important and most efficient to remove the entire viscera and place them in petri dishes of saline before the fish is placed in the body soak, so that the intestinal tract can be examined while the fish is soaking. The fish is orientated head-up in the soak, as marine transversotrematids will respond to the reduced salinity of the soak (saline vs. seawater), withdraw from under the scales, and sink to the bottom of the container; the worms are less likely to get free of the host if the head and scales of the fish are facing down. To ensure all transversotrematids have exited from under the scales, before the fish is removed the jar, it should be sealed and shaken vigorously. Once the body of the fish is removed, the wash is processed as described for any wash. Not infrequently, gut-infecting species will be found in the sediment of the body wash, dislodged from the rectum or oesophagus when the viscera was removed; based on the site specificity of most trematodes, these worms usually can be attributed to the correct infection location. As with the body cavity wash, this wash will likely also contain other classes of ectoparasites dislodged from the gills and body surface.

Other sites

There are a few further sites that rarely host trematodes that are usually neglected (at least by us) in an effort to reduce dissection time. Nontypical sites are usually a low-yield venture but are certainly worth searching if there is a smoking gun (records of trematodes from such sites from similar hosts). Almost all the nontypical sites we sometimes examine are typically infected by the Didymozoidae. We have found didymozoids encysted in sites such as the pelvic girdle, all surfaces of the buccal cavity including in and under the cartilage of the 'tongue', on the gill filaments and gill arches, in the fin rays and in the wall of the gut, in tissues around and behind the eye, in the nasal cavity, in the bases of fin spines, in the soft tissue of the kidney, liver, and spleen, and free in the interstitial spaces of the musculature. Additionally, the eye is often neglected in parasitological assessments; recent investigations have uncovered adult blood flukes from this site.

Searching all the sites that these worms could possibly infect would certainly extend the dissection time for each fish, and, as such, we suspect that most trematodologists (including ourselves) do not regularly search them systematically. Should a systematic investigation for didymozoids be of interest, however, we recommend being highly attentive to detail, particularly with regard to infection site. Didymozoids are often highly site-specific; a species infecting the gill arch may not be the same species as one found on the gill filaments. Didymozoids present as either relatively compact forms or highly elongate threadlike forms. Species with a compact form are often encysted in pairs that may show sexual differentiation. Threadlike forms may reach great lengths (e.g., > 1 m) and may or may not form cysts; teasing these long and fragile worms out from the host tissues in one piece is often impossible, but this is not always a problem as they would ultimately need to be cut into pieces for mounting. Didymozoid cysts are often fibrous and tough-walled, and rough tearing may result in squashing the worms; the use of fine-gauge insulin needles, blades from diabetic lancets, or entomological micro-pins mounted on probes is recommended. Pairs of individuals from the same cysts should ideally be fixed and stored together, each pair separate from the others.

Specimen storage

Handling live trematodes

As the dissection progresses, including the examination of tissues and the sediment of washes, encountered trematode specimens need to be collected and isolated prior to fixation and preservation. Trematodes, and indeed most parasites, should always be handled via pipette; using forceps to grasp the worm will almost always result in damage. For trematodes too large to fit in a pipette, a plastic transfer pipette can be cut to widen the opening, or blunt forceps may be used to lift (not grasp) the worms. As per the equipment

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section above, we use cavity blocks to temporarily hold collected specimens. Cavity blocks have sloping sides so that worms tend to aggregate in the centre of the block; this is generally an advantage for transferring worms to fixation or preservative but can also cause issues if the worms attach to one another. One practice that helps safeguard the morphology of the specimens, that does not especially add to dissection time, is to isolate trematodes with large and powerful suckers, nematodes and metacestodes from all other parasites. Trematodes with large suckers (e.g., hemiurids and opecoelids) will often attach to other trematodes in the cavity block and sometimes cannot be separated from them, even with heat fixation. If the worms are separated after fixation, there will usually be some morphological distortion created by the attachment. Metacestodes will also attach to trematodes, and although they are easier to dislodge, they will also often damage the worm. Nematodes (primarily cucullanids) will sometimes bite chunks from trematodes when stored together in a cavity block. An easy method of separating such trematodes is to pipette individuals into different positions in a larger, clean, flat-bottomed, saline-filled petri dish so that the trematodes are unlikely to encounter one another. If possible, it is also recommended that such worms are heat-fixed as they are found, rather than at the end of the dissection.

Fixation

The method of fixation (killing the worm) is a matter of debate. Flattening during fixation was historically predominate and is still commonly practised by some, whereas we, and others, consider it detrimental to morphological analysis. Flattening improves morphological examination and interpretation of specimens, especially certain fine details, and particularly for globular or thick-walled species. However, flattening distorts morphology (that is its goal), altering the relative positions and sizes of organs. Given how much species- and even genus-level trematode taxonomy is dependent on such features, we argue that this makes the marginal gains from flattening not worth it. In cases where we find it is difficult to get worms to fix consistently flat (e.g., blood flukes, which tend to coil or curve during fixation), we have obtained better results by gently flattening or straightening the worms *after* fixation, during preparation of morphological whole-mounts, and even then only using very mild coverslip pressure unabetted by additional direct application of weight.

We recommend fixing all live trematodes using heat; dead worms are rarely improved by fixing and can be preserved immediately. We pipette live worms into saline that has been brought to the boil and removed from the heat source. Heat fixation results in relatively uniformity within species, which we have found to be comparable between samples collected over temporal periods, overngeographic locations, and between research groups. Cribb and Bray (2010) recommended the boiling of saline in 5-ml or 10-ml heatproof beakers on a heat source. We now boil our saline in an electric kettle and pour this into the 10-ml heatproof beakers. Kettles have a limited life but are cheap, boil quicker, and allow multiple team members access to boiling saline simultaneously. The saline in the kettle should be changed and topped up regularly to ensure that the salinity is not increasing unduly. The specimens should be pipetted into the heated saline, rather than pipetting or pouring the saline onto the worms in the cavity block or petri dish; the latter approach tends to result in gradual and uneven fixation leading to misshapen worms.

It should be noted that heat fixation can be problematic for some finer bodied worms, larger worms, and those which feed on blood. We have had problems with fragile and fine worms like monorchiids and derogenids; if these worms are starting to die, they can explode when pipetted into boiled saline. One method to avoid such damage is to cool the boiled saline to a lower temperature; we suspect a fixation at around 60°C might work for the smaller and finer trematodes. Heat fixation of some of the larger bodied worms can lead to small ruptures in the tegument. We have observed small ruptures in the caeca of blood-feeding species (such as lecithasteridss), specifically when the caeca are engorged. Similarly, we have seen small ruptures in the uterus in large gorgoderids, specifically when the uterus is very full. On rare occasions, we have also observed heat fixation causing worms to forcibly regurgitate their gut contents or expel some of their eggs or sperm. Although this may seem alarming, this is not a completely bad outcome, as it does not compromise the integrity of the specimen, and indeed, losing some eggs and gut contents can actually help with interpretation of morphological features that would otherwise be obscured. Despite the issues listed above, the benefit of heat fixation far outweighs the negatives, and we strongly recommend this approach.

Preservation and storage

The preservation of worms has been one of the most dynamic aspects of our collection techniques. In the 1980s, all our trematode specimens were preserved in 10% formalin. In the 1990s, with the uptake of molecular sequencing techniques, we transitioned to storing most specimens (those intended for morphological study) in 10% formalin and some in 96–100% ethanol. In the 2000s we transitioned to storing everything in 70% ethanol, and now we preserve everything in 80% ethanol. Along the way, we have used other fixatives and preservatives for specific reasons (e.g., cold-fixed in 100% ethanol or RNAlater for genomic analyses). If intended downstream uses include analyses like SEM, TEM, or histology, other fixatives (such as Bouin's solution) are needed.

Overall, we have found that 80% ethanol proves entirely adequate for both morphological and at least basic molecular studies. A single preservation medium simplifies storage systems and, importantly, allows for parallel morphological and molecular analyses using hologenophores (Pleijel *et al.* 2008). There is an abundance of literature exploring the propensity for, and problems associated with, cryptic speciation in the Trematoda (e.g., Cribb *et al.* 2022; Cutmore *et al.* 2023; Pérez-Ponce de León and Nadler 2010; Pérez-Ponce de León and Poulin 2018; Poulin 2011); hologenophores should be routinely generated for all collections and should be regularly incorporated in type- and voucher series. Although formalin does produce superior specimens for morphological study, the improvement over ethanol specimens is slight. In addition, formalin is more hazardous. Thus, we no longer find the use of formalin worthwhile.

Ensure ethanol used is *non-denatured.* Although there is debate as to whether denatured ethanol (ethanol mixed with methanol) damages DNA, most of this debate refers to vertebrate tissue. We have had grim experiences, as have others to whom we have spoken, with denatured ethanol having a destructive impact on the DNA of trematodes. Although we understand that new technologies permit the amplification of genomic DNA from even highly degraded samples, such techniques are still far from perfect and are beyond the reach of most standard laboratory set-ups (including ours). We have found it difficult to near impossible to reliably PCR-amplify sequence data from small worms fixed in denatured ethanol.

Choice of vials for long-term storage is not trivial. We store all our specimens in 2-ml, screw-top (with O-ring cap), flat-bottomed,

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polypropylene tubes, transferring all the fixed worms into the tube, letting them settle to the bottom, removing most of the saline, and filling with 80% ethanol. We preserve with 80% ethanol knowing that there is always a small amount of saline left in the tube with the specimens, so that the final ethanol concentration will be somewhere between 70–80%. In cases where there are lots of trematodes (enough to occupy a third of a tube's volume), we recommend replacing the ethanol after a few hours because saline will leach from the worms and dilute the ethanol concentration. Tubes with a simple snap-seal cap risk evaporation of ethanol. For these same reasons of dilution and evaporation, we avoid vials smaller than 2 ml. Worms should be separated from as much associated debris as possible before preservation, as debris will stick to the worms and can ultimately damage them.

Mixed infections of trematodes are common. Typically, during the dissection process, we sort trematodes collected from a single fish to family or genus and allocate a single tube per group; this reduces the number of times any tube is removed from cold storage and allows for simple organisation by trematode family within the storage freezer. Attempting to sort to species-specific tubes adds time to the dissection for little, if any, additional utility; any future interest in a particular species is likely to extend to all congeners from the same host. Vials should be labelled internally, with, at minimum, the unique identifier for the individual host, host information, and trematode family written in pencil (not ink; mechanical pencils are ideal) on parchment or parchment-type paper (readily available from stationary suppliers). We have experimented with 'waterproof paper' (actually thin sheets of plastic coated with a matte laminate) and other plastic paper like tracing or drafting paper, but this should be avoided as cutting them creates excessively sharp edges and corners which can damage worms in the tubes. Moreover, the laminate layer on waterproof paper degrades over time in ethanol; we have experienced the entire layer (and all the information written upon it) crumbling into nothingness as we pulled the label out of the tube. Labelling the outsides of tubes, which needs to either be done in ink or using sticky label paper, is a recipe for disaster when working with ethanol. Samples should be moved to cold storage (-20°C) as soon as is practically possible. In our experience, storage at room temperature for the length of an intensive field trip (a week or two) has no impact on the viability of the DNA, though they should at least be kept out of the sun and in a refrigerator or air-conditioned space if conditions are obviously hot; leaving them in above-freezing temperatures for months or years will almost certainly lead to irreversible DNA degradation.

Record keeping

Host identification

The host is the habitat, and its identity is among the most important biological data associated with any parasite; correct host identification is critical. Although many fishes are readily identifiable, there are a great number for which accurate identification (by even experienced ichthyologists) is difficult at best and impossible at worst. Representatives of families like trevallies (carangids), hardyheads (atherinids), and mullet (mugilids) are so generally similar in morphology, especially as juveniles, that they are sometimes impossible to identify without specific training in scale or vertebral counts. We have undoubtedly misidentified some of the 20,000 fishes we have examined and have recently taken steps to futureproof our field identification. We now photograph each fish before dissection (with a scale and colour comparison palette) and preserve a tissue sample (preferably muscle) in 100% ethanol to enable later molecular verification if necessary. We recommend lodging these tissues in a state or federal repository (i.e., institutions that often have legislation in force to protect collections for perpetuity); we lodge ours in the tissue collection of the Queensland Museum. Making these host tissues publicly available through such an institution adds significant value to each host examined; beyond being used for downstream host identification, the tissue could provide a resource for a wide range of ichthyological research (e.g., population structure and connectivity and phylogenomic analyses).

Negative data

Record-keeping is self-evidently important. Our approach has certainly evolved and now provides us with a powerful repository of both positive and negative data. Although it is obviously essential to keep clear records of what was found and from where, we have found that it is not so common to keep records of uninfected hosts and what organs were examined but not infected. We emphasise that these negative data are just as important as positive data. Leaving aside reporting obligations for permits, keeping accurate documentation of negative host and organ information may illuminate compelling trends in the data. It is important to differentiate between instances where organs were not examined and when they were examined and genuinely negative for trematodes. Where the breadth of potential target hosts is substantial, knowing which taxa and organs to target is vital for effective field collection.

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