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Aquatic parasite cultures and their applications

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Abstract

In this era of unprecedented growth in aquaculture and trade, aquatic parasite cultures are essential to better understand emerging diseases and their implications for human and animal health. Yet culturing parasites presents multiple challenges, arising from their complex, often multi-host life cycles, multiple developmental stages, variable generation times and reproductive modes. Furthermore, the essential environmental requirements of most parasites remain enigmatic. Despite these inherent difficulties, *in vivo* and *in vitro* cultures are being developed for a small but growing number of aquatic pathogens. Expanding this resource will facilitate diagnostic capabilities and treatment trials, thus supporting the growth of sustainable aquatic commodities and communities.

Aquatic parasite cultures permit advances in human, animal and environmental health

Parasite **cultures** (see Glossary) facilitate the completion of life cycles over successive generations, either *in vivo* (i.e. on or in a host) or *in vitro* (i.e. in the absence of the host). Alternatively, if the full life cycle cannot be completed, some parasite development or extension of life span might be achieved. Ideally, cultures should have defined origins and depending on the application, it may be desirable for them to be genetically restricted and maintain the same restricted subset of genotypes across generations. The ultimate goal of a parasite culture is to provide readily manipulated material of defined life-stages for replicable experimental research. Aquatic cultures include the use of molluscan, crustacean, fish, amphibian, avian and mammalian hosts for a wide diversity of parasites (Table 1). Recent rapid growth in the mass production of aquatic animals for food, alongside growth in international trade, rapid domestication and application of new technologies, drive emergent

parasitic diseases. Furthermore, global change is predicted to have important effects on parasitism in both freshwater and marine ecosystems, through changes in parasite distribution and transmission rates [1]. Although advances in aquatic parasite culture lag behind the growth in aquaculture, culturing remains a valuable tool in medical and veterinary parasitology as it enables understanding of basic parasite biology, facilitates vaccine and chemotherapeutic development, and improves diagnoses. Parasite cultures offer valuable opportunities for fundamental research and permit manipulative studies that can shed light on various aspects of transmission ecology, host-specificity, life history, host exploitation and evolutionary biology. The aim of this review was to examine existing *in vivo* and *in vitro* approaches to aquatic parasites cultures and explore opportunities for future development. We consider the origins of aquatic parasite cultures, recent developments in the field, and the application of this technology for advancing animal health and scientific knowledge.

Origins, establishment, maintenance and ethics

The medicinal leech, *Hirudo medicinalis*, was one of the first aquatic parasites to be cultured. In the 18th century, large-scale use of wild-caught leeches in Europe for human medicine gradually led to depopulation and the subsequent development of hirudiniculture [2]. Historically, sick horses were used as hosts for *in vivo* culture, while today medicinal leeches are bred in outdoor ponds on amphibian hosts or cultured *in vitro* using blood from slaughtered livestock. Interest in developing *in vitro* parasite culture peaked in the 1960s and 70s, building on extensive biochemical and metabolic research on cestodes undertaken in the 1940s and 50s by Smyth and others [3]. The motivation was to characterise the basic conditions required to support parasite maintenance and growth, and to identify the stimuli

triggering developmental transitions [4]. With little restriction on host maintenance or collection from the wild at the time, few researchers considered *in vitro* culture as a tool for maintaining pathogens in the laboratory. Through the 1980s and 90s, however, a gradual shift occurred from *in vitro* experimentation to maintenance, and the majority of laboratories now working on pathogens such as *Plasmodium* or *Schistosoma* species, do so using *in vitro* maintenance and long-term cryopreservation [5]. Such approaches mimic best practice with model organisms such as nematodes (*Caenorhabditis*) or amoebae (*Dictyostelium*), and are essential where there is intention to attempt methodologies such as RNA interference (RNAi) or gene transformation.

The current impetus to culture parasites is driven by the need for fundamental and applied research to support human and animal health agendas. Successful, continuous aquatic parasite cultures can be maintained for years or even decades [6-11], yet short term cultures may also be valuable for targeted experiments or the production of parasite tissues free of host contaminants. Indeed, many successful helminth culture media were developed from short-term maintenance media used in biochemical experiments. As culture media do not include dynamic host-related factors related to an immunological response, *in vitro* cultured parasites can be manipulated in ways not possible within hosts, making such techniques well suited to the study of excretory/secretory (ES) factors or in demonstrating the potential of the pathogen to grow or reproduce. At the same time, *in vitro* culture represents a gross oversimplification of the native environment, limiting its value when studying epigenetic influences on pathogen phenotype or the impact of microbiome interactions on parasites.

Parasite cultures can be established from infected wild or farmed hosts, or purchased commercially. Culturing requires adherence to local and national biosecurity and animal ethics legislation. The World Organisation for Animal Health (OIE)ⁱ provides a list of notifiable diseases, including some aquatic parasite infections, that can only be maintained in accredited facilities. Equally, host welfare and prevention of environmental contamination must be considered in line with national policies. A key advantage of *in vitro* culture, where animal hosts are replaced by culture systems, is the positive impact on the 3Rs animal protection principles [12].

***In vivo* cultures**

Development of *in vivo* aquatic parasite models are largely governed by the propensity of the host organism to be maintained in captivity and the degree of parasite host-specificity. Establishing a new culture may involve co-habitation of laboratory animals with wild or infected farmed stock, although individual exposure to a known quantity of infective stages is preferred in the interest of animal welfare and to avoid **over-dispersion**, which can also compromise hosts [10]. Where possible, recipient laboratory hosts should have no previous history of infection. Investigators can subsequently manipulate infections through direct physical addition or removal of parasite life stages and/or hosts [13, 14]. Uncontrolled reduction in infection may occur from intraspecific competition, direct removal by host behaviours or host immune response. Hosts provide sophisticated micro-environments and it is challenging to replicate these precise cellular conditions *in vitro*. For example, artificial nutrition *in vitro* is an approximation of the parasite's requirements, whereas *in vivo* culture

ensures optimal nutrition and thus facilitates the production of high-quality, virulent parasite life stages [15].

Skin ectoparasites, including **copepods** and **monogeneans**, are important parasites of captive fisheries, and have become valuable *in vivo* models because their infections are readily quantifiable and/or experimentally amenable, and their hosts can be sampled non-destructively (Table 1, Box 1). The most intensively-studied monogenean taxa are the viviparous gyrodactylids, which give birth *in situ* on the host and have no free-living larval stage [7]. This can lead to an exponential increase in parasite numbers on individual hosts; parasites typically only transfer to a new host when there is physical host-host contact, but can be dislodged from their hosts and maintained for short periods for survival experiments [1, 16] or drug trials [8, 17]. Genetically defined strains of *Gyrodactylus turnbulli* have been successfully maintained on guppies (*Poecilia reticulata*) for more than 10 years [7, 18]. Similarly, continuous cultures of sea-lice, *Lepeophtheirus salmonis*, have been established, with a defined protocol enabling predictable hatching of larvae and infections of host fish [11]. This has permitted the culture of specific *L. salmonis* strains, with defined phenotypic and genetic characteristics, for 22 generations [11]. Subsequent research utilising the culture has resolved prior inaccuracies in the understanding of the parasite life cycle and ontogeny [19], permitted experimental examination of stock susceptibility [20] and determined the effectiveness of parasiticides [21].

In vivo culture methods are useful for experimental validation of *in vitro* drug screening trials because studies conducted *in vitro* can generate spurious results that do not match those obtained from parasites attached to the living host. Parasites maintained on or in hosts

have the ability to seek out specific microhabitats, which may provide physical buffers to environmental change or protection from host immune responses [22].

***In vitro* cultures**

In vitro culture systems replace the host(s) with defined or semi-defined media conditions that may include undefined host factor supplements (or host cells acting as feeder cells) to sustain the parasite. Simple or direct parasite life cycles can be relatively easy to replicate and mimic *in vitro*, leading to rapid developments; for example, culturing of the diplomonads *Spironucleus* spp. [23] has facilitated metabolic analyses [24-27], genome sequencing [28] and genetic transformation [29]. In contrast, the complex life cycles involving multiple stages and/or hosts (e.g. of apicomplexans, myxosporidians, some kinetoplastids, helminths and crustaceans) are complicated by the need to mimic host sensory-biochemical signals to coordinate the parasite developmental transformations required for life cycle progression. Hence, parasites that exhibit complex life cycles are often maintained using a 'hybrid' culture system of both *in vitro* and *in vivo* methods, which are widely utilised in aquatic helminth research. Schistosome adults are typically maintained *in vitro*, avoiding the ethical difficulties, cost and complications of maintaining them in mammals (Box 2). However, although the snail parasitic stages can be maintained *in vitro* [6], it is difficult and the life cycle is normally closed by cycling through living snails. Even here, however, intervention can expedite the experimental program; methods for cloning sporocysts and re-inoculating parasite material into naive snails allow long-term propagation of especially valuable genetic lines [30].

Emerging pathogens are often the fastest to have *in vitro* methods developed, because of the urgent need to minimise their spread and the availability of research funds associated

with emergency response. For example, the **ciliophoran** *Philasterides* spp. only emerged as a pathogen in the burgeoning turbot (*Scophthalmus*) aquaculture industry in the early 2000s. Already, *in vitro* methods for the species are well-established, a freezing storage protocol is available [31], the metabolism is being unravelled [32] and drug screens are routine [33]. Similarly, the **euglenozoid** *Azumiobodo hoyamushi* was unknown before 2012, but appeared as an emerging infectious disease following culture of the edible ascidian *Halocynthia roretzi* to ease pressure on wild stocks [34]. The pathogen proved straightforward to maintain *in vitro* [35], and the biology of the organism is now being investigated using a variety of methodologies, although cryopreservation methods have not been developed.

Complex life cycles present the greatest challenges to maintain pathogens *in vitro*. Moreover, the life cycle of several parasites that cause important aquatic diseases have only been elucidated relatively recently [18, 36-40]. Metazoan parasites especially can have complex sensory/behavioural requirements that lead to major changes in morphology and gene transcription that are difficult to replicate *in vitro*. Understandably, culturing parasitic helminths, which often have more than two hosts, lags behind that of single-celled pathogens. Yet, a small number of *in vitro* systems have been developed, often using hybrid *in vivo* / *in vitro* methodologies (Box 3, Table 1).

Despite the lack of direct economic or human health impact, the experimental amenability of the cestode *Schistocephalus solidus* model has led to its adoption as a leading aquatic system for studying the ecology and evolution of host-parasite interactions. This contrasts with other diphyllbothriidean parasites, including *Ligula intestinalis* and *Diphyllbothrium latum*, which are more readily justifiable on socioeconomic or human health grounds, but are less readily cultured [3, 41]. Although the complete *Schistocephalus*

life cycle can be generated *in vitro* [42], most researchers use living copepods and sticklebacks as intermediate hosts. Plerocercoids of *Schistocephalus solidus* can be matured to the sexually-reproducing adult form under *in vitro* physicochemical conditions that mimic the definitive avian host intestinal environment [43], making it ideally suited for the study of parasite reproductive biology [44-46].

Applications

Established laboratory strains permit a continuous supply of aquatic parasites at different stages of development and of known genetic background, and hence represent a significant resource for continued advances in experimental applications. Herein we provide examples of how parasite cultures have advanced our understanding of taxonomy and phylogeny, aquatic parasite ecology and evolutionary biology, and diagnosis and treatment of pathogens in aquaculture.

Taxonomy and phylogeny

An important function of *in vitro* culture has been to facilitate the study of important, taxonomically diverse, yet hitherto poorly understood, aquatic pathogens. For example, prior to the development of *in vitro* culture, the **amitochondriate** eukaryote diplomonads (e.g. *Spironucleus* spp.) were only known from environmental sampling. *In vitro* culture has allowed the confirmation of high-level taxonomic links previously only suggested by molecular phylogenetic evidence. The close relationship between apicomplexans (including *Plasmodium*, *Toxoplasma* and *Cryptosporidium*) and dinoflagellates (predominantly free-living algae) was confirmed following development of culture techniques for the **obligate**

dinoflagellates *Amyloodinium ocellatum* (Noga 1987) and *Hematodinium* sp. [47], the shellfish pathogen *Perkinsus* [48] and the photosynthetic chromerids [49]. Similarly, while the taxonomic affinities of the fish pathogen genera *Ichthyophonus* and *Dermocystidium* proved contentious for many years, it is now clear they are members of the Ichthyosporea or Mesomycetozoa – part of an **opisthokont** lineage, and an early diverging sister group to the Metazoa [50]. Representatives of this apparently exclusively parasitic group are relatively easy to culture *in vitro* [51], and this has facilitated research development; *Creolimax*, a recently discovered genus [52], already has an annotated genome and protocols for genetic transformation, and is suggested as a model organism for studying the origin of multicellularity in the Metazoa [53]. The status of the Microsporidia in relation to *in vitro* culture is important in this context. While the best known, and most economically-significant microsporidians are pathogens of terrestrial insects (e.g. *Nosema*), many other representatives of this poorly-known fungal group infect a range of metazoans and chromalveolates from ciliates to humans, and have aquatic life cycles. Microsporidians are obligate intracellular parasites, and *in vitro* culture methodologies have been awaiting the development of methods to infect cell monolayers with the pathogens, only recently available [54].

Ecology, host-parasite dynamics and evolutionary biology

Infective stages from a parasite culture can be used to address fundamental questions in ecology. Controlled experimental infections and mesocosm systems can be used to explore the effect of parasites on host population dynamics, elucidate parasite life cycles, and determine reproductive behaviour, life-history strategies, transmission dynamics and

invasion pathways. Cultures overcome the problems of working with life stages that are typically small, dispersed and extremely difficult to collect from the wild. For example, the provision and fluorescent labelling of ample monogenean larvae (oncomiracidia) enabled the exploration of invasion routes of passive, unciliated oncomiracidia on rays [55] and active, ciliated oncomiracidia on fish [22]. Similarly, continuous culture of the monogenean *Neobenedenia girellae* was vital for the collection of sufficient material to provide the first detailed quantitative biochemical information of a marine parasite species' eggs [56]. Much of our understanding of parasite ecology on coral reefs has arisen from experimental studies involving juvenile stages of gnathiid isopods. Gnathiids play a significant role in coral reef ecosystems through their interactions with marine fishes and by providing a substantial food source for cleaner organisms. They provide an experimentally amenable culture model given their relatively simple and short life cycle, low host specificity and large size (Box 3).

Experimental aquatic host-parasite systems are frequently used as models in evolutionary research to investigate basic and applied questions about co-evolutionary processes, speciation and the evolutionary basis of host specificity and parasite manipulation strategies [43]. One area of interest is how evolutionary processes shape virulence, and specifically how the lack of a shared co-evolutionary history impacts interactions between host and parasites. Given the frequency with which aquatic ecosystems are subject to species introductions, there is a need to better understand the likely consequences for subsequent interactions between introduced aquatic hosts and/or parasites. The ability to produce infective stages of known-provenance parasites and hosts, and to undertake controlled experimental challenges, provides an invaluable tool in such studies. Recently, Kalbe et al. [57] were able to use reciprocal cross-infection studies to demonstrate significant effects of

both host and parasite provenance, but no interaction, on growth of *Schistocephalus solidus* plerocercoids in stickleback hosts, a proxy for virulence in this system. Experimental studies made possible by the *in vivo* culture of cestode, nematode and acanthocephalan parasites have also examined the evolutionary basis of both sexual ornamentation and **major histocompatibility complex** (MHC)-based mate choice in fish, providing further evidence for the role of parasites in natural and sexual selection [58, 59]. More recently, this approach has shed light on the influence of parasite infections on the rate of MHC allele selection [60, 61].

The evolutionary basis of infection-associated behaviour change is another field of investigation being addressed using aquatic parasite culture. While distinguishing between the various competing hypotheses is challenging in naturally infected hosts, experimental infection studies allow parasites to be identified as the causal agents of behaviour change and provide a route into studying physiological and neurochemical mechanisms of manipulation [62, 63], which can subsequently inform its evolutionary basis [64].

Finally, the ability to culture parasites *in vitro* permits control over their reproduction, so for dioecious or hermaphroditic species this allows mechanisms including hybridization [65] between cryptic species or strains to be studied under controlled conditions. Cryptic speciation is common among aquatic parasites, with closely-related parasite taxa often infecting related hosts in communities [66-68]. Recent studies have shown that *in vitro* matings between adults of different cryptic hermaphrodite *Schistocephalus* species generated both hybrid and pure-species offspring, with hybrids showing less **host-specificity** than pure-species parasites [69]. Such studies raise further question about the fitness of parasite hybrids in wild host populations, and the maintenance of species boundaries.

Diagnosis and treatment of pathogenic agents in aquaculture

The emergence of new or unknown parasite agents is often associated with growth in aquaculture. Aquatic parasite cultures can provide specimens for traditional comparative morphology, molecular taxonomy, identification of etiological agents and determination of species susceptibility. Indeed, parasite identification can be particularly challenging in aquaculture, where several similar parasite species are present (e.g. *Chilodonella* spp. infecting *Lates calcarifer* [70] and *Paramoebae* spp. infecting *Salmo salar* [71]) or when a single parasite species exhibits several different morphologies [56, 72]. Parasite cultures provide a means to determine species diversity (e.g. cryptic species) through isolation, colonial culture and characterisation. For example, single individual parasites, isolated and up-scaled to single lines, enabled morphological and molecular information to be linked precisely for four *Chilodonella* species found on a single freshwater fish farm [70]. Furthermore, species monocultures can be used to identify etiological agents (or validating culture virulence) by determining whether **Koch's postulates** are fulfilled [73].

Parasite cultures provide a valuable resource to screen chemotherapeutants, identify targets for chemotherapy, test anti-parasitic drug resistance, conduct drug trials and explore vaccine development. Yet, the majority of current treatments for the management of aquatic parasites use drugs that are only effective in killing adult parasites on or in their hosts and are unable to prevent re-infection from the environment. To ensure the efficiency of treatments, strategic management practices that break parasite life cycles can be deduced from life cycle information [Box 1; 19, 74, 75]. Reliance on and extensive use of chemical treatments in mass treatment programs in human medicine and aquaculture has reduced efficacy and in some instances has led to drug resistance [76]. Subsequently new drugs, vaccines, or new

approaches are desirable. More socially acceptable parasite management techniques, such as the application of biocontrols against attached and benthic parasite life stages, can also be explored [77, 78].

Challenges and opportunities for further research

Continuous passage of parasites through culture presents some common challenges to *in vivo* and *in vitro* culture, including loss of virulence [79]. Where feasible, *in vitro* cultures should be stored, or periodically passaged in fish to maintain the virulence of the isolates, because multiple passages can result in cells that have little in common with the original reference strain. Whilst *in vivo* cultures provide an opportunity to observe the overall effects of infection on living subjects and potential loss of virulence can be quantified with each serial passage, they require constant maintenance and upkeep of animal husbandry and welfare. Oomycete pathogens, such as *Saprolegnia*, can be cultured *in vitro* on agar plates or in simple broth during the mycelial phase, but are subject to reduced virulence if kept off the host for substantial periods [80]. To maintain parasite infectivity, the zoospore stage must be induced and the parasite must complete the life cycle on fish. Since this decline in virulence cannot be related to genetic change, this raises interesting questions concerning the epigenetic control of virulence in these organisms.

A potential benefit of parasite culture is the capacity to generate isogenic stocks. However, as different pathogens have different breeding systems and reproductive biologies, the ease and value of developing of isogenic stocks differs between taxa. Apicomplexans and dinoflagellates, for example, are haploid throughout most of the life cycle, exposing all loci to selection; diplomonads such as *Spironucleus* have paired diploid nuclei (giving a normal ploidy

of 4N) which can undergo homologous recombination. Much of the early tapeworm research focused on their breeding biology; the proglottids are genetically identical, so tapeworms can either self- or cross-fertilise. *In vitro* culture of *Schistocephalus solidus* provides a fascinating and useful, though not yet fully exploited, experimental tool with which to study reproductive decision-making by **hermaphrodites** [81, 82]. Details of an organisms' breeding system can have important implications for gene modification technologies including DNA knockouts, transformation and RNAi approaches. In the case of **haplontic** organisms such as dinoflagellates or apicomplexans, interference with a gene causes 100% disruption of function. For pathogens such as *Spironucleus*, two diploid nuclei must be transfected. In fact, this is possible for *S. salmonicida* [83], but the method has not yet been fully exploited to probe the reproductive biology of this organism.

State-of-the-art *in vitro* methods are scarcely used in aquatic parasite research, but there is enormous potential for translating new tissue culture developments into this discipline. Primary, as well as stable, commercially available cell lines can be used for *in vitro* culture of fish pathogens [84, 85]. Furthermore, recent developments in induced pluripotent stem cell (iPSC)-derived culture systems will potentially change the landscape of parasitology research. Successful implementation of these systems, including **organoids**, has been shown for various human pathogens [86]. The development of long-term full fish skin and short-term fish scale cell culture [87] 'opens the doors' for *in vitro* culture of many ectoparasites, including economically important species in aquaculture and the ornamental trade, and enables biological, developmental and environmental investigations of pathogens. If methodologies are robust and adapted for wide-scale screening, it would also facilitate fast and relatively inexpensive drug testing.

Continuous flow cultures permit the maintenance of cells and organisms that need stable conditions, and have been used successfully for culturing economically important dinoflagellates on industrial scales [88]. Many different types of continuous flow cell culture equipment are widely used, for example for pulmonary or intestinal bacterial diseases [89]. Recently, the hollow-fibre system was successfully implemented for *in vitro* culturing of the water-borne pathogen *Cryptosporidium* using host cells [90]. As this system is biphasic, it can be adapted for parasites that develop in the presence of host cells but require different environmental conditions to the host tissue (i.e. intestinal or gill microparasites). The constant flow of media through the system provides a stable environment and allows for optimisation to specific pathogens.

Concluding remarks

The continued pursuit to optimise aquatic parasite culture techniques will enable researchers to tackle important questions about the impacts of parasites on the economics of a primary growth industry, and on ecosystem functioning and evolution. The availability of commercial cell lines presents exciting new possibilities for *in vitro* culturing and the maintenance of various aquatic parasite species (see Outstanding Questions). Future advances in aquatic biosecurity and aquaculture technology may reduce parasitic disease emergence and facilitate complete exclusion from aquaculture environments, particularly those that have limited connectivity with the wild (e.g. recirculating land-based aquaculture systems), thus changing the landscape with respect to priority species targeted for culture.

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Glossary

Amitochondriates: Eukaryotes lacking a mitochondrial organelle

Ciliophorans: members of the protozoan phylum Ciliophora

Copepods: a group of crustaceans found in aquatic habitats

Culture: growing of animals, microbes or tissue cells on specially prepared media

Euglenozoids: a large group of unicellular flagellate excavates

Facultative parasite: an organism that lives independently of a host but may occasionally be parasitic under certain conditions; it does not rely on its host to continue its life cycle

Hermaphrodite: An organism that produces both male and female gametes and is capable of sexual reproduction either by self-fertilisation (i.e. without the requirement for a mate) or by out-crossing (i.e. mating with another individual)

Haplontic: alternation of dominant haploid stage with short-lived diploid stage

Host-specificity: the infectivity of a particular parasite species to a certain species or group of hosts

***In vitro*:** Latin for 'within the glass', refers to studies that have been isolated from usual biological surroundings

***In vivo*:** Latin for 'in the living', refers to studies in which the effects of various biological entities are tested on whole, living organisms or cells, usually animals

Koch's postulates: four criteria designed to establish a causative relationship between a microbe and a disease

Major histocompatibility complex (MHC): is a set of cell surface proteins essential for the acquired immune system to recognize foreign molecules in vertebrates, which in turn determines histocompatibility

Monogeneans: a Class of ectoparasitic platyhelminths or flatworms commonly found on the skin, gills or fins of fish

Obligate parasite: a parasitic organism that cannot complete its life cycle without exploiting a suitable host, cf. facultative parasite

Opisthokonts: a broad group of eukaryotes, including both the animal and fungus kingdoms

Organoids: clusters of cells, grown from tissue cells, that organise themselves into mini versions of organs

Over-dispersion: aggregated parasite distribution

Box 1: Cultures permit treatment strategies for monogenean parasite infections in aquaculture

Monogeneans can be problematic pathogens in finfish aquaculture and the ornamental trade. *In vitro* and *in vivo* monogenean cultures have been used to examine the efficacy of commercial [91] and natural products [17, 92, 93] as well as biocontrols [94]. *In vivo* culture enabled parasite life cycle parameters to be examined in response to varied environmental parameters which were used to develop strategic treatment regimens on aquaculture farms [10, 74]. For example, life cycle parameters including egg embryonation period, oncomiracidia longevity, infection success and time to sexual maturity have been evaluated from cultures of *Neobenedenia girellae* on Asian seabass (or barramundi, *Lates calcarifer*, Latidae; Figure 1). Administration of bath or in-feed treatments can be specifically tailored to reduce parasite reinfection given specific farm conditions (i.e. water temperature). An initial treatment kills adult parasite populations on fish, followed by a second treatment to kill immature parasites that have recruited as larvae from eggs around the farm.

Figure 1. *In vivo* culture of the fish monogenean *Neobenedenia girellae* infecting barramundi, *Lates calcarifer*, determined key biological parameters (A) egg embryonation period; (B) larva longevity and (C) time to sexual maturity for strategic management of infestations in aquaculture [10, 74]. An initial treatment (1) kills parasites attached to fish and a second, timed treatment (2), kills new parasite recruits before they reach sexual maturity and re-contaminate the system. Schematic, not to scale.

Box 2: Human flukes: advances in *in vitro* cultivation of intramolluscan stages of trematodes

The World Health Organization list of neglected tropical diseases includes several human trematodes, including blood, liver, lung and intestinal flukes. Infections affect more than one billion people and cost economies billions of dollars each year. For *in vivo* culture of the blood fluke *Schistosoma mansoni*, adult parasites are produced using mammalian hosts (i.e. mouse, hamster, rabbit and sheep; [95]) with asexual reproduction in natural aquatic molluscan hosts (i.e. snails; Table 1; Figure I). Early attempts *in vitro* using culture medium produced few, infertile eggs. However, the use of an embryonic snail cell line from its natural host, *Biomphalaria glabrata*, dramatically improved *in vitro* culture and provides adequate nutritional requirements and development-signalling factors to support sequential asexual propagation (i.e. from miracidium to cercariae; Figure I, [96]).

Figure I. The life cycle of human blood flukes, *Schistosoma mansoni* in nature and replicated for *in vivo* and *in vitro* cultures. Maturation (schistosomulae, juveniles and adults) occurs within humans in nature (A), laboratory mammals *in vivo* (B) and culture medium *in vitro* (C). The natural snail host *Biomphalaria glabrata* is used for *in vivo* cultures of the asexual stages and is also the primary source of embryonic snail cell lines *in vitro* (facilitating miracidia to sporocyst development). Schematic, not to scale.

Box 3. Gnathiid isopod cultures inform many aspects of parasites and host dynamics in coral reef ecosystems

The availability of an effective gnathiid culture system has permitted a wide range of repeatable laboratory experiments studying the biology, ecology and evolution of host-parasite interactions in barrier reef fishes.

Parasite biology. Insights into the taxonomy and the development of the model species *Gnathia aureamaculosa* (Figure 1; [97, 98]), gnathiid feeding ecology [99], and effect of host identity on gnathiid males' moulting duration and survival [98] were facilitated by *in vivo* culture techniques. Genetic studies of Great Barrier Reef species, including *G. aureamaculosa*, show gnathiids have undergone evolutionary diversification, accompanied by changes in morphology and behaviour [100].

Host-parasite interactions. The primary host of the haemogregarine fish blood parasite *Haemogregarina balistapi* and a potential vector of *H. bigemina* were identified as *Gnathia aureamaculosa* [see 101]. High gnathiid infestation causes extensive mucus shedding in adult host gills and death [102], whereas low exposure in juvenile fish causes size-selective host mortality at settlement [103, 104], reduced growth [105], and decreased swimming performance and oxygen consumption [106]. That gnathiids harm juveniles supports the hypothesis that fishes' pelagic phase allows larvae to avoid gnathiids. Yet, gnathiids affect fish species with and without a pelagic phase similarly, indicating the latter avoid gnathiids in other ways [106]. Fish skin toxin gland distribution influences gnathiid attachment site, suggesting toxins deter parasites [107]. Sleeping parrotfish without mucous cocoons were attacked more by gnathiids than ones with cocoons, showing cocoons protect fish against gnathiids [108].

Fish cleaning interactions. Changes in gnathiid load affected success of cleaner fish mimics indicating changes in net benefits influence aggressive mimicry systems [109]. Infestation by [110] and physiological responses to gnathiids, such as host haematocrit [111] and blood cortisol [112], suggest several proximate mechanisms influence fish client's cleaning behaviour. That cleaners prefer client mucus over gnathiids, which is more nutritious [113], suggest a cleaner-client conflict and the need for partner control in this mutualism [114]. That cleaners showed no preference for fed over unfed gnathiids, however, showed no cleaner-client conflict occurs over which gnathiids should be eaten [114]. Clients exposed to gnathiids spent more time seeking cleaners, showing parasite infection is a proximate cause of cleaning [110]. Client visual discrimination was reduced in gnathiid-exposed fish, providing a mechanism for how long-term cleaner presence in the wild similarly affected clients [9].

Figure 1. *Gnathia aureamaculosa* adapt well to laboratory culture *in vivo*. The life cycle is biphasic with a haematophagous fish-parasitic larval phase (A, B and C) which attaches and feeds on the host reef fish, *Hemigymnus melapterus*, before dropping off to moult into its next stage and a non-feeding adult phase (D) which is spent in the benthos. Schematic, not to scale.

Table 1. A selection of examples of aquatic parasite cultures and applications.

Species	<i>In vivo</i> (model organism)	Application(s)	Ref.
	<i>In vitro</i> (original host and/or cell line)		
Fungi			
<i>Saprolegnia parasitica</i>	<i>in vivo</i> (<i>Gasterosteus aculeatus</i>)	Culture techniques	[14]
	<i>in vitro</i> (<i>Oncorhynchus mykiss</i> cell line)	Molecular and cellular mechanisms of infection	[115]
<i>Anncaliia algerae</i>	<i>in vitro</i> (fish cell lines)	Culture techniques	[54]
Alveolates			
<i>Amyloodinium ocellatum</i>	<i>in vivo</i> (<i>Amphiprion ocellaris</i>)	Drug screening	[116]
	<i>in vitro</i> (fish cell line)	Culture techniques	[117]
<i>Chilodonella</i> spp.	<i>in vitro</i> (<i>Lates calcarifer</i>)	Species differentiation	[70]
<i>Cryptocaryon irritans</i>	<i>in vivo</i> (<i>Lates calcarifer</i>)	Infection dynamics	[118]
	<i>in vitro</i> (fish cell line)	Culture techniques	[119]
<i>Ichthyophthirius multifiliis</i>	<i>in vitro</i> (<i>Oncorhynchus mykiss</i> ; fish cell line)	Culture techniques	[120]
<i>Philasterides dicentrarchi</i>	<i>in vitro</i> (<i>Scophthalmus</i> spp.)	Cryopreservation, metabolism, characterisation	[31-33]
Euglenozoids			

<i>Azumiobodo hoyamushi</i>	<i>in vivo / in vitro (Halocynthia roretzi)</i>	Drug screening	[121]
<i>Trypanosoma carassii</i>	<i>in vitro (Cyprinus carpio)</i>	Biochemistry, immune response	[122]
<i>Hematodinium</i> spp.	<i>in vitro (Nephrops norvegicus)</i>	Culture techniques	[47]
Metamonadans			
<i>Spironucleus salmonis</i>	<i>in vitro (Salmonidae)</i>	Biology	[23]
Amoebozoans			
<i>Paramoeba perurans</i>	<i>in vivo (Salmo salar)</i>	Aquaculture management	[123]
	<i>in vitro</i>	Virulence testing	[73]
Myxozoa			
<i>Tetracapsuloides bryosalmonae</i>	<i>in vivo (Salmonidae and Fredericella sultana)</i>	Susceptibility, transmission	[124, 125]
Platyhelminthes - Cestoda			
<i>Schistocephalus solidus</i>	<i>in vivo / in vitro (Gasterosteus aculeatus and cyclopoid copepods)</i>	Ecology, evolution and reproductive biology	[43, 126]
Platyhelminthes - Monogenea			
<i>Discocotyle sagittata</i>	<i>in vivo (Oncorhynchus mykiss)</i>	Behaviour, reproductive biology	[127]

<i>Gyrodactylus</i> spp.	<i>in vivo</i> (<i>Poecilia reticulata</i> ; <i>Gasterosteus aculeatus</i> ; <i>Salmo salar</i>)	Biology, treatment	[7, 8, 14] 110, 111[1 28, 129]
<i>Entobdella solea</i>	<i>in vivo</i> (<i>Solea solea</i>)	Biology, life history	[130]
<i>Neobenedenia girellae</i>	<i>in vivo</i> (<i>Verasper variegatus</i> ; <i>Lates calcarifer</i>)	Behaviour, reproductive biology, aquaculture management (see Box 1)	[10, 131]
<i>Zeuxapta seriolae</i>	<i>in vivo</i> (<i>Seriola dumerili</i> ; <i>Seriola lalandi</i>)	Aquaculture management	[132, 133]
<i>Branchotenthes octohamatus</i>	<i>in vivo</i> (<i>Trygonorrhina fasciata</i>)	Biology, taxonomy	[55]
<i>Polystoma</i> spp.	<i>in vivo</i> (<i>Kassina senegalensis</i> ; <i>Kassina wealii</i> ; <i>Hyla meridionalis</i>)	Ecology, evolution reproductive biology	[134- 137]
Platyhelminthes - Trematoda			
<i>Diplostomum</i> spp.	<i>in vivo</i> (<i>Lymnaea stagnalis</i> ; <i>Gasterosteus aculeatus</i> ; <i>Larus argentatus</i>)	Reproductive biology	[138]
<i>Maritrema novaezealandensis</i>	<i>in vitro</i> (<i>Macrophthalmus hirtipes</i> and <i>Halicarcinus whitei</i>)	Culture techniques	[139]

<i>Philophthalmus</i> spp.	<i>in vitro</i> (<i>Zeacumantus subcarinatus</i>)	Experimental ecology	[140]
<i>Schistosoma</i> spp.	<i>in vitro</i> (molluscan cell lines)	Culture techniques (see Box 2)	[6]
	<i>in vivo</i> (various molluscs and mammals)	Chemotherapy (see Box 2)	[95]
<i>Fasciola hepatica</i>	<i>in vitro</i> (<i>Ovis aries</i>)	Chemotherapeutic resistance	[141]
<i>Echinostoma</i> spp.	<i>in vivo</i> (various mammals)	Biology	[142]
Crustacea			
<i>Lepeophtheirus salmonis</i>	<i>in vivo</i> (<i>Salmo salar</i>)	Biology	[11, 19]
<i>Argulus</i> spp.	<i>in vivo</i> (<i>Gasterosteus aculeatus</i>)	Culture techniques	[14]
<i>Gnathia aureamaculosa</i>	<i>in vivo</i> (<i>Hemigymnus melapterus</i>)	Ecology (see Box 3)	[9, 110]
Hirudinae			
<i>Hirudo medicinalis</i>	<i>in vivo</i> (various frogs, toads and newts)	Human and agriculture medicine	[2]
<i>Zeylanicobdella arugamensis</i>	<i>in vivo</i> (<i>Epinephelus lanceolatus</i>)	Aquaculture management	[13, 143]

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ⁱ <http://www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2018/>