Advances, challenges, and applications of laboratory culture of digenean trematode parasites

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Contents

1	Introduction	120
2.	Historical overview of trematode parasite cultures	126
	2.1 Data compilation	126
	2.2 History of trematode cultures	126
3.	Sporocyst or redia stages	127
	3.1 Parasite handling	127
	3.2 In vitro	129
	3.3 In vivo	130
	3.4 In ovo	131
4.	Metacercariae stage	132
	4.1 Parasite handling	132
	4.2 In vitro	134
	4.3 In vivo	136
	4.4 In ovo	137
5.	Adult stage	138
	5.1 Parasite handling	138
	5.2 In vitro	139
	5.3 In vivo	140
	5.4 In ovo	141
6.	Egg collection and handling	141
7.	Conclusions	144
Ac	knowledgements	146
	ferences	146

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Abstract

The development of parasite cultures has long been pivotal in advancing parasitology, with broad applications in medicine, veterinary science, and biology. Laboratory cultures are invaluable tools for studying parasite biology, host-parasite interactions, and the development of treatments and vaccines. However, cultures of digenean trematodes under laboratory conditions remain a challenging yet critical endeavour in parasitology. These parasites hold significant importance to both human health and ecological systems. Nevertheless, trematodes exhibit a complex life cycle involving multiple hosts, which demands innovative culture methods. This review aims to provide a comprehensive overview of laboratory culture techniques for digenean trematodes, covering in vitro, in vivo, and in ovo approaches. These techniques are discussed in order across the different parasitic life stages of trematodes, from sporocyst/redia to adults, as well as the handling of trematode eggs, with a focus on optimising media composition, host-cell integration, and environmental parameters. In vitro approaches, particularly those using host-cell-based media or host-derived sera, have shown promise for certain zoonotic species. However, cultures of marine trematodes often face limitations due to suboptimal media protocols. On the other hand, in vivo and in ovo methods, while generally achieving higher success rates, raise ethical and logistical concerns. Despite notable progress, the standardisation of protocols and the adaptation of techniques for a broader range of species remain significant challenges in digenean trematode cultures. Future research should prioritise the development of host-cell-based media, innovative culture technologies, and integrative molecular and proteomic tools to address these limitations and further our understanding of trematode biology.

1. Introduction

The development and use of parasite cultures have long been crucial tools to advance knowledge in parasitology, with broad application in medicine, veterinary science, and biology (Ahmed, 2014; Hutson, et al., 2018b). Cultures enable the easy handling of parasites under controlled conditions, and, more importantly, outside their natural host which is paramount to achieve a better understanding of the particularities of parasite infection processes (e.g., at a genetic level, where it helps identify genes and regions of resistance in the host (Ren et al., 2014)), or of parasite biology, such as behavioural adaptations like egg-hatching rhythms (Gannicott and Tinsley, 1996), or the effects of temperature on parasite growth (Sterud, 1998). The evolution of such applied research tools can have a strong positive impact on the development of treatments

and vaccines, leading to better disease management (Ahmed, 2014; Matile and Pink, 1990; Olivo et al., 2023). For instance, the in vitro culture of the trematode Paragonimus kellicotti allowed the identification of extracellular vesicles and their associated proteins, namely cysteine protease-6, a protein implicated in parasite-host interaction and a potential biomarker for diagnostic and therapeutic development (Di Maggio et al., 2023). From a fundamental research perspective, parasite cultures provide valuable insights into life cycles. For example, in vitro culture of Atriophallophorus winterbourni enabled the morphological and molecular characterization of this digenean species, contributing to resolve its taxonomy when hosts are not identified (Blasco-Costa et al., 2020). It can also provide critical information on host-parasite interactions. For instance, studies on the interaction of Fasciola hepatica newly excysted juveniles with host intestinal cells through in vitro models have revealed key molecular mechanisms such as the involvement of cathepsins and immunoregulatory proteins during early infection stages (Becerro-Recio et al., 2022). Similarly, the excretory-secretory products of Echinostoma paraensei sporocysts were shown to interfere with haemocyte functions of Biomphalaria glabrata, shedding light on immune evasion strategies (Loker et al., 1992). Thus, laboratory cultures of parasites can result in a comprehensive knowledge of parasite biology, ecology and evolution with subsequent improvement to epidemiological studies and disease outbreak management.

Trematoda is the most abundant class of parasitic flatworms, comprising two subclasses, Digenea and Aspidogastrea, with over 18,000 known species across four orders and more than 140 families (de León and Henández-Mena, 2019). Given that the vast majority of trematodes are within the subclass Digenea, the focus hereafter is almost exclusively on digeneans. Digenean trematodes can infect a wide range of hosts, including terrestrial, aquatic, and amphibious organisms. Regardless, their life cycle almost always includes an aquatic phase. The life cycle of these parasites is highly complex, alternating between free-living and parasitic stages, consisting of both asexual and sexual reproductive phases. These complex life cycles typically include three hosts but can vary from two up to four different hosts (Poulin and Cribb, 2002). Generally, the trematode life cycle starts with an egg that hatches and releases a larva (miracidium) which infects a first intermediate host, a mollusc (except in rare cases involving polychaetes). Inside the mollusc, the miracidium

develops into either a sporocyst or a redia, an advanced form with a mouth, multiplying asexually to produce cercariae, *i.e.* free-living nonfeeding infective larvae. Cercariae emerge from the first intermediate host and actively swim to infect a second intermediate host, which may be either an invertebrate or vertebrate depending on the trematode species, where they develop into metacercariae. The final host, a vertebrate (fish, amphibians, reptiles, birds, or mammals), becomes infected through predation of an infected second intermediate host. In this final host, the parasite matures into an adult stage, reproducing sexually to release eggs and initiate a new life cycle (Bartoli and Gibson, 2007) (Table 1).

The study of digenean trematodes is crucial due to their significance in public health (Chai and Jung, 2022) and ecological systems (Hatcher and Dunn, 2011; Johnson et al., 2010; Preston et al., 2021). Trematodes are responsible for major human parasitic diseases (Chai and Jung, 2022; Wanger et al., 2007), particularly in under-resourced regions where neglected tropical diseases remain a persistent challenge (Hotez et al., 2008). Ecologically, trematodes play vital roles in influencing community biodiversity composition (Mouritsen and Poulin, 2002), host population dynamics (Granovitch and Maximovich, 2013; Lafferty, 1993), and energy flow within ecosystems (Johnson et al., 2010; Thieltges et al., 2008). Laboratory studies can shed light on how these parasites interact with their hosts across different life stages and can provide critical insights into their adaptability to environmental changes, such as habitat degradation and climate change scenarios (Poulin et al., 2024; Selbach and Poulin, 2020), concerns of growing relevance. Advancements in trematode culture methods can bridge parasitology, disease management control, and ecological studies, offering a holistic approach to address both human and ecological health challenges. However, studies on trematodes are particularly challenging, given the complexity of their life cycles, which require multiple hosts with speciesspecific environmental needs, such as salinity, temperature, and light conditions (Hutson et al., 2018a). Unlike parasites with direct transmission (Hutson et al., 2018a), trematodes demand more intricate culture protocols, making standardisation across freshwater and marine systems difficult. A case study illustrating these challenges is the attempt to culture Bucephalus minimus sporocysts, a marine trematode that infects bivalves as its first intermediate host (see Box).

Table 1 Typical morphological and functional characteristics of the parasitic life stages of trematodes. Sporocyst Redia	logical and functions Sporocyst	al characteristics of th Redia	ne parasitic life stages Metacercariae	s of trematodes. Adult	Egg
Size range (µm)	100–1500	100–1500	100–300	1000–35 mm	30–150
Shape	Elongated Sac-like	Elongated Sac-like	Spherical Encysted	Flattened Leaf-shaped	Oval Operculated
Reproduction	Asexual	Asexual	N/A	Sexual	N/A
Feeding Mechanism	Absorbs nutrients	Ingest tissue	Stored nutrients	Ingest tissue	Yolk reserves
Environment Resistance	Host dependent	Host dependent	High (cyst wall)	Host dependent	Resistant to desiccation
Key Features	Lacks digestive gland	Developed pharynx	Thick cyst wall	Complex reproductive system	Operculum, protective shell
Function	Multiply	Multiply	Infect final host	Egg production	Transmission to first host

Box

Bucephalus minimus is a trematode parasite that infects the cockle Cerastoderma edule (Mollusc: Bivalvia) as first intermediate host (Magalhães et al., 2015). In the cockle, the parasite initially targets the digestive gland and quickly spreads throughout the whole body (Desclaux et al., 2002) (Fig. B1).

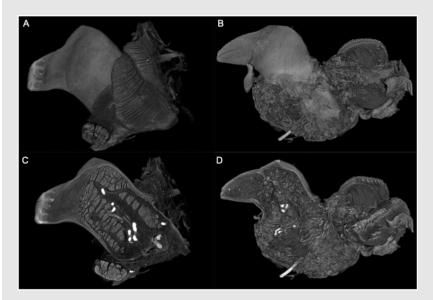


Fig. B1 Micro-CT scan of the cockle *Cerastoderma edule*. A) Frontal section of non-infected cockle; B) Frontal section of cockle infected with *Bucephalus minimus*; C) Dorsal cross section of non-infected cockle; D) Dorsal cross section of cockle infected with *B. minimus*.

For *in vitro* culture, *B. minimus* sporocysts were collected by dissecting cockles in a sterile artificial seawater medium with antibiotics (100 μg·ml⁻¹ gentamicin) under a stereomicroscope. Using fine tip tweezers, sporocysts were carefully extracted and placed in new sterile artificial seawater with antibiotics and put through three consecutive washes. After washing, sporocysts were individually placed in one well of 24-well plates with 1.5 ml of one of the three tested media: L15 (Lloyd & Poulin, 2011), DME/F-12 (West et al., 2014), and Medium F (Lloyd & Poulin, 2011), adjusted to 780 mOsm, supplemented with 0, 20 or 30% bovine serum albumin, and incubated at three different temperatures (16°C, 20°C, and 25°C). The media were refreshed every three days, and individuals showing no movement or body degradation were considered dead. The L15 medium at 20°C (regardless of supplements) showed the best potential to cultivate *B. minimus* sporocysts, resulting in the highest survival

rate, followed by medium L15 incubated at 16°C and supplemented with 30% bovine serum and DME/F-12 also incubated at 16°C and supplemented with 30% bovine serum. Nonetheless, no cercariae or daughter sporocysts development was observed. Besides, after 20 days, a 100% mortality rate was recorded, with severe degradation of *B. minimus* sporocyst tegument (Fig. B2).

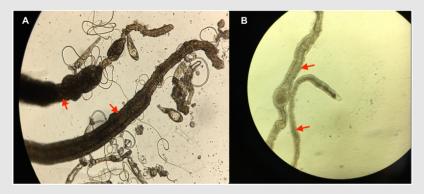


Fig. B2 *Bucephalus minimus* sporocysts under microscopy. A) Newly cultivated sporocyst displaying intact morphology; B) Sporocyst after one week of cultivation, showing degradation of the tegument. Red arrows indicate the sporocysts.

Collecting these sporocysts from the host tissue proved to be very challenging due to their large size and the difficulty in identifying and isolating a single individual. This process might have damaged the tegument, which is a crucial structure for trematode survival (Overstreet, 2024) (e.g., the most effective vaccine against *Schistosoma* sp. infections targets their tegumental structures (Cardoso et al., 2008; Loukas et al., 2007; Tran et al., 2006)). The use of alternative methods to extract the parasite (e.g., trypsin acid) could improve the laboratory cultivation of the first parasitic stages of trematodes (sporocyst), particularly for larger specimens where distinguishing their morphology is challenging.

The motivation for this review arises from the need for an updated and comprehensive overview of laboratory culture methodologies used to establish and maintain digenean trematodes under controlled laboratory conditions. This review is organised into *in vitro* (culture in a controlled, sterile environment outside the host), *in vivo* (culture within a living host), and *in ovo* (culture within embryonated chicken eggs). These techniques are discussed in order across the various parasitic life stages of trematodes, beginning with the first parasitic stage (sporocyst/redia), followed by the metacercaria stage, adult stage, and concluding with handling of trematode

eggs. The most effective methods are highlighted, and recommendations are provided to enhance the success rates of these cultures.



2. Historical overview of trematode parasite cultures

2.1 Data compilation

A literature search was conducted in the Web of Science® database to identify papers on trematode culture methods published before January 2025. The search keywords included "('in vivo' OR 'in ovo' OR 'in vitro') AND ('cult*') AND ('trematod*' OR 'digen*')". Abstracts and proceeding papers were excluded. The remaining scientific papers were filtered to focus on those addressing the topic of interest, namely the development or improvement (rather than the application) of protocols for cultivating trematodes under laboratory conditions. This resulted in a total of 43 relevant scientific papers out of an initial total of 149.

Among the remaining papers on trematode cultures (from the initial 149), studies applying culture methods focused on several aspects such as the development, growth and physiology of the parasite (Sulaiman et al., 2016), gene expression (Ren et al., 2014), and host immunological responses related to the parasite (Cortés et al., 2019; Zawistowska-Deniziak et al., 2022). Trematode cultures have also been used in studies describing trematode species (Blasco-Costa et al., 2020) and testing drugs and compounds for vaccine development or identifying substances harmful to the parasite (Arbabi et al., 2023; Olivo et al., 2023) (Fig. 1).

Similar literature searches were performed using comparable keywords, replacing "('trematod*' OR 'digen*')" with terms for other well-studied and significant parasite phyla, such as protozoans ('protozoa' OR 'protozoan'), nematodes ('nematod*'), apicomplexa ('apicomplexa'), and platyhelminths (cestodes ('cestoda' OR 'cestode') and monogenean ('monogen*')). Due to the large number of scientific papers retrieved in these searches, papers were not individually reviewed or filtered according to whether they focused on the development or application of protocols.

2.2 History of trematode cultures

The first successful parasite culture was reported in the early 20th century, notably in 1912, when (Bass and Johns, 1912) attempted an *in vitro* culture of *Plasmodium falciparum*, the protozoan parasite responsible for malaria in humans. Protozoan parasites have since been one of the most extensively

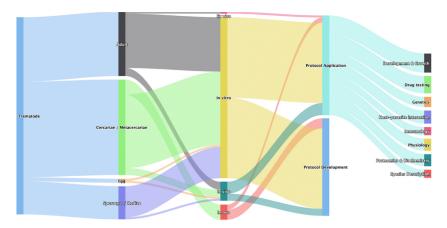


Fig. 1 Sankey diagram illustrating the focus of trematode culture research by "Life cycle stage", "Type of culture used", and "Protocol approach". The diagram distinguishes between studies that developed new culture protocols and those that applied existing protocols, with further categorization by research area. The thickness of the colour bands is proportional to the relative number of publications in each area.

studied parasites in terms of protocol development and usage of species from laboratory cultures (Fig. 2).

The first cultivation study involving trematode parasites was reported in 1948 by Goodchild (1948), focusing on *Schistosoma mansoni*, a human-infecting trematode that causes schistosomiasis. Since then, the number of studies on the development or improvement of trematode cultures has gradually increased, reaching a peak during the 2011–2020 decade with nine papers published (Fig. 2 – barplot). However, research on trematode cultures has remained limited compared to other parasite taxa (Fig. 2 – lineplot). The complexity of trematode life cycles may have contributed to a slower and limited development of culture protocols and may have led to the repeated use of similar protocols across different studies.



3. Sporocyst or redia stages

3.1 Parasite handling

Hosts infected with the sporocyst or redia stage (the occurrence of one or the other depends on the trematode species) can usually be identified by exposing the host to a temperature boost, which prompts cercarial emergence (de Montaudouin et al., 2016; Prokofiev et al., 2024). This

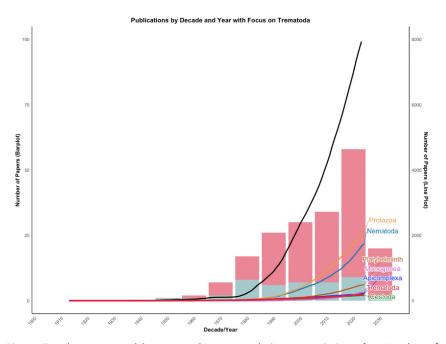


Fig. 2 Trends in parasite laboratory culture research (1900–2024). **Bar plot:** Number of papers per decade published specifically on trematodes. Blue indicates publications focused on protocol development, while red represents publications in which culture protocols were used in research. **Line plot:** Cumulative number of publications on laboratory cultures across different parasite phyla or classes. The black line represents the total number of publications across all parasite taxa. Data sourced from Web of Science database.

method, although widely used, might not be the most reliable for identifying infected hosts. First, the low prevalence of this life stage, likely because it is the most deleterious stage of a trematode's life cycle (Galaktionov and Dobrovolskij, 2003; Magalhaes et al., 2020), makes detection challenging. Second, stimulation of cercarial emergence may not always be successful, leading to misidentification. Consequently, this procedure can be time and resource intensive. Another approach to identify trematodes in the first intermediate host involves using a syringe to collect interstitial or gonadal fluid and observe the presence of cercariae under a stereomicroscope (Brian and Aldridge, 2021).

When infected, hosts are typically washed with sterile seawater or freshwater, depending on host habitat, and supplemented with antibiotics, for example $100\,\mu\mathrm{g\cdot ml}^{-1}$ gentamycin (Gorbushin and Shaposhnikova, 2002; Lloyd and Poulin, 2011). For hosts with shells, the host shell is usually wiped with ethanol (Lloyd and Poulin, 2011; Loker et al., 1999) before processing

with the antibiotic-treated water. Once cleaned, the host is dissected to extract the parasites. Some studies, however, soaked the host in water containing 1 % penicillin, streptomycin, and fungizone (Loker et al., 1999) before dissection.

After dissection, parasites are individually extracted and washed at least three times (or up to 10 times (Gorbushin and Shaposhnikova, 2002)) in antibiotic-treated water, as previously described, before being transferred to the culture medium (Gorbushin and Borisova, 2014; Lloyd and Poulin, 2011; Shirakashi et al., 2020). Nonetheless, solutions containing penicillin and streptomycin are also commonly used to wash parasites before transfer. For instance, Laursen and Yoshino (1999) washed the parasites five times in freshwater containing 60 μg·ml⁻¹ Penicillin G and 50 μg·ml⁻¹ Streptomycin sulfate. Similarly, DiConza and Basch (1974) used a solution of penicillin (300 μg·ml⁻¹), streptomycin (500 μg·ml⁻¹), and gentamicin (33 μg·ml⁻¹) to wash the parasites prior to transfer to the culture medium.

3.2 In vitro

The development of *in vitro* cultures for trematode sporocysts and rediae has focused primarily on two groups: freshwater species with significant impact on human or livestock health and welfare, and marine species with seabirds as final host. The first group of studies have in common the first intermediate host, the freshwater snail *Biomphalaria glabrata*, and includes species such as *Echinostoma caproni* (Ataev et al., 1998; Loker et al., 1999), *Fascioloides magna* (Laursen and Yoshino, 1999), and *Schistosoma mansoni* (DiConza and Basch, 1974). The second group of studies focused mainly on *Himasthla elongata* (marine trematode), whose first intermediate host is *Littorina littorea* (marine gastropod) (Gorbushin and Shaposhnikova, 2002), and four additional trematode species that use *Zeacumantus subcarinatus* (marine gastropod) as first intermediate host (Lloyd and Poulin, 2011).

For freshwater trematodes like *S. mansoni*, cultures used monolayers of *B. glabrata* (Bge) cells. DiConza and Basch (1974) developed an axenic medium for *S. mansoni* sporocysts, which included balanced salts, glucose, amino acids, vitamins, organic acids, and HEPES buffer with 20% inactivated human serum. In this medium, sporocysts grew 3 to 4 times in length, developing germ balls (stem cell-like structures that divide to produce subsequent generations) within 14 days at 25 °C, with serum from fasting donors yielding the best results. Laursen and Yoshino (1999), in turn, found that *F. magna* required Bge cell-conditioned medium for transformation from miracidia to sporocysts, achieving rates of 67% to 83% compared to less than

5% in unconditioned media. A similar pattern was found for *E. caproni* sporocysts, that showed limited survival in cell-free medium but survived up to 17 weeks in Bge medium with 10% fetal bovine serum (Ataev et al., 1998). However, their development into embryos was restricted to ~100 cells before degenerating. (Loker et al., 1999) found that *E. caproni* rediae cultured in Medium 199 with Bge cells supported better early development, however Bge medium supported longer survival despite some initial abnormalities.

For marine trematodes such as *H. elongata* and those infecting *Z. sub-carinatus*, an adapted Leibovitz-15 (L15) medium (GIBCO), supplemented with antibiotics at 780 mOsm, pH 7.8, and incubated at 14 °C, was used for cultivation (Gorbushin and Shaposhnikova, 2002). This medium, effective for cultivating marine invertebrate cells (Odintsova et al., 2001), allowed the development of cercariae and daughter sporocysts. However, (Lloyd and Poulin, 2011) further tested alternative osmolarities, serum concentrations, and other media (NCTC 135 (GIBCO), Medium 199 (GIBCO), and Medium F). They found that Medium F and L15 with 954 mOsm, supplemented with 20 % chicken serum and antibiotics, yielded the highest survival rates, with trematodes surviving up to 60 days. *Himasthla elongata* survived up to 160 days, though 50 % of rediae died within 70 days (Gorbushin and Shaposhnikova, 2002).

The most successful *in vitro* cultures combined appropriate serum supplements with host-derived cells, resulting in improved survival and development of trematode sporocysts or rediae *in vitro* (Coustau and Yoshino, 2000). Nonetheless, host cell-based media have only been developed for species that have the snail *B. glabrata* as first intermediate host (Laursen and Yoshino, 1999). On the other hand, more recent studies focusing on marine trematodes have been using other commercially available media, namely L15 with some modifications, which have not achieved long-term survival (Lloyd and Poulin, 2011). Limited survival in sporocyst and redia studies may reflect the specific culture media used or also the extraction method, which can be crucial for parasite viability (see Box).

3.3 In vivo

Only three studies attempted *in vivo* cultures for the first parasitic life stage of trematodes (*i.e.*, sporocyst or redia) by implanting trematode individuals into new, uninfected hosts. In the first study, conducted by Gorbushin and Borisova (2014), *Himasthla elongata* rediae were introduced into the

cephalopedal sinus of the snail hosts. However, the host immune response encapsulated and killed the rediae within days.

The other two studies focused on the genus *Cardicola*, where sporocysts were injected near the host's muscle tissue to avoid damaging the intestinal or nervous areas of the host (Shirakashi et al., 2020; Sugihara et al., 2017). These studies achieved better success rates, with observed sporocyst development and multiplication within 30 days, though with high host mortality (Sugihara et al., 2017).

In all studies, the trematode specimens were collected and washed as described in the 'Parasite Handling' Section (i.e., prior to dissection, hosts were washed in filtered seawater treated with antibiotics, and the parasites were washed again before introduction), and then introduced into the host using a 26 G to 30 G syringe with filtered, sterile seawater. Additionally, sporocysts that could not be injected immediately were stored at 4 °C for 24 h, during which they remained viable under these conditions (Shirakashi et al., 2020).

In vivo cultures of trematodes' first life stage have proven to be the most successful method to achieve sporocyst/redia multiplication under laboratory conditions, highlighting the critical importance of host environment for the survival and proliferation of the parasite. While sporocyst development and multiplication were successfully observed in some cases, the high host mortality and immune responses against the parasites present significant obstacles. Refining these techniques, such as optimising injection sites, improving host-parasite compatibility, and/or developing methods to suppress the host immune response, could increase the success rates of *in vivo* cultures. However, the ethical implications of using live hosts for *in vivo* cultivation should not be overlooked, highlighting the importance of exploring alternatives, particularly replicating the host environment, to address the current limitations of *in vitro* studies (such as using host-derived cell media).

3.4 In ovo

In ovo cultivation of early-stage trematodes has not yet been reported. However, it could be a promising approach for studying specific life stages in a controlled environment. For instance, further exploration of germ balls development as an infective stage using unfertilized chicken eggs could improve our understanding of the dynamics and asexual multiplication of this trematode stage and enhance cultivation efficiency. If successful, this approach could also provide an alternative to *in vivo* cultures, eliminating the need for a host and thereby reducing ethical concerns (Andersson et al., 2015).



4. Metacercariae stage

4.1 Parasite handling

Metacercariae, the second parasitic stage in the trematode life cycle, have been the most studied stage for developing trematode cultures in the laboratory. Metacercariae cultures are typically established either by encysting cercariae into metacercariae or by collecting metacercariae from the second intermediate host. Once obtained, metacercariae are excysted into a medium to promote their development into adults, with the aim of producing adults capable of laying viable eggs.

If starting with cercariae, similar to the methods used for 'Sporocyst and Redia Parasite Handling', infected hosts are typically identified, and cercariae are collected by exposing the infected first intermediate host to a temperature boost that stimulates cercarial emergence. This method has been commonly used for collecting cercariae (Basch and DiConza, 1977; Basch and O'Toole, 1982; Milligan and Jolly, 2011). Snails are placed in warm water to stimulate cercarial emergence, after which cercariae are promptly collected with a pipette and transferred to culture media (Milligan and Jolly, 2011). Alternatively, water containing cercariae is collected, centrifuged, and then washed in medium containing antibiotics before cultivation (Basch, 1981). For instance, (Basch, 1981) exposed infected Biomphalaria glabrata snails to 29 °C to stimulate cercarial release. The water containing cercariae was centrifuged at 1000 rpm for 30 to 60 s at 10 °C to concentrate the cercariae and remove excess water, and the cercariae were then washed in BME medium with 15 mM HEPES buffer, 180 µg·ml⁻¹ penicillin, 300 µg·ml⁻¹ streptomycin, and 160 µg·ml⁻¹ gentamicin.

Although species of the *Schistosoma* genus do not have metacercariae in their life cycle, they have a post-cercarial stage known as a schistosomula (Nelwan, 2019). Since this stage occurs between the cercarial and adult stages, it has been included in this section for practical purposes. In one particular study testing the viability of immature cercariae of *Schistosoma mansoni* to survive and develop into the adult stage, Basch and DiConza (1977) experimentally infected snails with miracidia. They maintained the snails at 24 °C for 14 days to develop sporocyst infections and subsequently slowed their development by maintaining the snails at 18 °C. The snails were then dissected in sterile water containing 300 µg·ml⁻¹ penicillin, 500 µg·ml⁻¹ streptomycin sulfate, and 50 µg·ml⁻¹ gentamicin per 10 ml of water. The immature cercariae were washed twice in the antibiotic bath to remove host debris before being transferred to culture media.

In contrast, metacercariae are generally more prevalent and abundant than the first parasitic stage (Magalhães et al., 2018) or the adult stage (Kuris et al., 2008; Rodríguez-González and Vidal-Martínez, 2008), making them easier to find and collect. However, this often requires dissecting multiple hosts to examine target tissues for metacercarial cysts or conducting experimental laboratory infections using cercariae from the first intermediate host. Most studies involved the collection of metacercariae from intermediate hosts (Chaithong et al., 2001; Daczkowski and Pung, 2016; Huesca-Guillén et al., 2007; Yasuraoka and Kojima, 1970). However, extraction methods were tailored to specific parasite-host systems. The most common extraction methods involved acid-pepsin digestion (Chaithong et al., 2001; Yasuraoka and Kojima, 1970), followed by trypsin to excyst the metacercariae.

For example, *Haplorchis taichui* metacercariae were extracted from the fish *Thynnichthys thynnoides* using pepsin acid digestion, followed by excystment in 1% trypsin at 39 °C (Chaithong et al., 2001). Fried and Johnson (2005) used a bile-trypsin medium (containing bile salts and acid pepsin) to excyst *Ribeiroia ondatrae* metacercariae obtained from the frog *Rana pipiens*.

Nonetheless, there are some other methods applying saline solutions. For instance, Gynaecotyla adunca metacercariae from Uca pugnax crabs were excysted by shaking in saline and then incubated in Hank's balanced salt solution with trypsin (Daczkowski and Pung, 2016). Similarly, Cotylurus erraticus metacercariae from trout hearts were excysted in Hank's saline solution containing trypsin and sodium taurocholate at 41 °C (Mitchell et al., 1978). For Microphalloides japonicus, a 0.85 % NaCl solution at 37 °C achieved a high excystation success rate (greater than 80%) (Fujino et al., 1977). Metacercariae of Maritrema gratiosum, obtained from barnacle second intermediate host, were excysted in a phosphate-buffered saline solution at 40 °C, achieving 97.5 % excystation within 120 min (Chuang et al., 2025). In all cases, antibiotics such as penicillin and streptomycin were added to excystment media to prevent contamination (Fujino et al., 1977; Yasuraoka and Kojima, 1970). These results also demonstrate the importance of temperature as an effective excystation trigger (Chuang et al., 2025; Mitchell et al., 1978).

The use of digestive enzymes (pepsin and trypsin) has been widely employed to help isolate metacercariae from host tissues. While this technique has not been applied to sporocyst or redia stages, it could be highly beneficial for isolating larger trematode species (e.g., Bucephalus minimus; see Box), as it may enhance the effectiveness of parasite isolation. However, it could also risk damaging the sporocyst integument.

4.2 In vitro

In vitro cultivation of metacercariae aims to replicate host environments to support the development of excysted metacercariae into egg-shedding adults. RPMI-1640 medium (GIBCO), a commercial medium frequently used for mammalian cell culture (Arora, 2013), supplemented with specific sera (often horse or calf), has been widely used for this life cycle stage. For example, Microphallus turgidus metacercariae developed successfully in RPMI-1640 with horse serum at 37 °C, producing egg-bearing adults and viable eggs capable of infecting snails (Pung et al., 2009; Pung et al., 2011). Similarly, Huesca-Guillén et al. (2007) used the RPMI-1640 culture medium to test the survival of Paramphistomum spp. However, for this species, the Hedon-Fleig medium was found to support the longest survival of immature stages (up to 10 days) compared to RPMI-1640, which enabled survival for up to four days (Huesca-Guillén et al., 2007).

Another commonly used medium was NCTC 109 media (GIBCO), specially for trematodes of the Microphallidae family. For example, Microphalloides japonicus responded well to Eagle's MEM (GIBCO) and NCTC 109 (ThermoFisher Scientific) media, both supplemented with 20 % calf serum. These media supported better egg production compared to basic saline solutions, with NCTC 109 achieving the highest success in complete egg formation. However, in vitro development was slower and less successful compared to in vivo conditions (see Metacercariae In Vivo Section) (Fujino et al., 1977). Similarly, Maritrema novaezealandensis also showed favourable responses to cultivation in NCTC 109. Excystment of M. novaezealandensis metacercariae was notably high when cultured in NCTC 109, irrespective of serum supplementation, demonstrating the medium's effectiveness in promoting excystation compared to less effective saline solutions. Furthermore, maximum egg production was observed after 48 h in adults derived from cultured excysted metacercariae in chicken serum-enriched NCTC 109 media (Fredensborg and Poulin, 2005). Maritrema gratiosum metacercariae developed into egg-laying adults when cultured in NCTC 109 enriched with 40 % chicken serum, maintaining a 50% survival rate after 10 days (Chuang et al., 2025). Eggs were observed within 48 h, with maximum production recorded on the fourth day.

Beyond the Microphallidae family, species from other trematode families have also been successfully cultured using NCTC 109. For instance, *Metagonimus yokogawai* was cultured in a specialised medium composed of chick embryo extract, human serum, and NCTC 109 (4:3:3 ratio) under 8 %

 CO_2 conditions. This medium supported full maturation of M. yokogawai into the adult stage with egg production, although the egg shells were imperfectly formed (Yasuraoka and Kojima, 1970).

Other studies have also highlighted the utility of NCTC 135 medium (GIBCO), supplemented with different additives, namely egg yolk, for achieving metacercarial development. For instance, metacercariae of Leucochloridiomorpha constantiae, when cultured in NCTC 135 supplemented with 20 % chicken egg yolk, developed into ovigerous adult stages within nine days. Cultures supplemented with lower-nutrient solutions, such as Locke's solution with albumen, extended survival but only supported minimal adult development (Fried and Contos, 1973). Similarly, Amblosoma suwaense achieved ovigerous adult stages in just four days when cultured in NCTC 135 with 20 % yolk (Schnier and Fried, 1980). Furthermore, Bucephaloides gracilescens metacercariae excysted in 0.5 % pepsin at pH 2 and subsequently cultured in NCTC 135 supplemented with chicken serum, albumen, and yolk, reached sexual maturity and began egg production within 14 days (Halton and Johnston, 1983). Nonetheless, metacercariae of Fasciola hepatica showed better growth in vitro when cultured with RPMI-1640 medium supplemented with human serum and red blood cells compared to NCTC 135 (Smith and Clegg, 1981).

Natural saline-based media have also been effective for certain species. Orthocoelium scoliocoelium showed extended survival (15 days) and normal egg production in a saline medium composed of 0.9 % NaCl and filtered rumen fluid with 60 µg·ml⁻¹ penicillin and 100 µg·ml⁻¹ streptomycin (Sharma and Sharma, 1982).

Trematode metacercariae maintained in minimal setups, such as saline or Hank's solution, typically survived for only 1 to 3 days and exhibited limited development (Fujino et al., 1977; Huesca-Guillén et al., 2007). However, under optimised conditions, certain species achieved complete *in vitro* life cycles. For instance, *Microphallus turgidus* metacercariae not only matured into egg-bearing adults but also produced viable eggs that could infect natural hosts, effectively completing its life cycle (Pung et al., 2011). Similarly, *Microphalloides japonicus* and *Metagonimus yokogawai* metacercariae reached sexual maturity after developing into adults and produced eggs under favorable conditions (Fujino et al., 1977; Yasuraoka and Kojima, 1970). Egg production varied significantly depending on the media and supplements used. For example, *Microphalloides japonicus* cultured in Eagle's MEM with serum produced eggs with complete shells (Fujino et al., 1977), whereas *Metagonimus yokogawai*, after the metacercariae successfully excysted and

transformed into adults, produced eggs that were morphologically incomplete *in vitro* but appeared normal when cultivated in host systems (Yasuraoka and Kojima, 1970).

Despite these advances, several challenges remain for *in vitro* cultivation. Variability in development is a frequent issue, even within the same culture, with some individuals exhibiting incomplete gametogenesis (Huesca-Guillén et al., 2007; Fujino et al., 1977). Reproducibility also poses a significant challenge, as even minor changes in gas composition or serum quality can drastically affect outcomes (Pung et al., 2011; Yasuraoka and Kojima, 1970). Across studies, serum supplementation has consistently proven crucial for achieving sexual maturity and egg viability, particularly when derived from the parasite's natural host. Acid-pepsin digestion followed by trypsin has emerged as the most effective method for excysting metacercariae across diverse species.

Environmental factors such as temperature and gas composition play a pivotal role in successful cultivation. Optimal temperatures typically range from 37 °C to 42 °C, closely mimicking body conditions of avian and mammalian hosts (Graczyk and Shiff, 1993; Yasuraoka and Kojima, 1970). While ambient air suffices for many species, specific requirements have been noted, such as the 8 % CO₂ atmosphere that significantly improved gametogenesis in *M. yokogawai* (Yasuraoka and Kojima, 1970). Dynamic culture systems, such as roller-drum setups (Berntzen and Macy, 1969), have been shown to enhance parasite development by improving gas exchange, while static cultures often require frequent media changes to maintain viability (Basch and DiConza, 1977; Yasuraoka and Kojima, 1970). Species-specific challenges also arise, with some parasites requiring co-culture with host cells, such as Bge cells for *Schistosoma mansoni*, or unique environments like unfertilized chicken eggs for *Cymatocarpus solearis* (Grano-Maldonado and Álvarez-Cadena, 2010).

4.3 In vivo

In vivo cultivation of metacercariae has not been a common technique for cultivating this stage under laboratory conditions. Nevertheless, it has achieved significant success when employing trematode parasites to infect alternative hosts, such as laboratory model organisms like mice, as substitutes for their natural definitive hosts.

For example, *Microphalloides japonicus*, a trematode parasite that typically uses birds as its final host, showed rapid development in mice *in vivo*. Within 12 h post-infection, sperm and incomplete eggs appeared, and

maximum egg production was reached within three days. In contrast, the best *in vitro* conditions for this species required 16 days to achieve similar development, producing fewer and less mature eggs (Fujino et al., 1977).

Similarly, *Metagonimus yokogawai* cultured in mice demonstrated faster growth rates and higher egg production compared to *in vitro* conditions. In mice, the worms rapidly reached maturity and produced fully developed eggs within a week. Conversely, *in vitro* cultures using NCTC 109 medium supplemented with human serum and chick embryo extract required 15–17 days to achieve sexual maturity, and the eggs produced were often incomplete, with fragile or malformed shells (Yasuraoka and Kojima, 1970).

These results indicate a higher efficiency of *in vivo* cultivation for achieving rapid and complete trematode development, whereas *in vitro* approaches, despite being more controlled, often require extensive optimization and often fail to fully mimic the natural host environment. However, *in vitro* approaches allow direct observation of growing worms without requiring experimentally infected hosts to be dissected; for different research purposes, *in vitro* approaches may be preferable despite their limitations.

4.4 In ovo

In ovo cultivation of trematode metacercariae, primarily using fertile chicken eggs, has proven highly effective for species that typically utilise birds as their final host. This method provides a nutrient-rich environment that closely mimics the trematode natural host, allowing for faster maturation and higher egg yields compared to *in vitro* cultures.

For example, *Microphallus primas* metacercariae, chemically excysted and placed under the chorioallantoic membrane of nine-day-old chicken eggs, matured into ovigerous adults within 5 days, producing up to 941 eggs per fluke. These eggs successfully infected snails, completing the parasite's life cycle and establishing a laboratory model for this species (Saville and Irwin, 1991). Similarly, *Clinostomum marginatum* metacercariae were successfully placed on the chorioallantoic membrane of chicken eggs. While eggbearing adults developed, the success rate varied significantly, with only 8 out of 123 worms becoming ovigerous after 4–7 days. Most worms migrated to the albumen, and the eggs produced were fewer and often malformed compared to *in vivo* models (Larson and Uglem, 1990). Metacercariae of *Leucochloridiomorpha constantiae* cultured on the chorioallantoic membrane of three-day-old chicken eggs also developed into

ovigerous adults within 4–8 days. In this species, however, no evidence of cross-fertilization was observed and, as with *Clinostomum marginatum*, worms in the albumen failed to develop (Fried and Holmes, 1979).

In addition to these studies, *in ovo* cultivation has been applied to other trematodes from bird hosts. *Echinostoma caproni*, for instance, showed significant postmetacercarial development on the chorioallantoic membrane of chicken eggs. Ovigerous adults were first observed 8 days post-inoculation, with egg counts reaching up to 100 per worm by day 12. However, the eggs laid on the membrane failed to embryonate, possibly due to the absence of insemination (Fried and Rosa-Brunet, 1991). Similarly, *Amblosoma suwaense* metacercariae developed into ovigerous adults within 4 days, with worms attaching to both the albumen and the chorioallantoic membrane. While the body area of these flukes decreased during cultivation, the gonadal and vitelline areas increased significantly, indicating effective sexual maturation (Fried et al., 1981).

Unfertilized chicken eggs were successfully used as a medium for *Cymatocarpus solearis*, yielding mature adults without the need for a definitive host (Grano-Maldonado and Álvarez-Cadena, 2010).

These studies highlight the effectiveness of *in ovo* cultivation for producing mature trematodes and viable eggs, though outcomes can vary depending on the species and experimental conditions. Compared to *in vitro* methods, *in ovo* cultivation provides a more supportive environment for trematode development, though it still faces challenges, such as ensuring egg viability and uniform development.



5. Adult stage

5.1 Parasite handling

Adult trematodes represent the final stage in their life cycle, where parasites reach sexual maturity and reproduce sexually. Progenetic trematodes, which mature sexually shortly after excysting from metacercariae, are particularly suited for laboratory cultures, especially *in vitro*, as they bypass the need for extended culture periods to develop into egg-producing adults (Irwin, 1997). However, cultivating adult trematodes in laboratory conditions, especially the need to use the final vertebrate hosts to collect the parasites, poses significant ethical and logistical challenges, particularly for procedures like dissection and infection (necessary to identify infection or producing *in vivo* cultures). Consequently, fewer studies focus on adult

trematodes compared to earlier life stages. Instead, the use of metacercariae chemically or mechanically excysted and cultured *in vitro* to develop into sexually mature adults capable of egg production is more frequent (see Section *Metacercariae stage*).

In studies performed with adult trematodes, the adult worms were collected from definitive hosts which had been naturally (Arbabi et al., 2021; Nizami and Siddiqi, 1974) or experimentally infected (Huesca-Guillén et al., 2007; Schou et al., 1997) and then washed several times using the same solution that would be later used to cultivate the parasites (in the case of in vitro culture). When the definitive host is a mammal, the most commonly used medium was the RPMI-1640 (Arbabi et al., 2021; Huesca-Guillén et al., 2007; Schou et al., 1997). For example, adult worms of Schistosoma japonicum were recovered by perfusing the mesenteric veins of mice with citrate saline solution. Following their extraction, the worms were washed in RPMI-1640 culture medium before undergoing surgical transfer or further in vitro experimentation (Schou et al., 1997). In the case of the liver fluke, Dicrocoelium dendriticum, specimens were collected from the bile ducts and gallbladders of sheep during necropsy and the worms were carefully washed three times in sterile RPMI-1640 culture medium before cultivating (Arbabi et al., 2021).

In the case of the trematode *Isoparorchis hypselobagri*, commonly found in the swim bladder of the freshwater fish *Wallago attu*, worms were extracted from their fish host and thoroughly washed three times in a laboratory-prepared balanced salt solution used as culture medium to ensure sterility before *in vitro* experimentation. Antibiotics used included penicillin and streptomycin (typically in concentrations between 30–60 µg·ml⁻¹ penicillin, and 50 µg·ml⁻¹ streptomycin) (Arbabi et al., 2021; Nizami and Siddiqi, 1974).

5.2 In vitro

Optimal conditions for *in vitro* cultures vary among trematode species, depending on their specific requirements, particularly with regard to their final host. RPMI-1640 has been widely used when the trematode's final host was a mammal, due to its adaptability across species. Supplementation with fetal bovine serum (FBS), glucose, and sheep red blood cells (RBCs) has been shown to enhance survival and maintenance in various trematode species. For example, *Dicrocoelium dendriticum* adult cultures maintained in RPMI-1640 supplemented with 50 % FBS and 2 % sheep RBCs demonstrated survival for up to 25 days (Arbabi et al., 2021). However, other media, such as Hedon-Fleig, NCTC109

or NCTC 135, have proven to be viable and, in some cases, more suitable options. For instance, *Paramphistomum* spp. adults survived longer (10–11 days) in Hedon-Fleig medium compared to RPMI-1640 (Huesca-Guillén et al., 2007). In contrast, *Schistosoma japonicum* exhibited better short-term survival in NCTC 135 medium supplemented with 10% heat-inactivated pig serum at room temperature (Schou et al., 1997).

The success of *in vitro* cultures of adult trematodes often depends on achieving a balance of pH, adequate nutrient supplementation, and optimal temperature conditions (usually 37 °C or room temperature showed better results). For instance, *Gynaecotyla adunca* egg production was optimised using Hank's balanced salt solution (HBSS) at 37 °C and pH 7, followed by transfer to DME/F-12 medium (GIBCO) enriched with horse serum (Daczkowski and Pung, 2016).

For species using non-mammal hosts, such as the trematode *Isoparorchis hypselobagri*, a balanced salt solution with glucose and salts at pH 7.0 was used under aerobic conditions. The addition of blood to a balanced salt solution significantly reduced weight loss and supported parasite viability for up to 49 days, although gradual degradation occurred over time (Nizami and Siddiqi, 1974).

While RPMI-1640 remains a common choice for mammalian host trematodes, the exploration of alternative media has proven essential for optimising survival and functional outcomes in various species. For instance, comparisons of Hedon-Fleig, RPMI-1640, and Rohrbacher media for *Paramphistomum* spp. revealed Hedon-Fleig as the most effective medium (Huesca-Guillén et al., 2007). Additionally, *S. japonicum* cultures demonstrated varying results across multiple media, including NCTC 135, NCTC 109, RPMI-1640, and physiological saline, emphasising the importance of species-specific adaptations in culture protocols (Schou et al., 1997).

5.3 In vivo

In addition to the *in vitro* techniques, (Schou et al., 1997) also studied *Schistosoma japonicum* through *in vivo* cultivation. Adult worms of *S. japonicum* were transplanted into pigs by injecting them into caecal veins under sterile conditions after being briefly maintained in NCTC 135 medium supplemented with pig serum. This procedure achieved successful post-transfer survival and recovery rates ranging from 14 % to 36 % after three weeks.

Nevertheless, in vivo studies involving adult trematodes remain relatively rare due to the significant logistical and ethical challenges associated

with using vertebrate hosts, which serve as the definitive hosts for trematodes (Keiser, 2010; Sutrave and Richter, 2023). These studies require the vertebrate hosts to provide the adult worm and to establish infections. Moreover, maintaining parasites in animal models over extended periods can be technically demanding, resource-intensive, and raises ethical concerns regarding animal welfare (Sutrave and Richter, 2023).

5.4 In ovo

No *in ovo* culture study of the adult stage of trematodes has been reported to date.

Previous reports of successful *in ovo* reproduction of adult trematodes were obtained using excysted metacercariae (see section: Metacercariae stage *In ovo*). However, starting directly by transferring adult trematodes from the definitive host into a chicken egg has not been a scientific priority. This is likely due to the logistical and ethical challenges associated with collecting parasites from vertebrate definitive hosts. Consequently, the focus has shifted towards developing *in vitro* culture techniques that eliminate the need for living hosts, provided trematode adult specimens are available.

6. Egg collection and handling

Egg collection and handling in trematodes vary depending on the trematode species and final host, but all involve retrieving eggs from infected hosts and maintaining them under controlled conditions to ensure survival and embryonic development. Trematodes are oviparous organisms that produce eggs during their adult stage (Bartoli and Gibson, 2007), generally expelled from the host through its faeces. These eggs can be collected from infected hosts either by faecal sampling or by dissecting the final host to retrieve adult individuals, allowing for egg collection directly from the internal organs where adult worms reside. For instance, Echinostoma caproni eggs were collected by dissecting infected mice, washing the worms in Locke's solution with antibiotics and incubating them (Ataev et al., 1998). Similarly, eggs of Echinostoma paraensei were obtained from the uteri of adult worms after dissecting naturally infected water rats (Lelis et al., 2014), while Fascioloides magna and Schistosoma mansoni were extracted from the liver or digestive tracts of infected animals, such as hamsters or other definitive hosts, to collect the worms and extract the eggs (Campbell, 1961; Laursen and Yoshino, 1999).

When faecal samples are collected, sedimentation methods are often employed to separate trematode eggs from organic material, with the eggs settling to the bottom due to their density, thus facilitating isolation (Rinca et al., 2019). An example of this method's application was demonstrated by (DiConza and Basch, 1974), who harvested eggs of *S. mansoni* from the livers of infected hamsters using homogenization and sedimentation in saline solution, followed by light exposure to induce miracidia hatching.

Once collected, the eggs are washed and maintained under controlled laboratory conditions that promote survival and support embryonic development. Eggs are typically placed in aerated water and kept at stable temperatures, most commonly around 25–26 °C (Campbell, 1961; Jurberg et al., 2008), although successful embryonation has also been observed at 37 °C in some cases (Ataev et al., 1998). Nonetheless, long-term viability of eggs can also be optimised under specific storage conditions. If faecal samples are not used immediately, storing them at 4 °C can maintain egg viability for an extended period (Rinca et al., 2019). Additionally, *F. magna* eggs were shown to remain viable for over a year when washed, incubated for 2 to 3 weeks at 26 °C, and then stored in water with activated charcoal at 4 °C (Laursen and Yoshino, 1999).

Some trematode species require specific conditions or additional factors to stimulate transformation into larvae. *Echinostoma caproni* eggs, for example, were incubated in the dark at 26 °C in tissue culture plates, while *F. magna* eggs required an initial incubation at 26 °C for 2–3 weeks before further storage (Ataev et al., 1998; Laursen and Yoshino, 1999). In certain cases, additional components, such as Bge cells, can enhance embryonic development and improve hatching rates. Co-culture with Bge cells has been shown to support the efficient transformation of *F. magna* eggs into miracidia by providing essential nutrients and environmental cues (Laursen and Yoshino, 1999).

Hatching is typically induced through environmental triggers, such as exposure to light and controlled water conditions, once the eggs have matured (Jurberg et al., 2008). For *E. caproni*, light exposure stimulates miracidia hatching after a 3-week incubation, resulting in an 80% infection rate in exposed snails (Ataev et al., 1998). Similarly, *S. mansoni* miracidia were hatched after light exposure and subsequently transformed into sporocysts during axenic cultivation with human serum at 25 °C, although sporocysts began deteriorating after 14 days (DiConza and Basch, 1974). In *F. magna*, hatching miracidia were transferred to a co-culture system with Bge cells, which supported further development of sporocysts and rediae

Table 2 Summary of trematode species used for *in vitro* cultivation, including the life cycle stages and the most commonly used culture media.

Life cycle stage	Trematode species	Culture medium	Key reference
Sporocyst/	Acanthoparyphium sp	L15	Lloyd and Poulin (2011)
Redia	Echinostoma caproni	Bge	Ataev et al. (1998)
	Fascioloides magna	CBSS + Bge	Laursen and Yoshino (1999)
	Galactosomum sp.	L15	Lloyd and Poulin (2011)
	Himasthla elongata	L15	Gorbushin and Shaposhnikova (2002)
	Maritrema novaezealandensis	L15	Lloyd and Poulin (2011)
	Philophthalmus sp.	L15	Lloyd and Poulin (2011)
	Schistosoma mansoni	Saline solution + Bge	DiConza and Basch (1974)
Metacercaria	Amblosoma suwaense	NCTC 135	Schnier and Fried (1980)
	Bucephaloides gracilescens	NCTC 135	Halton and Johnston (1983)
	Cotylurus erraticus	NCTC 135	Mitchell et al. (1978)
	Cotylurus lutzi	NCTC 135	Basch et al. (1973)
	Fasciola hepatica	RPMI-1640	Smith and Clegg (1981)
	Gynaecotyla adunca	DMEM F-12	West et al. (2014)
	Haplorchis taichui	RPMI-1640	Chaithong et al. (2001)
	Leucochloridiomorpha constantiae	NCTC 135	Fried and Contos (1973)
	Maritrema gratiosum	NCTC 109	Chuang et al. (2025)
	Maritrema novaezealandensis	NCTC 109	Fredensborg and Poulin (2005)
	Metagonimus yokogawai	NCTC 109	Yasuraoka and Kojima (1970)

(continued)

Table 2 Summary of trematode species used for *in vitro* cultivation, including the life cycle stages and the most commonly used culture media. (*cont'd*)

Life cycle stage	Trematode species	Culture medium	Key reference
	Microphalloides japonicus	NCTC 109	Fujino et al. (1977)
	Microphallus turgidus	RPMI-1640	Pung et al. (2009)
	Orthocoelium scoliocoelium	Saline medium	Sharma and Sharma (1982)
	Paramphistomum spp.	Hedon-Fleig	Huesca-Guillén et al. (2007)
	Schistosoma mansoni ^a	RPMI-1640	Basch (1981)
	Schistosomatium douthitti ^a	Medium 169	Basch and O'Toole (1982)
	Sphaeridiotrema globulus	NCTC 109	Berntzen and Macy (1969)
Adult	Dicrocoelium dendriticum	RPMI-1640	Arbabi et al. (2021)
	Gynaecotyla adunca	DMEM F-12	Daczkowski and Pung (2016)
	Isoparorchis hypselobagri	Saline solution	Nizami and Siddiqi (1974)
	Orthocoelium scoliocoelium	Natural media	Sharma and Sharma (1982)
	Schistosoma japonicum	RPMI-1640	Schou et al. (1997)

^aSpecies with schistosomulae as post-cercarial stage instead of metacercariae.

for up to 60 days (Laursen and Yoshino, 1999). Furthermore, rediae of *E. caproni* developed from cultured eggs survived for over 30 days when cultured with Bge cells in a medium supplemented with fetal calf serum and antibiotics, compared to shorter survival times in the absence of co-culture (Loker et al., 1999).

7. Conclusions

The cultivation of trematode parasites under laboratory conditions remains a challenging yet crucial area of research, with direct applications in parasitology, epidemiology, and the development of anti-parasitic drugs and vaccines. The complexity of trematode life cycles, often involving multiple host species and specific environmental requirements, has limited the success and scope of culture techniques compared to other parasitic taxa. However, advances in *in vitro* (see detailed summary in Table 2), *in vivo*, and *in ovo* culture methods have gradually expanded the possibilities for studying various trematode life stages in controlled environments.

Despite these advances, several key challenges persist. First, the difficulty in replicating the exact conditions found in natural hosts, particularly for marine trematodes, often limits the longevity and developmental success of cultured parasites. Host cell-based media have shown promise for freshwater species, yet marine trematodes still lack similarly optimised solutions. Second, standardising protocols across species is challenging due to the wide range of host-parasite interactions and the specific physiological requirements of each species. For example, while media such as RPMI-1640 and NCTC-135 have supported certain developmental stages in some species, others require specialised conditions such as the addition of animal serum or host-derived cells.

Future research should prioritise the development of methodologies and techniques that, while adaptable to the specific habitat requirements (e.g., freshwater or marine), and most probably through different culture media, aim to establish universally applicable systems, such as the inclusion of host cells as medium or serum, to accommodate a broader range of trematode species. Integrating novel cell culture technologies, such as the use of host stem cells or organoids (Duque-Correa et al., 2020), may offer new avenues for mimicking host environments more effectively. Additionally, advancing genetic, transcriptomic, and proteomic tools could provide insights into the molecular requirements of each developmental stage, further refining culture conditions.

This review highlights the importance of tailored methodologies for each life stage, from egg collection and hatching to the cultivation of sporocysts, rediae, cercariae, metacercariae, and adult stages. In conclusion, while the cultivation of trematodes remains a labour-intensive and technically demanding field, continued efforts to optimise and standardise protocols hold promise for significant advancements. Expanding our understanding of trematode biology through improved culture techniques will not only enhance parasitological research but also contribute to developing more effective treatments and control strategies for trematode-borne diseases.

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