Hook, line and infection: a guide to culturing parasites, establishing infections and assessing immune responses in the three-spined stickleback

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1.0 Abstract

The three-spined stickleback (*Gasterosteus aculeatus*) is a model organism with an extremely well-characterised ecology, evolutionary history, behavioural repertoire and parasitology that is coupled with published genomic data. These small temperate zone fish therefore provide an ideal experimental system to study common diseases of cold water fish, including those of aquacultural importance. However, detailed information on the culture of stickleback parasites, the establishment and maintenance of infections and the quantification of host responses is scattered between primary and grey literature resources, some of which is not readily accessible. Our aim is to lay out a framework of techniques based on our experience in order to inform new and established laboratories about culture techniques and recent advances in the field. Here, essential knowledge on the biology, capture and laboratory maintenance of sticklebacks, and their commonly studied parasites is drawn together, highlighting recent advances in our understanding of the associated immune responses. In compiling this guide on the maintenance of sticklebacks and a range of common, taxonomically diverse parasites in the laboratory, we aim to engage a broader inter-

disciplinary community to consider this highly tractable model when addressing pressing questions in evolution, infection and aquaculture.

2.0 Introduction

Aquaculture is currently the fastest growing animal food-producing sector, increasing by 6% annually in the 2000s (The World Bank, 2013a). In 2014, 73.8 million tonnes of fish were farmed, rising from 55.7 million tonnes in 2009 (FAO, 2016). In order to maintain the current level of consumption, whilst compensating for shortfalls from fisheries that have reached their maximum potential output, global aquaculture production will have to reach 93 million tonnes by 2030 (The World Bank, 2013b). As with agriculture, fish production can be increased via two main approaches: increasing the area turned over to the industry or improving yields. With the use of terrestrial and aquatic environments reaching their sustainable maximum, the focus of aquaculture is now firmly set on yield improvement via selective breeding, genetic modification and feed conversion efficiency (Myhr and Dalmo, 2005; FAO, 2016; Janssen et al., 2016). These goals, however, must be coupled with a better understanding of host-parasite interactions and improved disease prevention, since a major inhibitory factor to fisheries' yield improvement are losses to infectious diseases, many of which are caused by parasitic organisms (Meyer, 1991).

Teleosts diverged from other vertebrates some 333-285 million years ago (Near et al., 2012) and are the largest group of vertebrates (ca. 30,000 species) with a diverse range of morphological and behavioural characteristics (Near et al., 2012). This diversity is attributed, in part, to a suspected whole-genome duplication event ca. 320-404 million years ago, after the divergence of ray-finned and lobe-finned fish, but prior to the teleost radiation (Amores et al., 1998; Hoegg et al., 2004). Such diversity makes the establishment of suitable teleost models challenging. While the zebrafish (*Danio rerio*) has been adopted by many research communities and is especially suitable for developmental biology, embryology and genetic disease research (e.g. Parng et al., 2002; Wienholds et al., 2005; Zon and Peterson, 2005; Lieschke and Currie, 2007), it does not sufficiently resemble economically-important food fish such as salmon that tend to be temperate, ancestrally marine and omnivorous.

One candidate model species is the three-spined stickleback (*Gasterosteus aculeatus*) hereafter referred to as the 'stickleback', which has been described as a supermodel for ecological, evolutionary and genomic studies (Shapiro et al., 2004; Colosimo et al., 2005;

Gibson, 2005; Barber and Nettleship, 2010; Jones et al., 2012; Barber, 2013). This ancestrally marine fish occurs in coastal marine, brackish and freshwater environments north of 30°N latitude. Sticklebacks have been utilised as a model of adaptive radiation due to their remarkable morphological diversity, including variation in size, shape and protective armour, which has arisen following the post-glacial colonisation of innumerable freshwaters from marine refugia (Schluter, 1993; Reimchen, 1994; Walker, 1997; Colosimo et al., 2005; Jones et al., 2012). The reproductive isolation of populations inhabiting a wide variety of habitat types and exploiting diverse resources are generally thought to be the primary causes of stickleback adaptive radiation (Schluter, 1993; Lackey and Boughman, 2016); with phenologic differences among morphotypes being linked to idiosyncratic genome variation (Jones et al., 2012; Feulner et al., 2015; Marques et al., 2016; reviewed in Lackey and Boughman, 2016) and at least partially controlled by the epigenome (Smith et al., 2015a). Of particular interest are the Canadian limnetic-benthic 'species pairs' (that inhabit the pelagic and littoral zones respectively) and the river-lake morphs of sticklebacks which, despite that fact that hybridization is possible both in nature and the laboratory, display high levels of reproductive isolation (McPhail, 1992; Gow et al., 2006; Berner et al., 2009; Eizaguirre et al., 2011). In the case of the limnetic-benthic pairs, both forms are thought to have evolved from independent marine ancestors (McPhail, 1992), while a mixed pattern of morphotypes is likely the cause of the river-lake differentiation (Reusch et al., 2001a; Berner et al., 2008). Supporting predictions of adaptive radiation, the limnetic and benthic stickleback forms each have growth advantages in their native habitats, which are lost in the alternative habitat, while hybrids are intermediate; the efficiency of this exploitation matches the observed morphological differences (Schluter, 1993, 1995). The same holds true for river-lake fish ecotypes, which are locally adapted and suffer from translocations in non-native habitats (Eizaguirre et al., 2012a; Räsänen and Hendry, 2014; Stutz et al., 2015).

In addition to their wide geographic range and diverse morphology, the stickleback has many amenable features that make it ideal for experimental studies of host-parasite interactions. First, sticklebacks are easily maintained and bred in the laboratory as a result of their general hardiness, small size and low maintenance cost. Second, within their habitat range, sticklebacks can be collected easily from the wild. Third, unlike many vertebrates, there is comprehensive knowledge of stickleback parasitology (Arme and Owen, 1967; Kalbe et al., 2002; Barber and Scharsack, 2010; MacNab and Barber, 2012), natural history and ecology (Wootton, 1976, 1984a; Östlund-Nilsson et al., 2006), evolutionary history (Schluter, 1996;

Taylor and McPhail, 1999; Mckinnon and Rundle, 2002; MacColl, 2009), physiology (Taylor and McPhail, 1986; Pottinger et al., 2002) and behaviour (Tinbergen and van Iersel, 1947; Giles, 1983; Milinski, 1985, 1987; Milinski and Bakker, 1990; Reusch et al., 2001b; Barber et al., 2004). Fourth, publication of the stickleback genome (Kingsley, 2003; Hubbard et al., 2007; Jones et al., 2012) coupled with advanced post-genomic techniques makes this fish an ideal model for molecular study, including host immunology (Kurtz et al., 2004; Hibbeler et al., 2008; Brown et al., 2016; Hablützel et al., 2016). All of this allows one to focus, not on a single aspect of the system, but to take a holistic systems approach to studying host-parasite interactions.

The regional parasitic fauna of sticklebacks is remarkably diverse, covering nine phyla to date (Kalbe et al., 2002; Wegner et al., 2003b; Barber, 2007; Eizaguirre et al., 2011), largely as a result of the host's wide geographical distribution, diverse habitat exploitation, varied diet and central position in food webs. Virtually all niches of the stickleback have been exploited by at least one parasite species, including the skin and fins, gills, muscle, eye lens and humour, body cavity, swim bladder, liver, intestine, kidney and urinary bladder (e.g. Kalbe et al., 2002). Over 200 parasite species have been described infecting the stickleback, although many of these are cross-species infections from other teleosts (for complete list see Barber, 2007). Following the recent surge of interest relating variation in the gut microbiome to disease progression (Holmes et al., 2011), the stickleback's microbiome appears to be largely determined by genetic and sex dependant factors rather than transient environmental effects (Bolnick et al., 2014; Smith et al., 2015b); although differences in gut microbiota are also correlated with variation in diet (Bolnick et al., 2014). Heightened innate immune responses also appear to result in a less diverse microbiota (Milligan-Myhre et al., 2016); however, the reciprocal relationship between microbiota and parasites has yet to be studied in this system.

The impact of infection on host behaviour is well documented (Giles, 1983; Milinski, 1985, 1990; Milinski and Bakker, 1990; Poulin, 1995; Urdal et al., 1995; Barber et al., 2004; Spagnoli et al., 2016) but uncontrolled parasitic infections may confound results (as recently demonstrated in zebrafish; Spagnoli et al., 2016). Parasitic contamination applies not only to behavioural studies but to all research (immunological, parasitological, molecular etc.) where uncontrolled parasite infections other than those under investigation may have confounding effects, via stimulation of the immune system or interactions with co-infecting parasites.

While pharmaceutical treatments may be useful to control or limit confounding parasitic factors, their use is a double-edged sword bringing other problems linked to the severity of the treatment (Buchmann et al., 2004; Srivastava et al., 2004) and it can never be assumed that such treatments have 100% efficacy (Schelkle et al., 2009). It is also increasingly important that infection models can conform to a ‘wild’ or ‘uncaged’ state (Leslie, 2010) in order to understand the complex interaction of parasites, host immunological responses and ecological variation that are the prevailing state. The immune systems of wild animals and humans are rarely naïve and co-infection is the norm (e.g. Lello et al., 2004; Behnke et al., 2005, 2009; Benesh and Kalbe, 2016), partly explaining the many inconsistencies between laboratory models and wild animals.

The difficulty and importance of maintaining parasite populations in the laboratory is often underestimated and partly hampered by the lack of published practical information on establishing and maintaining host-parasite systems. In addition, molecular (drug) and immunological (vaccine) based approaches are increasingly needed for mitigating the impacts of disease. Effective models of aquaculture fish species are limited: the zebrafish, although ideal for molecular studies, is unrepresentative in terms of habitat, evolutionary history and parasitology. In this respect the stickleback provides a useful study species, being susceptible to a range of problematic aquaculture diseases, including those caused by the oomycete *Saprolegnia parasitica*, *Diplostomum* trematodes and *Gyrodactylus* monogeneans, as well other parasites closely related to aquaculture-relevant species. This review first covers the basic husbandry of the three-spined stickleback and then focuses on the parasites that are most frequently used in research projects: *Argulus* spp., *Camallanus lacustris, Diplostomum* spp., *Gyrodactylus* spp., *Saprolegnia parasitica* and *Schistocephalus solidus*. For each taxon, culture methods, experimental infection techniques and host immune responses are outlined. *Glugea anomala*, although not widely used experimentally, is a common infection of sticklebacks and is included in this review to stimulate future research. Whilst all of these parasites are common, until now there has been no single resource that summarizes all available culture methods. We also provide an overview of the host’s immunological responses to these parasites, and to put these studies in a wider context we recommend reviews of vertebrate (Murphy 2012; Owen et al., 2013) and teleost immunology (see Miller, 1998; Morvan et al., 1998; Press and Evensen, 1999; Claire et al., 2002; Watts et al., 2008; Takano et al., 2011; Forn-Cuni et al., 2014). Overall, we aim to provide a comprehensive and standardised approach to support new research utilising the three-spined stickleback as a model for experimental parasitology and immunology, while increasing awareness of the impact of any infections for non-parasitological studies.

3.0 Stickleback husbandry

Here, methods for the collection, maintenance and breeding of three-spined sticklebacks are described. In some instances multiple methods are provided, the suitability of which is dependent on the focus of a particular study.

3.1 Ethics

All protocols carried out are subject to the relevant regulatory authority. Care, maintenance and infection of protected animals in UK laboratories are governed by local animal ethics committees and the Home Office under The Animals Scientific Procedures Act 1986. EU member states are subject to Directive 2010/63/EU on the protection of animals used for scientific purposes. The Animals Scientific Procedures Act outlines humane methods for animal euthanasia referred to as 'Schedule 1 Procedures'. This nomenclature is used throughout the manual, but different guidelines are in place for other regulatory authorities. All experimental parasite research carried out at Cardiff University was approved by Cardiff University Ethics Committee and performed under Home Office Licence PPL 302357.

3.2 Collection

While some experiments require naïve hosts, for others, previous experience of endemic infections or specific ecotypes might be critical; information on fish provenance, parasite history and exposure to anti-parasitic treatments is therefore essential for most studies (see Giles, 1983; Poulin, 1995; Urdal et al., 1995; Barber et al., 2004; Spagnoli et al., 2016). When acquiring sticklebacks from wild populations, we advise multiple screens for ectoparasites and dissection for macroparasites (e.g. Kalbe et al., 2002); although the latter may not be necessary, particularly for breeding, as many macroparasites often require the presence of intermediate hosts to persist. Regardless, the presence of parasites should be reported for any study, and it should never be assumed that an animal is uninfected unless bred in specific pathogen free conditions.

Sticklebacks may be acquired from other researchers actively breeding these fish, possibly holding multiple inbred and/or outbred lines (e.g. Mazzi et al., 2002; Aeschlimann et al., 2003; Frommen and Bakker, 2006). Alternatively, they may be purchased from a commercial fish supplier (e.g. Katsiadaki et al., 2002a). Given the diversity and abundance of stickleback parasites, the principal of 'buyer beware' must apply, as rarely can a supplier guarantee ‘parasite-free’ fish and most fish will have been treated chemically (e.g. Giles, 1983; Poulin, 1995; Urdal et al., 1995; Barber et al., 2004; Spagnoli et al., 2016). Fish suppliers or researchers may be willing to provide infected sticklebacks for research or teaching, particularly in the case of overt infections, such as *Glugea anomala* or *Schistocephalus solidus*. A third option is to collect wild fish and use them directly (e.g. Bakker, 1993; Cresko et al., 2004; Bernhardt et al., 2006) after treating for infections (e.g. Soleng and Bakke, 1998; Ernst and Whittington, 2001; Cable et al., 2002a; Morrell et al., 2012; Anaya-Rojas et al., 2016; Hablützel et al., 2016) or breeding from these wild fish (e.g. Mazzi et al., 2002; Aeschlimann et al., 2003; Wegner et al., 2003a; Frommen and Bakker, 2006; Eizaguirre et al., 2012b).

Most institutions in Europe and continental North America neighbour a water body containing sticklebacks, particularly around coastal regions. Sticklebacks can be captured in commercial (e.g. Hendry et al., 2002; Gow et al., 2007; MacColl et al., 2013) or hand-made minnow traps constructed from 2-3 L soft drinks bottles. Each bottle, with holes in the sides, is cut such that the spout may be inverted and reattached using cable ties to resemble a minnow trap and partially filled with pebbles so it remains immersed. Typically, the traps are placed into water with one end secured by string to a concealed marker. Bait is not normally required as sticklebacks are inquisitive and catching one fish entices others. The trap is left for a maximum of 24 h to prevent fish becoming overly stressed. Dip-netting, using a hand net, is also effective (e.g. Gow et al., 2007; Brown et al., 2016), especially targeting areas of vegetation along the bank or under bridges where sticklebacks shoal and hide (Wootton, 1976). Permission should be sought from the landowner and appropriate regulatory authority before using traps or nets and these should be of a design so as not to endanger other aquatic organisms. Most wild sticklebacks will be infected with parasites (Barber, 2007) and appropriate measures must be taken to limit mortality (see Section 5). Importantly, ‘trapping’ stresses fish and compromises the immune system, but ‘netting’ can be used to sample fish in their natural state if euthanized immediately (e.g. Brown et al., 2016).

3.3 Maintenance

Sticklebacks are normally kept at densities not exceeding 1 fish/L to reduce fish stress (e.g. Mazzi et al., 2002; Aeschlimann et al., 2003; Barber, 2005; de Roij et al., 2011). Dechlorinated water is always used: 0.1-0.3 parts per million (ppm) of chlorine is lethal to the majority of fish (Wedemeyer, 1996), although brief exposure to chlorinated water (1-2 h) can be beneficial in removing some parasites (Johnson et al., 2003; Ferguson et al., 2007). Dechlorinated water is typically obtained either through an activated charcoal filter, commercially available dechlorinating and water conditioning solutions (follow manufacturer's instructions) or vigorous aeration of tap water for 24 h before use. Dechlorinated water should not be fed through copper pipes as high concentrations of copper ions can kill fish (Cardeilhac and Whitaker, 1988; Sellin et al., 2005; Grosell et al., 2007). Although sticklebacks are normally kept in fresh water, routine addition of 0.5-1 % salt water (aquarium or marine grade) inhibits some infections (e.g. Cresko et al., 2004; Bernhardt et al., 2006; Schluter, 2016). Freshwater captured sticklebacks are exceptionally salt tolerant, even tolerating sea water levels (3% salt), by means of differential gene expression; particularly those associated with hypertension including MAP3K15 (Wang et al., 2014). Care should be taken to adjust salinity levels gradually over a period of several days to avoid osmotic shock. Aeration to each tank is often provided by means of an air stone or filter. The physiological temperature range of sticklebacks is 0-34.6°C (Jordan and Garside, 1972; Wootton, 1984b); fish in our laboratories are typically maintained between 10-20°C, 15-18°C being optimal (e.g. Cresko et al., 2004; Barber, 2005; Scharsack and Kalbe, 2014; Kalbe et al., 2016). Lower (5-7°C) and warmer (18-20°C) temperatures are often used to induce a winter- or summer-like state (Bakker and Milinski, 1991; Barber and Arnott, 2000; Katsiadaki et al., 2002b; Kalbe and Kurtz, 2006; Hopkins et al., 2011; Eizaguirre et al., 2012b). Fish exposed to lower temperatures display growth rates that can be up to 60% slower (Lefébure et al., 2011), whereas those at temperatures above 20°C are subject to higher stochastic mortality. Sticklebacks are typically kept on a summer 14-16 h light: 8-10 h dark cycle (e.g. Barber, 2005; MacNab and Barber, 2012; Scharsack and Kalbe, 2014), which is altered to induce breeding (see Section 3.4).

Adult sticklebacks are most commonly fed on live, frozen or freeze-dried bloodworm (larvae of the non-biting midge in the Family Chironomidae), *Tubifex* spp. (also commercially referred to as bloodworm) or *Daphnia* spp. The preferred laboratory food is frozen bloodworm, which is easily stored and the most nutrient dense (Wouters et al., 2001), but should be defrosted and rinsed in a strainer before use to maintain water quality. Due to dietary conservatism (Thomas et al., 2010), wild fish prefer live food and may not feed immediately after capture but will begin eating defrosted bloodworm after 48 h. Commercial flake food can be used to supplement the diet, particularly if used during fish rearing (e.g.

Katsiadaki et al., 2002a). Optimal diets for stickleback fry are outlined in Table 1. Precautions should be taken with live food that may contain parasites (e.g. copepods are the intermediate host for *Schistocephalus solidus* and *Camallanus lacustris*), although laboratory culture and gamma irradiated food will remove many of these risks. For experimental protocols, sticklebacks can be isolated in tanks at 1 fish/L, with 90% water changes at least every 48 h to prevent increased ammonia and nitrite levels (e.g. de Roij et al., 2011). Chemical cleaning products, particularly those containing chlorine, should be avoided or chosen carefully as they may impact parasite infections and fish health (Brungs, 1973; Finlay, 1978).

[**Insert table 1 here**]

3.4 Breeding sticklebacks in vivo and in vitro

Breeding sticklebacks has a major advantage in that it can produce naïve fish that are free from macroparasite infections, mitigating the risks associated with uncontrolled infections; however, it is time consuming and resource demanding. Females carrying eggs are identifiable by their swollen abdomens, sharply angled in the region of the cloaca, sometimes with a single egg protruding from the cloaca. Male stickleback breeding condition is apparent when the eye sclera is blue and the jaw and abdomen are bright orange-red (Wootton, 1984c).

Photoperiod is considered an important stimulus in stickleback breeding, although this is dependent on the latitudinal origin of each fish population (Yeates-Burghart et al., 2009). Sticklebacks are typically exposed to a winter light cycle (8 h light: 16 h dark) for 2-3 months, before the length of daylight is increased to a summer light cycle (15-16 h light: 7-8 h dark) (Wootton, 1976; Bakker and Milinski, 1991; Barber and Arnott, 2000; Katsiadaki et al., 2002b; Kalbe and Kurtz, 2006; Hopkins et al., 2011); although Wootton (1984c) describes additional light cycles to induce reproduction. Temperature is also a major factor in inducing breeding condition (Borg, 1982; Sokołowska and Kulczykowska, 2009). We suggest a summer light cycle (see above) and a temperature of 18-20°C to be the most conducive for bringing fish into breeding condition. For both *in vivo* and *in vitro* breeding in the laboratory, a male and low density of females can be initially separated by sex in a tank divided with a mesh net, thus allowing reciprocal visual and chemical stimulation without direct contact. If males and females are housed in the same tank for *in vitro* breeding, the most gravid individuals are selected for fertilisation, and/or any males that become aggressive separated or euthanised for fertilisation. Alternatively, a female enclosed in a water filled transparent container can be placed into a tank containing males twice daily for approximately 30 min (e.g. Barber and Arnott, 2000). The fish should be fed at least 2-3 times a day on bloodworm; unrestricted feeding will also allow the sticklebacks to compensate for infection (Barber et al., 2008). Extra care should be taken to clean these tanks regularly, as a result of extra food waste and faeces.

Breeding *in vivo* is a common practice that does not require euthanasia of fish: eggs and fry are often raised in hatcheries to inhibit parasite transmission (e.g. Aeschlimann et al., 2003; Frommen and Bakker, 2006; Kalbe and Kurtz, 2006; Kim and Velando, 2015). All aquaria should be equipped with environmental enrichment, such as gravel, rocks and pipes or plant pots for refugia. Males must be provided with a submerged Petri dish containing aquarium-grade sand or gravel and 50-100 cotton threads (5 cm long), which they use for nest building (e.g. Kalbe and Kurtz, 2006; Little et al., 2008; Hopkins et al., 2011; Morrell et al., 2012). Alternatively, pondweed and other natural nest building material can be provided (see Jakobsson et al., 1999; Katsiadaki et al., 2002b; Östlund-Nilsson and Holmlund, 2003), but this may introduce unwanted pathogens or plant growth into the tank. Once the nest is built, once or twice a day the most egg bound female is introduced into the male tank for 30 min; if breeding does not occur within this period it is unlikely to do so. Stickleback courtship goes through a series of stages (see Wootton, 1984c; Östlund-Nilsson et al., 2006), then after the female has laid eggs she will swim out of the nest and the male will immediately enter, fertilise the eggs and proceed to chase away the female. At this stage, the female is removed from the tank and the male left to raise the clutch of eggs until they hatch or the eggs are removed into a hatchery (e.g. Barber and Arnott, 2000; Kalbe and Kurtz, 2006; Pike et al., 2009). The use of a hatchery reduces the likelihood of pathogen transmission between the parent and offspring. The male may be used again for breeding by supplying it with more nest building material allowing generation of half-siblings.

For *in vitro* breeding, the female is stripped of eggs, typically using a gloved hand dipped in Stress Coat® (API Fishcare), by gently squeezing the abdomen of a gravid female, moving fingers posteriorly from the pectoral girdle to the cloaca, and allowing the eggs to be collected in a 25 mm sterile Petri dish. Hanks' solution without phenol red (Hank' balanced salt solution, HBSS) may be added to the Petri dish to irrigate the eggs but this can reduce fertilisation rates (see Table 2). The eggs are released easily if the female is fully gravid, if not, the female should be replaced for a further 24 h to prevent damage by excessive force.

The released eggs should form a clump if fully developed, if the egg mass dissociates then they should be discarded. Using an approved euthanization procedure (see Section 3.1), sperm is collected from a male in breeding condition. An incision is made from the pelvic girdle cutting posteriorly, or at the anus cutting anteriorly, and a second incision just behind the operculum, pulling the flap off tissue back to expose the gut. An incision in the vas deferens is then made to remove the testes (Figure 1), which should be placed in sterile HBSS solution.

Sperm may be stored by shredding the testes into multiple pieces using forceps, releasing the sperm into a small dish of HBSS or adjusted Ginsburg's ringer solution and transferring it to an Eppendorf microtube containing HBSS. The sperm can then be stored at 4°C for 2-3 days with HBSS or 2 weeks in Ginsburg's solution if it is refreshed after 7 days (see Schluter, 2016 for Ginsberg's). Large testes can be cut into 2-3 sections using a sterile blade and the egg mass divided using artists' fine paint brushes in order to perform multiple fertilisations and produce half siblings (Barber and Arnott, 2000). Similarly, sperm from different males can be combined for sperm competition assays (Kaufmann et al., 2015; Mehlis et al., 2015). Fertilisations are carried out by stirring the shredded testes around the egg mass or adding a portion of the stored sperm; the testes are then removed after a few minutes replacing the lid of the Petri dish. Testes may also be macerated in 300 µl of HBSS and 50 µl added to a 'dry' Petri dish containing eggs for fertilisation; maceration can be conducted using a 40 µm cell strainer to avoid contamination with the tissue (Kaufmann et al., 2014). After 30 min at 15°C, the eggs can be checked for successful fertilisation, as indicated by separation of the inner and outer membranes, using a low power microscope (x10-60). Cell division should begin within 45-60 min, after which the egg mass is transferred to a hatchery (described below). Breeding *in vitro* is more reliable than *in vivo* breeding, requiring less time, and allows generation of maternal half siblings (e.g. Barber and Arnott, 2000; Pike et al., 2009; de Roij et al., 2011; MacNab and Barber, 2012).

[**Insert Figure 1**]

[**Insert table 2 here**]

3.5 Hatchery

For the hatchery, a small tank is used (20-30 x 40-50 x 10-20 cm deep) containing Hatchery Water (Table 2), which inhibits bacterial, fungal and oomycete growth, particularly *Saprolegnia declina* (e.g. Barber and Arnott, 2000; Pike et al., 2009). Methylene blue fades over time and should be replenished until the water is again a pale blue. Malachite green, at a concentration of 0.1 ppm, may be used as an alternative preventative measure (e.g. Kalbe and Kurtz, 2006). Hatcheries should be cleaned and re-made every 2-3 weeks to reduce infection risk. Newly fertilised eggs derived from *in vivo* or *in vitro* breeding can be placed in the hatchery within plastic cups suspended from the edge of the tank with the rims out of the water (Figure 2). The bottom of each cup is replaced with a fine mesh (0.5 mm) so that the eggs are suspended with sufficient water circulation. The mesh can be sandwiched between two cups or attached to a cup with aquarium silicone sealant. Air stones positioned under the cups provide oxygen and water circulation, but fine streams of bubbles that cause the egg mass to float and dry out must be avoided. Eggs will hatch in 7-8 days at 15°C, after which the cups are transferred and suspended from the edge of a standard 100 L tank containing a low salt concentration and methylene blue to inhibit infection of the fry (see Table 2). If eggs become infected with *S. declina*, the infected egg batch is removed, and all remaining eggs in the hatchery can be treated with malachite green (see low concentration bath; Section 5) (e.g. Barber and Arnott, 2000). Newly hatched fry fall through the mesh or can be tilted out of the hatching cups. The fry initially sink to the tank bottom where they remain for 1-3 days before establishing neutral buoyancy and they will then shoal in tank corners or around environmental enrichment. To prevent young fry being drawn into tank filters, they should be covered in a mesh or sponge and run at the lowest setting, or turned off entirely until 1-2 weeks post-hatching. Newly emerged fry are fed as indicated in Table 2 (e.g. Barber, 2005; Kalbe and Kurtz, 2006; de Roij et al., 2011; Schluter, 2016).

[**Insert Figure 2 here.]**

4.0 Common Stickleback Parasite Cultures

Here we provide updated culture methods for the parasites most commonly used in stickleback research that cover a broad range of phyla. Although not covered here, we recommend LaBauve and Wargo (2012) for information on *Pseudomonas aeruginosa* culture and Nielsen and Buchmann (2000) for *Ichthyophthirius multifiliis* culture.

4.1 *Argulus foliaceus*

4.1.1 Introduction

Argulus foliaceus (Linnaeus, 1758) is an ectoparasitic crustacean of the sub-class Branchiura (Figure 3 A-C). It is a generalist parasite with a widespread distribution across much of Europe and is recorded on most freshwater fishes including: common carp (*Cyprinus carpio*), bream (*Abramis brama*), brown trout (*Salmo trutta*), pike (*Esox lucius*), rainbow trout

(*Oncorhynchus mykiss*) and roach (*Rutilus rutilus*) in addition to sticklebacks (*Gasterosteus* spp.) (see Bower-Shore, 1940). According to Kearn (2004), *Argulus foliaceus* may parasitise any freshwater British fish species. At high infection intensities, major fish stock losses have resulted in the closure of some fisheries (Northcott et al., 1997; Gault et al., 2002). When attaching to the host *A. foliaceus* makes use of circular sucking disks (see Figures 3 and 4), with contraction of disk muscles resulting in adhesion (Møller et al., 2008). Alternate relaxation and contraction of these two disks allows the parasite to move around the host's surface. Further support is provided by a series of spines on the underside and edges of the carapace (Figure 4A). Individual *A. foliaceus* have two compound eyes for vision alongside olfaction and mechanoreceptors used for ambush detection of the host in light conditions (Mikheev et al., 2000). This behaviour switches in the dark to a 'cruising search strategy' accompanied by increased swimming speed, allowing the parasite to cover an area 3-4 times greater (Mikheev et al., 2000). Argulids feed using a stylet (Figure 4A) and proboscis (Figure 4B), the latter possessing serrated mandibles surrounding the mouth. During feeding, the spine-like stylet is inserted into the host's skin. Whilst the role of the stylet is still unclear, it is thought to involve injection of cytolytic substances that aid breakdown of tissues (Hoffman, 1977; Walker et al., 2011; Møller, 2012). This action with the rasping mouthparts and grazing behaviour of the parasite can inflict considerable damage to the skin of infected fish, particularly during heavy infection. Partly because of its feeding mechanism, *A. foliaceus* may act as a vector for viruses, bacteria and flagellates, including Spring Viremia Carp Virus (Ahne, 1985; Ahne et al., 2002). Depending on fish species, argulids will detach from their host and spend some time in the water column (Mikheev et al., 2015).

[**Insert Figure 3 & 4**]

Egg-laying of argulids is seasonal in the wild, being most active between July and August, but can occur all year round in the laboratory (Pasternak et al., 2000; Harrison et al., 2006). The first life stage is the nauplius, which depending on *Argulus* spp., develops to the metanauplius or first pre-adult stage prior to hatching (some authors refer to these stages as a 'copepodids' because of the historical inclusion of the *Argulus* genus in the Copepoda subclass). After hatching, 7 pre-adult stages occur before adulthood (Hoffman, 1977). Males are generally smaller than females and both moult frequently once sexually mature. Once adult, sexes can be easily distinguished through examination of the abdominal lobes (Fryer, 1982).

4.1.2 Source, culture and infection

All life stages of *A. foliaceus* can be maintained in the laboratory: although the methodology outlined below refers specifically to this species, it probably applies to most *Argulus* species (e.g. *A. coregoni* see Hakalahti et al., 2004).

As a generalist parasite *A. foliaceus* may be sampled from numerous freshwater fish species, although carp are a good source in the UK. Individual lice should be sexed, males have a larger and darker region defining the testes (Figure 3A), while the abdominal lobes of females possess small black spermathecae. In gravid females, the pale eggs (Figure 3B) may also be visible within the ovary running along the underside of the parasite. Although adult female *A. foliaceus* are generally too large for sticklebacks to eat (see Figure 3C), the swimming style makes them vulnerable to predation and fish will readily attack detached individuals. Therefore, abundant refugia (plant pots, fake or real weed, netting and/or plastic pipes) are necessary for shelter. Reduced lighting can also help reduce predation of parasites and may aid egg laying.

Infections with all *A. foliaceus* life stages can be performed by anaesthetising a stickleback in 0.02% MS222, transferring the fish to 100 ml of dechlorinated water and adding argulids. Alternatively, argulids can be allowed to infect fish naturally (e.g. Ruane et al., 1999; Forlenza et al., 2008; Kar et al., 2015); although we suggest placing the fish in the dark and adding refugia to reduce predation, which works well with metanauplii and pre-adults. To improve attachment, argulids can be starved for up to 24 h before exposure to a potential host.

For *A. foliaceus* breeding, infected fish are kept at 15-25°C (optimally 20°C), with one adult male and female *Argulus* per host; temperatures below 8-10°C cause egg laying to cease (Hoffman, 1977; Pasternak et al., 2000; Gault et al., 2002; Harrison et al., 2006; Taylor et al., 2009). Mating occurs on the host and then the female detaches to lay eggs, often in shaded areas on a hard substrate, such as the underside of rocks, stones or wood (Pasternak et al., 2000; Taylor et al., 2009; Sahoo et al., 2013). The eggs are laid in 2-4 rows with between 20 and 300 eggs per string (Figure 5A). Each egg is 0.3-0.6 mm in length and coated in cement, which anchors it firmly to the substrate. Tanks should be regularly checked for eggs to prevent unwanted infections when nauplii hatch. Eggs laid directly on the walls or bottom of the tank can be collected, but it is easier to transfer the infected fish to a new 1 L pot, as the eggs can be damaged even if carefully removed using a cell scrapper. Alternatively, fertilised female argulids can be removed from the fish when they develop large ovaries and placed into a Petri dish (90 mm dia.) containing dechlorinated water for 24 h allowing them to lay their eggs.

Egg hatching time varies with parasite species and temperature (Table 3). *Argulus* spp. eggs can be stored at 4-5ºC, which arrests embryo development, causing the nauplii to go into an 'over winter' state (Shimura, 1983; Gault et al., 2002; Harrison et al., 2006; Taylor et al., 2009). Photoperiod may also alter hatching in *A. siamensis* (see Bai, 1981), but has not been fully explored in other species. As a result of the temperature range and potential photoperiod required for hatching, a domestic fridge (4°C) provides ideal storage conditions. It is unknown how long eggs can be maintained in an arrested state, but successful hatching of eggs up to 4 months old has been achieved in our Cardiff aquarium. To induce hatching, eggs are transferred to a 1 L container of freshwater with aeration (Table 3). Egg development can be monitored by examining the egg string under a low power microscope (x10-40) the conspicuous eye spots of the developing metanauplii are easily seen, along with increased movement prior to hatching. Once hatched the metanauplii (Figure 5B) can survive off the host for 2-3 days. The metanauplii and pre-adults can be kept on sticklebacks (maximum of 5) or carp (20 max. on a 20 g fish). Infected fish should be maintained at 15-20°C; warmer temperatures will increase *A. foliaceus* growth rate but also stochastic fish mortality. To reduce pathology when argulids reach the later pre-adult and adult stages, all but two argulids should be removed, by gently encouraging them off the fish with a pipette tip or blunt forceps, and then excess detached argulids can be used to infect other fish.

[**Insert Figure 5 here**]

[**Insert table 3 here**]

The intensity of *Argulus* spp. is simply determined by counting the number present on the fish (e.g. Saurabh et al., 2010; Kar et al., 2015), sometimes adjusted for fish mass (Ruane et al., 1999). Given the range of sizes that this parasite can attain at different life cycle stages, measuring mass or size of the parasite is also beneficial. The size of the lesions (characterised by thinning of the epithelium, oedema and haemorrhaging) produced by *Argulus* spp. and behavioural lethargy of the fish may be useful measures of infection pathology (see Walker et al., 2004).

4.1.3 Immunology

Argulids induce a consistent innate response with the addition of an adaptive response approximately 7-10 days post-infection. The immunology of *A. foliaceus* infection has been little studied; there are however some closely related species for which the host immune phenotype has been documented. The majority of these studies have focused on sea lice of the genus *Lepeophtheirus* which, despite belonging to a different sub-class of the Copepoda, exhibit a similar life cycle to argulids. Typically, these studies have found constant increases in expression of *il-1*β, *tnf*-α and MHC II throughout the course of the experiment (9-40 days post-infection) (Fast et al., 2006a, b). Over a 6 day period *A. japonicus*, which infects common carp, produces a similar response to that of sea lice including up-regulation of *tnf-α* and the chemokines *CXCa* and *CXCR1* in the skin (Forlenza et al., 2008). Infections of rohu (*Labeo rohita*) with *A. siamensis* also demonstrate increased expression in the skin, particularly of innate responses, including *tnf*-α (although later at 15 days-post infection), lysozyme and natural killer cell enhancing factor (Saurabh et al., 2011; Kar et al., 2015). Kar et al. (2015) demonstrated a further role for adaptive immunity as IgM and β_2M also appear to be upregulated in the head kidney, although not consistently, from 0.5 to 15 days post-infection. Of further interest is the downregulation of TLR22 early in infection, complement and α2M more or less consistently across experiments, demonstrating that *A. siamensis* has the ability to modulate the immune system and other biological responses (Saurabh et al., 2010, 2011; Shailesh and Sahoo, 2010; Kar et al., 2015). Downregulation of the coagulation inhibitor α2M suggests a strategy that allows the argulid to inhibit clotting, making feeding easier. A key problem interpreting these studies is the harvesting of different organs and tissues, (skin, head kidney, kidney, serum and/or liver) for extraction of genetic material or immunological assays. While harvesting of the skin was performed in the majority of these studies, the range of other tissues taken and differences in methodology makes correlations between studies difficult to assess.

4.2 *Camallanus lacustris*

4.2.1 Introduction

The nematode *Camallanus lacustris* (Zoega, 1776) is a parasite of predatory fish, primarily perch but also pike, eels, and sticklebacks as a paratenic host (Kalbe et al., 2002; Krobbach et al., 2007). As adults, camallanids attach to the blind sacs and anterior intestine causing an inflammatory reaction (Meguid and Eure, 1996) and exhibit a seasonally reproductive life cycle with first stage larvae (L1s) only produced during the summer months (Skorping, 1980;

Nie and Kennedy, 1991). Gravid female nematodes may contain several thousand active L1 larvae, which are free moving, visibly coiling and uncoiling in the parental uterus. These larvae are shed from the vulva into the environment within fish faeces. Free-living L1s are viable in water for 12 days at 22°C and 80 days at 7°C (Campana-Rouget, 1961). They are ingested by a range of Cyclopidae copepods that act as intermediate hosts in which the larvae develop into L2s after 3 days at 25°C or 5 days at 20°C. For *C. lacustris* the second moult into the L3 stage occurs after 6 days at 25°C or 10-12 days at 20°C (Campana-Rouget, 1961). This is similar for other species within the genus, with *C. oxycephalus* reaching the L3 nine days post-infection at 25°C (Stromberg and Crites, 1974, 1975). Only at the L3 stage, coiled in the haemocoel of the copepod after migration from the digestive tract (De, 1999), is the camallanid larva infective to the definitive host on ingestion of the intermediate host (Moravec, 1969). These L3 larvae are relatively large within the haemocoel and at high intensities (>3 worms per copepod) copepod survival is reduced in a sex dependant manner (Benesh, 2011); smaller copepod species likely suffer reduced survival at lower infection intensities. Infected copepods are at a greater risk of predation upon attainment of *C. lacustris* infectivity (Wedekind and Milinski, 1996; Hafer and Milinski, 2016). Direct transmission from the copepod to the definitive host may occur by ingestion (Chubb, 1982), although more likely the copepods are first eaten by planktivorous fish, such as sticklebacks. When these paratenic hosts are predated, the camallanid reaches adulthood, producing *in utero* L1s within 69 days (Chubb, 1982).

4.2.2 Source, culture and infection

Gravid *C. lacustris* adults can be collected from the intestinal tract of perch (*Perca fluviatilis*) during summer in the UK; although Salmonidae, Gadidae, Esocidae and Siluridae may also act as hosts (Moravec, 1971). Parasites attach between the intestinal folds and may be easily removed by means of forceps. *C. lacustris* may be distinguished from other intestinal nematodes by the presence of a scallop-shaped buccal capsule and scheloritised tridents (Moravec, 2013) (Figures 6A & B).

The characteristic red adult *Camallanus* worms (Figure 6A) survive for 1-2 weeks *in vitro* at 4°C in 50% PBS. L1s can be removed from the adult worm (Figure 6C), held in a watch glass with 50% PBS, by puncturing the uterus with watchmakers forceps and allowing uterine contractions to force out the larvae. The L1s are visible using a dissection microscope (x10-60) and are conspicuous due to their high motility (Figure 6D), which is likely an adaption to increase predation. L1s survive for a minimum of 2-3 days *in vitro* at 4°C in tank water. They can be transferred using a *Caenorhabditis elegans* worm pick or P2 pipette to a non-treated culture dish or watch glass with lid containing copepods from the Family Cyclopidae. For larger infections 100 copepods are kept in beakers (250-500 ml) with 500 L1 larvae for ~10 days, changing the water 3 days post-infection. Larvae within the copepod should be counted before infection (see below). Previous experiments have used many copepod species as hosts for camallanids, including *Mesocyclops*, *Thermocyclops* (see Bashirullah and Ahmed, 1976), *Macrocyclops* (see Krobbach et al., 2007), *Acanthocyclops* (see Chubb, 1982) and *Cyclops* spp. The larger of the *Macrocyclops* spp. have been used as a host for up to six larvae of *Camallanus lacustris* (see Krobbach et al., 2007). Smaller copepod species may be less able to survive such a high infection. Female copepods are also subject to increased mortality at high infection intensities in comparison to males (Benesh, 2011).

[**Insert Figures 6 A-D here**]

Macrocyclops spp. should be fed on *Artemia* spp. (see Krobbach et al., 2007) although species such as *Cyclops strenuus* survive well on a daily mixture of *Spirulina* and yeast (approximately 1 ml per 10 L tank of copepods; see Table 2). For copepods kept in culture dishes, half their water should be removed and replaced with a dilute feed mixture (100 μl in 100 ml) every 2-3 days.

Development of *Camallanus lacustris* into the L3 takes approximately two weeks at 15-18°C on a 16:8 h light: dark cycle. Infectivity of the L3 can be checked using a recently deceased host, squashing the copepod onto a glass slide with a cover slip and a drop of water and viewing under a compound microscope (x40). Live copepods may also be checked individually by putting them on a slide with as little water as possible and rapidly counting the larvae under a compound microscope; this also allows dose determination (e.g. Eizaguirre et al., 2012b; Lenz et al., 2013). Striations on the buccal capsule are characteristic of the L3 (Figures 6A & B), but may only be visible through microscopic examination of squash preparations of the whole copepod host; the buccal capsule itself is apparent first in the L2 larvae. Prior to infection, sticklebacks should be acclimated to feeding on copepods. To infect sticklebacks with *C. lacustris*, the fish are starved for 24 h and then infected copepods are released into a crystallising dish containing the intended host. The optimal number of camallanids to feed each stickleback is six, which will give an infection rate of 40-50%

(Krobbach et al., 2007) with *C. lacustris* intensity measured by the number of individuals in the host's gut (e.g. Krobbach et al., 2007; Lenz et al., 2013).

4.2.3 Immunology

The cellular immunological responses of the stickleback to *C. lacustris* infection are largely unknown. However, a role has been described for the MHC, pivotal for activation and control of the adaptive immune response by presenting parasite- and self-antigen to T-cells. Eizaguirre et al. (2012b) identified a link between *C. lacustris* infection and a shift in adaptive MHC allele frequency with selection for specific haplotypes conferring resistance in the offspring of parents exposed to the infection. Such a rapid change in frequency highlights the important role of the adaptive immune response in this infection system. Granulocyte/lymphocytes ratios were elevated during high intensity parasite infections, but with no elevation in respiratory burst and leucocyte responses (Krobbach et al., 2007).

Within vertebrates the mucosal-associated lymphoid tissues direct immune responses at mucosal sites including the gut. The teleost gut-associated lymphoid tissue contains two predominate immune cell populations; lamina propria leukocytes (including granulocytes, macrophages, lymphocytes and plasma cells) and intraepithelial lymphocytes (T and B-cells found among epithelial cells) (see Rombout et al., 2014; Parra et al., 2015). In trout the T-cell receptor β was found to be relatively diverse and polyclonal, in comparison to the restricted diversity observed in mammals, an attribute possibly linked to the lack of Peyer's patches and mesenteric lymph nodes in fish (Bernard et al., 2006). Additionally, while both IgM and IgT are found within the gut-associated lymphoid tissues IgT+ B-cells make up the predominate cellular repertoire, particularly in response to intestinal parasites (Zhang et al., 2010). Given the high degree of conservation in the vertebrate immune system, it is possible that a gastrointestinal nematode infection in teleosts will, as in mammals, stimulate a response involving T-helper cell type 2 (T_H2) cells. In mammals T_H2 responses are characterised by increased expression of signature cytokines such as IL-4, IL-5 and IL-13 resulting in eosinophilia, mast cell activity, IgE production and mucosal changes (Jackson et al., 2009). While the teleost immune system is relatively understudied, T_H2-like cells and functional responses (involving teleost il4/il13) have been observed in zebrafish and salmonids (see Balla et al., 2010; Takizawa et al., 2011; Hammarén et al., 2014) and might be predicted to also occur in the stickleback.

4.3 *Diplostomum* spp.

4.3.1 Introduction

Trematodes of the genus *Diplostomum* (von Nordmann, 1832) are some of the most common parasite infections in sticklebacks (e.g. Pennycuick, 1971; Karvonen et al., 2013, 2015), especially for populations inhabiting lentic environments (Kalbe et al., 2002). Historically, three *Diplostomum* species have been frequently recorded; *D. spathaceum* (Rudolphi, 1819), *D. pseudospathaceum* (Niewiadomska, 1984) and *D. gasterostei* (Williams, 1966). Molecular approaches, however, have revealed an expanding assemblage of *Diplostomum* species complexes spanning the geographic range of sticklebacks (e.g. Locke et al., 2010; Georgieva et al., 2013; Blasco-Costa et al., 2014). Mitochondrial genomes and nuclear rDNA sequences for *D. spathaceum* and *D. pseudospathaceum* (see Brabec et al., 2015) now provide tools for landscape genetic mapping of these parasites.

Diplostomum utilises a complex, three stage life cycle comprising freshwater snails (Family Lymnaeidae) as the first intermediate host, fish as second intermediate hosts and a range of piscivorous birds as definitive hosts (e.g. common gulls *Larus canus*; see Karvonen et al., 2006a). Sticklebacks obtain *Diplostomum* infections by encountering free-swimming cercariae (Figure 7A) shed from infected snails, commonly of the genera *Lymnaea* or *Radix*. Whilst *Diplostomum* are typically described as eye flukes in the fish host, forming metacercariae (Figure 7B) in the lens, vitreous humour, and/or retina; specific lineages may also be present in brain tissue (see Blasco-Costa et al., 2014; Faltýnková et al., 2014). Although not covered here, Rieger et al. (2013) provide details for maintaining the parasite through its complete life cycle including the intermediate and definitive hosts *Lymnaea stagnalis* and the herring gulls (*Larus argentatus*) respectively.

[Insert figures 7 A&B here]

4.3.2 Source, culture and infection

If an infection of *Diplostomum* has been identified in a stickleback population, it is highly likely that *Lymnaea* or *Radix* snails from the same habitat will be infected. The prevalence of *Diplostomum,* however, varies considerably between seasons, localities and snail species (e.g. Karvonen et al., 2006b, c; Rieger et al., 2013; Faltýnková et al., 2014). To optimise *Diplostomum* collection, individual snails of larger size classes (e.g. *Lymnaea stagnalis* shell length > 40 mm) should be selected during late summer/early autumn to coincide with high prevalence and fully developed cercarial infections (Karvonen et al., 2006b). Infected snail populations can be maintained in laboratory aquaria containing continuously aerated water (dechlorinated tap or filtered from source locality), fed *ad libitum* on washed lettuce in controlled climate facilities (reflecting source environment or 18 h light: 6 h dark cycle, ca. 15°C). Light stress is commonly used to stimulate cercarial release, by placing snails individually into beakers of water (ca. 100 ml) at 10-20°C under a light source (e.g. Scharsack and Kalbe, 2014). Cercariae will be shed within 2-4 h, provided that fully developed *Diplostomum* cercarial infections are present, at a rate of 400-2400 cercariae/ h depending on temperature (Lyholt and Buchmann, 1996).

Identification of cercariae released from snails is necessary since aquatic snails may harbour single or multiple infections of other trematode species. Whilst *Diplostomum* cercariae can be distinguished from other cercariae based on their morphology and resting posture (see Niewiadomska, 1986) at x100 under a compound microscope, molecular techniques are essential to identify species and/or lineages of *Diplostomum*. Multiple lineages may be present in natural snail populations, which vary in their capacity to infect sticklebacks or other sympatric fish species (see Blasco-Costa et al., 2014; Faltýnková et al., 2014).

Sticklebacks can be infected individually in ~ 1 L water containing freshly emerged cercariae; typical exposure doses range from 20-220 cercariae per fish (Brassard et al., 1982; Lyholt and Buchmann, 1996; Kalbe and Kurtz, 2006; Scharsack and Kalbe, 2014; Haase et al., 2016) to 5,000-10,000 for other fish species (Sweeting, 1974; Rintamäki-Kinnunen et al., 2004). Whilst the parasite rapidly reaches the ocular tissues (within 24 h post-infection; Chappell et al. 1994), *D. pseudospathaceum* metacercariae establishment is best assessed after 1 week, since low numbers of early infections may be overlooked (Rauch et al., 2006). Kalbe and Kurtz (2006) have, however, demonstrated that 2 day and 8 week old metacercariae may be identified when sticklebacks are exposed to repeated cercarial infections. *Diplostomum* spp. infections are determined by counting the number of metacercariae in the eye tissues but this necessarily involves destructive sampling (e.g. Bortz et al., 1984; Lyholt and Buchmann, 1996; Kalbe and Kurtz, 2006; Locke et al., 2010; Scharsack and Kalbe, 2014).

4.3.3 Immunology

The eyes of teleosts are assumed to have the same immune privileged status of mammals (i.e. no localised immune response; Niederkorn, 2006; Sitjà-Bobadilla, 2008), thus for parasites invading the eye such as *Diplostomum*, we assume the immune response is limited to the migratory period between epidermal penetration of the cercariae and their arrival in the eye. Given this short window of vulnerability, it is generally acknowledged that the classical adaptive response plays no role in resistance against a primary parasite infection (Rauch *et al.,* 2006). Instead, oxidative burst and reactive oxygen species are thought to be the key components of the innate immune response against these pathogens. Head kidney lymphocyte respiratory burst activity is upregulated in fish 1.5 days post-infection but not from 5 days post-infection (Kalbe & Kurtz, 2006; Scharsack & Kalbe, 2014), while macrophages produce reactive oxygen species that are capable of killing larval *Diplostomum* (see Whyte et al., 1989). The phagocytic activity of granulocytes and monocytes has also been cited as inhibiting *Diplostomum* migration into the eye (Erasmus, 1959; Ratanarat-Brockelman, 1974). Despite this apparent bias towards the innate response against this parasite, a recent transcriptomic study identified antibody mediated responses and increased MHC and *il-4r* expression (a gene in mammals associated with adaptive helminth resistance) in response to infection (Haase et al., 2016). Such results support the notion that the innate and adaptive immune systems cannot be considered in isolation but must be viewed as a fluid and versatile network (Magnadóttir, 2006). There is also a level of concomitant immunity as sticklebacks that receive a primary infection of *D. pseudospathaceum* acquire lower levels of metacercariae in a secondary infection in contrast to the primary infection (Scharsack & Kalbe, 2014). In addition, sonicated metacercariae injected into sticklebacks induce antibody responses capable of providing immunity to subsequent infection (Bortz et al., 1984; Whyte et al., 1987); suggesting that the adaptive response may play a role in concomitant immunity if not the primary immune response.

While the host genotype, particularly that of the MHC, is cited as a major factor in resistance and susceptibility, the parasite’s genotype is also involved in determining infection outcome, with differential gene expression in different *Diplostomum* clones (Haase et al., 2014). As with MHC experiments that find homozygous individuals to be more susceptible to infection (see Wegner et al., 2003a, b), infections using a single clone of *Diplostomum* were less successful than mixed infections (Haase et al., 2014). Lake ecotype sticklebacks carry heavier and more diverse infections than their riverine ecotype counterparts (Kalbe et al., 2002; Scharsack et al., 2007a), with lake fish demonstrating a heightened level of resistance to *Diplostomum* infection (Scharsack et al., 2007a; Scharsack and Kalbe, 2014), in part due to selection within the MHC (Kalbe and Kurtz, 2006; Eizaguirre et al., 2011). In addition, lakes typically harbour a greater diversity of snails making the presence of the intermediate host more likely, but also making a greater range of parasite genotypes available, which may account for some of the ecotype variation (Karvonen et al., 2012).

4.4 *Glugea anomala*

4.4.1 Introduction

Glugea anomala (Moniez, 1887) is a microsporidian pathogen that causes white tumour-like growths, ca. 1-4 mm dia., known as the xenoparasitic complex (Chatton, 1920; Lom and Dyková, 2005). This complex is formed of many polypoid host cells (Figure 8), in which the microsporidian replicates and grows, by stimulation of hypertrophic growth of host tissue (Lom and Dyková, 2005). For *G. anomala* infecting sticklebacks, the xenoparasitic complex was re-named the 'xenoma' (Weissenberg, 1968). Nutrients are acquired by *G. anomala* through production of a hyposome with rhizoids that extend into the host cell cytoplasm (Lom and Dyková, 2005). Species can be positively identified via ribosomal DNA sequencing (see Cecile et al., 2000). Infection with *G. anomala* is linked to a reduction in feeding optimisation (Milinski, 1984, 1985) as well as exerting a metabolic cost and increasing the host's tendency to shoal (Ward et al., 2005).

[**Insert figure 8 here**]

4.4.2 Source, culture and infection

There are multiple published methods for infection of fish with *G. anomala* and other microsporidians (Olson, 1976; Shaw and Kent, 1999; Kurtz et al., 2004; Lom and Dyková, 2005), including *Tetramicra brevifilum* (see Figueras et al., 1992). It is assumed that *G. anomala* is transmitted orally during cohabitation of infected and uninfected fish (Lom and Dyková, 2005). In theory infection can be achieved experimentally by exposing fish to a spore suspension produced from infected fish (Kurtz et al., 2004), intraperitoneal, intramuscular or intravascular injection, and anal or oral gavage (Shaw and Kent, 1999). Crustaceans, including *Artemia salina* (brine shrimp) and *Corophium spinocorne* (amphipod), may also act as intermediate hosts for *G. stephani* (see Olson, 1976). However, preliminary testing of several infection methods in our Cardiff laboratory (oral transmission of extracted spores in the water column, oral gavage, intramuscular injection, co-habitation of infected and uninfected fish and exposure of putative intermediate hosts (*Artemia salina*, *Cyclops strenuous* and *Daphnia magna* to *Glugea* spores for 48 h) to date, has not resulted in parasite transmission 90 days post-treatment, despite xenomas reportedly developing 3-4 weeks post-infection (Lom and Dyková, 2005). The intensity of *G. anomala* can be measured by the number and size of xenoma visible externally (e.g. Schmahl et al., 1990; Lom et al., 1995; Dezfuli et al., 2004; Kurtz et al., 2004), internal zenomas may occur and these can be identified during dissection (e.g.Dezfuli et al., 2004).

4.4.3 Immunology

To date, there is only preliminary data on the immune response to *Glugea*. There is little or no detectable host response to the microsporidian until the xenoma is fully developed. Macrophage aggregates occur around the outside of the xenoma wall with eosinophils and neutrophils being recruited to reduce the mass of spores within the xenoma (Dezfuli et al., 2004; Lom and Dyková, 2005). Intermediate levels of individual allelic diversity in the MHC class *IIB* have been linked with increased *G. anomala* resistance (Kurtz et al., 2004).

4.5 *Gyrodactylus* spp.

4.5.1 Introduction

Gyrodactylus species are ubiquitous monogenean parasites of teleosts with over 400 described species (Harris et al., 2008). Identification of species is commonly conducted by rDNA internal transcribed spacer (ITS) region sequencing supplemented by the morphological characteristics of the marginal hooks and hamuli (Shinn et al., 2010), although mtDNA gene sequencing may also be necessary to reveal cryptic species (Xavier et al., 2015). The viviparous nature of their reproductive life cycle means that they are capable of uncontrolled infrapopulation growth that at high densities become pathogenic (e.g. Scott and Anderson, 1984; Bakke et al., 1990), although this is limited in most species by thermally-dependent host immune responses (e.g. Bakke et al., 1992; Harris et al., 1998; Lindenstrøm et al., 2004; Lindenstrøm et al., 2006; Kania et al., 2010) and hosts may seek elevated temperatures to ‘self-medicate’ (Mohammed et al. 2016).

Gyrodactylus salaris (Malmberg, 1957) is of particular economic importance as it infects salmonids and has been the focus of intensive eradication schemes particularly in Norway since the 1980s (Linaker et al., 2012). As such, *G. salaris* has a published genome (Hahn et al., 2014). Studies on salmon are often costly and their fry are particularly sensitive to stressors (Barton et al., 1986). Therefore, many studies have used model fish, including the guppy and stickleback (reviews by Cable, 2011; Barber, 2013, respectively) to assess potential ecological, pathological or immunological effects of these parasites on tropical and temperate fish species (Bakke et al., 2007). Because the parasites infect the gills, body and/or fins of the host, and most detached parasites have no swimming ability (a notable exception being *G. rysavji* Ergens, 1973 see El-Naggar et al., 2004), transmission typically occurs during host contact. Some parasite species, though, may drift or hang in the water column or attach to the substrate if detached from the host (Bakke et al., 1992; Soleng et al., 1999; Cable et al., 2002b), adopting a 'sit-and-wait' re-infection strategy. In high host density aquaculture systems, gyrodactylid infections can spread quickly with devastating consequences.

4.5.2 Source, culture and infection

Stickleback *Gyrodactylus* spp. may be obtained from research institutions or the wild. The two common species found infecting sticklebacks are: *G. gasterostei* (Glaser, 1974) and *G. arcuatus* (Bychowsky, 1933); *G. alexanderi* (Mizelle & Kritsky, 1967) and *G. branchicus* (Malmberg, 1964) are rare, whereas other species such as *G. salaris* or *G. pungitii* (Malmberg, 1964) may infect the three-spined stickleback but are not specialists; for a full list see Harris et al. (2008). Using a dissection microscope with fibre optic illumination, sticklebacks can be experimentally infected by anesthetizing a donor and recipient fish in 0.02% MS222 and allowing *Gyrodactylus* worms to cross from one fish to another by overlapping the stickleback caudal fins. Infections can also be performed by removing parasites on a fin clip or scale, or gently dislodging the worms from donors using an insect pin (Buchmann and Bresciani, 1997; Buchmann and Uldal, 1997), and then bringing a known number of parasites into close contact with a recipient fish. Alternatively, infections can be performed by co-habitation of recipient and donor fish (e.g. Lindenstrøm et al., 2006; Kania et al., 2010; Ramírez et al., 2015), but this results in inconsistent starting infection intensities. For controlled infections, typically one or two worms are added to the caudal fin to initiate an infection (e.g. Cable et al., 2000; van Oosterhout et al., 2003; Cable and van Oosterhout, 2007; de Roij et al., 2011; Konijnendijk et al., 2013; Smallbone et al., 2016a), but up to four have been used (Anaya-Rojas et al., 2016).

To produce an isogenic culture of any *Gyrodactylus* species, fish are infected with a single gyrodactylid worm. Several fish should be infected as the *Gyrodactylus* worms may be at the natural end of their short life-span. The infected fish are left for a week at 15-20°C to allow the parasite to reproduce *in situ*. One fish infected with an isogenic line should be transferred to a tank with at least three other fish to allow natural transmission and maintenance of the line. Fish should be kept at densities of one fish per litre for adults or one juvenile (<20 mm standard length) per 250 ml. To avoid parasite extinction, 2-3 tanks of the culture are often maintained with at least 4 fish in each, adding new naïve fish in the event of host mortality

(Schelkle et al., 2009). Additionally, in order that infections do not reach their pathogenic maximum, every 2 weeks the fish should be screened to count the parasites by anaesthetising each fish in 0.02% MS222 under a dissection microscope with fibre optic illumination. If additional tank replicates are needed, 1-2 fish with a total of 40 parasites can be removed from the screened tank and placed in a fresh tank with sufficient naïve fish to make the numbers up to four. If there are greater than 40 parasites per fish, the fish should be treated to prevent mortality (see Schelkle et al., 2009). Water should be changed regularly, every 48 h if unfiltered, as nitrates and nitrites can have a detrimental effect on *Gyrodactylus* survival (Smallbone et al., 2016b).

Measuring the infection intensity of some gyrodactylid species is remarkably simple given its ectoparasitic nature. It is, however, important to note that some gyrodactylid species of the three-spined stickleback, e.g. *G. arcuatus*, infect the gills and therefore cannot be counted without autopsy (Harris, 1982; Raeymaekers et al., 2008). When using a species such as *G. gasterostei*, which is predominantly found on the skin and fins (Harris, 1982), the infection trajectory can be monitored non-invasively (e.g. Buchmann and Uldal, 1997; Cable et al., 2000; Kania et al., 2010; Raeymaekers et al., 2011; Ramírez et al., 2015).

4.5.3 Immunology

Much of the immunological work conducted on gyrodactylids has been performed on *Gyrodactylus salaris* infected salmon, particularly the susceptible Norwegian salmon and resistant Baltic salmon (Bakke et al., 1990; Dalgaard et al., 2003; Lindenstrøm et al., 2006; Kania et al., 2010). There are some intermediate populations (see Bakke et al., 2004) but these have not yet been studied immunologically. Like other gyrodactylids there is also considerable variation among strains (Hansen et al., 2003; van Oosterhout et al., 2006). As with other parasite systems the MHC plays an important role in *Gyrodactylus* spp. resistance (e.g. Eizaguirre et al., 2009). Specific alleles of MHC class *IIB* genes in guppies, when present in high copy numbers, afford the host a measure of protection by reducing infection intensity (Fraser and Neff, 2009; Fraser et al., 2009, 2010). Furthermore, this protection is ecotype specific: river fish tend to be more resistant to infection than lake fish, probably because they are exposed to a narrower range of parasites and therefore are able to target specific parasites (Eizaguirre et al., 2011).

Immunity to *Gyrodactylus* spp. is primarily mediated by a ‘scorched earth strategy’, whereby parasites are starved of nutrients and exposed to increased expression of host complement (Buchmann, 1998; Harris et al., 1998; Kania et al., 2010). As such, resistant salmon show no increase in the mucus secretgogue *il-1β* while susceptible salmon show a marked increase in *il-1β* 24 h post-infection (Lindenstrøm et al., 2006; Kania et al., 2010). Likewise rainbow trout (*Oncorhynchus mykiss*), exposed to primary *G. derjavini* infections and then a secondary infection 35 days after parasite clearance, demonstrated susceptibility in the primary infections linked with increased *il-1β* transcript in the skin while resistant secondarily infected fish showed no increase in *il-1β* (Lindenstrøm et al., 2003). Gyrodactylids feeding on the mucus and epithelium will therefore be at a disadvantage on any host able to suppress the increase in *il-1β* production. Indeed, a reduction in the density of mucous cells is also associated with infection (Buchmann and Uldal, 1997; Dalgaard et al., 2003), however, this relationship may reverse later in infection as the mucous begins to contain higher concentrations of anthelminthic effectors (Buchmann and Bresciani, 1997). The major effector associated with resistance is alternatively activated complement present in both the serum and mucus (Buchmann, 1998; Harris et al., 1998). Immuno-cytochemical assays demonstrated binding of C3 to the cephalic gland opening, body and hamulus sheath of the parasite but found no immunoglobulin binding (Buchmann, 1998). Resistant salmon also have increased *il-10, mhc II* and *serum amyloid A* transcript 3-6 weeks post-infection in the epidermis of infected fins (Kania et al., 2010). The immune response to gyrodactylids can therefore be separated into two distinct stages: the passive stage where mucus production is inhibited to restrict parasite population growth and the immunologically active stage where complement and other effectors reduce the intensity of infection allowing host recovery. In infections with *Gyrodactylus* spp. it is therefore possible to infer the point at which the immune system is most active by virtue of the declining parasite population. For example, on *G. salaris* infected Baltic salmon and *G. gasterostei* infected sticklebacks, population reduction occurs at 2-3 weeks post infection at 12°C (see Bakke et al., 2002; de Roij et al., 2011; Raeymaekers et al., 2011), although such data may be confounded by the death of heavily infected fish during this time period.

4.6 *Saprolegnia parasitica*

4.6.1 Introduction

Oomycetes present a major threat to food security in aquaculture, but also terrestrial food sources, the most prominent being *Phytophthora infestans*, which caused the 19th Century

Irish potato famine (Haverkort *et al.*, 2008). In freshwaters, oomycetes from the genera *Saprolegnia*, *Achlya* and *Aphanomyces* (Order Saprolegniales, Sub-class Saprolegniomycetidae) are responsible for significant losses of fish (Jeney and Jeney, 1995; van West, 2006). As fungal-like heterotrophs they have branching tip-growing mycelia, typically thicker than fungi at 10 μm diameter, and unlike fungi they have cellulose and only a little chitin in their cell wall. Chitin synthases are present in the genome but are thought only to have a role in hyphal tip growth (Baldauf et al., 2000; Guerriero et al., 2010; Beakes et al., 2012; Jiang et al., 2013). Species identification typically depends on sequencing of the rDNA Internal Transcribed Spacer (ITS) region (Sandoval-Sierra et al., 2014). A full genome sequence is available for *S. parasitica* isolate CBS223.65 (Jiang et al., 2013).

The *Saprolegnia* lifecycle, as with other oomycetes, has an asexual stage including the development of sporangia and zoospores, and a sexual stage resulting in the production of oospores (see van West, 2006). The asexual stage is the primary method of infecting new hosts as free-swimming zoospores are released into the environment (Hatai and Hoshiai, 1994; Willoughby, 1994; Bruno and Wood, 1999). The sexual production of oospores is thought to enhance survival under acute stress conditions, such as temperature extremes or desiccation, until conditions become more favourable. Some *Saprolegnia* species (including most strains of *S. parasitica* Coker 1923), however, seem to lack a sexual cycle and do not produce oospores, at least under laboratory conditions.

Two of the major oomycetes of fish *S. parasitica* and *S. diclina* infect adults and eggs respectively (van den Berg et al., 2013). *Saprolegnia* species were controlled using the organic dye malachite green until 2002 when it was banned in aquaculture because of its carcinogenic properties. Formalin, although also notionally carcinogenic, is still currently permitted as a treatment (Srivastava et al., 2004; van West, 2006; Sudova et al., 2007). Current control methods for salmonid eggs include formalin, salt and ozone water treatment (Fornerisa et al., 2003; Khodabandeh and Abtahi, 2006; van West, 2006) of which formalin can also be used to treat or reduce mortality in fry, parr, smolts and adult fish (Ali, 2005; Gieseker et al., 2006).

During infection, *S. parasitica* secretes a SpHtp1 protein, which is able to translocate independently into fish cells via an interaction with a host cell surface tyrosine-O-sulphated molecule (van West et al., 2010; Wawra et al., 2012). The precise function of SpHtp1 is unknown, but it likely plays a role in the infection process. This finding and the immunomodulation capabilities of *S. parasitica* (see Belmonte et al., 2014) suggest that the interaction is more complex than previously considered. It is now becoming clear that *S. parasitica* is a primary pathogen rather than a secondary opportunistic pathogen as has often been assumed (e.g. Hoole et al., 2001).

4.6.2 Source, culture and infection

Cultivated strains of *S. parasitica* are held at various institutions but the parasite can also be isolated from wild fish. The mycelia can be maintained on potato dextrose agar (PDA) (e.g. van West et al., 2010; Belmonte et al., 2014; Sun et al., 2014; Parra-Laca et al., 2015) (Table 2) in 140 mm Petri dishes indefinitely at 15-25°C (light cycle and humidity unimportant). Cultures should be re-plated every month, to protect against bacterial and fungal contamination, by transferring a 5 mm dia. plug of healthy (white/grey in colour with no yellowing or other fungal growth) mycelium from one Petri dish to another. Cultures held on PDA should also be passaged though fish or cell lines every few generations in order to maintain virulence (Songe et al., 2014). To isolate a wild strain, mycelia are scrapped off an infected fish and inoculated onto a potato dextrose agar plate containing chloramphenicol at 50mg/ml to inhibit contamination (e.g. Songe et al., 2014; Kalatehjari et al., 2015; Thoen et al., 2015); chloramphenicol should not be used to maintain the culture as it is fungistatic (Rooke and Shattock, 1983). The *Saprolegnia* mycelium should then be re-plated (typically 2-5 times), taking 5 mm dia. plugs from the leading edge until a pure culture is obtained devoid of bacteria and fungi. The *Saprolegnia* mycelium is cotton-like and white/grey in colour, all other growth should be avoided when taking the plug for culture.

To infect sticklebacks from a stock PDA culture, three mycelium plugs (5 mm dia.) should be taken from the PDA stock and placed on a 140 mm Petri dish with 70 ml of pea broth (Table 2) for 72 h at 25°C. Following incubation, agar plugs are removed using sterile forceps and the pea broth withdrawn using a sterile syringe or pipette. The mycelium is then washed three times with 70 ml of a 50/50 mixture of distilled and tank water in the Petri dish. During each wash, after the addition of the water mix, the mycelium should be agitated before the water mix is removed. Finally, 30 ml of the 50/50 distilled and tank water mixture is added to the Petri dish and before it is incubated for a further 24-48 h at 15°C (Powell et al., 1972; Riberio, 1983). Alternatively, cleaned mycelium can be dispensed from one Petri dish into 500 ml of 50/50 distilled and tank water, incubating for 24-48 h at 15°C. The cultures should be checked for spore production under a microscope (x100), and the spores isolated by straining the *Saprolegnia* though a 40 μm cell strainer using a cell scraper to remove encysted spores from the Petri dish. Spore density is calculated using a haemocytometer, if necessary concentrating the sample by centrifuging at 3000 g for 5 min at room temperature, removing the excess supernatant and re-suspending the spores in distilled water. Fish are infected using the ami-momi technique, in which salmonids are typically shaken in a net for 2 min (Hatai and Hoshiai, 1994), this duration of shaking is excessive for sticklebacks instead we recommend 30 sec. Shaken fish are then exposed, ideally individually, to $3x10^5$ spores per litre (e.g. Belmonte et al., 2014), consistent with spore concentrations found in fish farms (Thoen et al., 2010).

The infection intensity of *S. parasitica* can be crudely analysed by photographing an infected fish and calculating the total body coverage of erupted hyphae (e.g. Fregeneda Grandes et al., 2001), but qPCR methods are being developed (van West et al. unpublished). Given the rapid time to mortality for infected fish, morbidity and prevalence of infection can also be used as a measure of *S. parasitica* virulence (e.g. Pickering and Duston, 1983; Hussein and Hatai, 2002; Gieseker et al., 2006).

4.6.3 Immunology

With true fungal infections it is generally accepted that cellular mediated immunity, particularly T-helper cell type 1 (T_H1) responses, are required for clearance of an infection (Blanco and Garcia, 2008). In general, hosts infected with oomycetes induce innate immune responses to infection, but some aspects of humoral immunity have also been found (see Roberge et al., 2007; Blanco and Garcia, 2008; Belmonte et al., 2014; Minor et al., 2014). Of particular interest is the humoral response towards the protein SpSsp1, which may provide a novel target for vaccine development (Minor et al., 2014). Given the rapid and destructive progression of *S. parasitica* infections, immune responses must likewise be fast acting and avid. Upon infection with *S. parasitica*, fish undergo a rapid acute response including upregulation of genes transcripts involved in all three complement pathways (classical, alternative and lectin) (Roberge et al., 2007). Upregulation of C1r, C2, mannose-binding lectin (MBL) indicate involvement of the alternative and lectin pathways, while substantial up regulation of C3 and C6, beyond what might be expected from just classical and MBL pathway activation, is postulated as the main reason for involvement of the alternative pathway (Roberge et al., 2007). Other immune related genes including *ATP-binding cassette* *transporter* (required for MHC class I antigen presentation), and the cytokine receptors *CXCR4* (chemokine of importance in humoral immunity) and *cd63* (cell development and growth of multiple immune cells) are upregulated (Roberge et al., 2007). Fish also produce a response to tissue damage caused by *S. parasitica*, including induction of proinflammatory genes such as *il-1β, il-6, tnf-α* and *cox2* (Kales et al., 2007; de Bruijn et al., 2012; Belmonte et al., 2014). In addition to upregulation of inflammatory genes, the parasite is capable of immunomodulation by means of prostaglandin E_2 causing suppression of cellular immunity, including a reduction in *cd8a* and *ifn-γ* transcripts (Belmonte et al., 2014). Proinflammatory genes are also upregulated by prostaglandin E_2 (IL-6, IL-8, IL-17) (Belmonte et al., 2014); an expression profile that in fungal infections is permissive to infection (Traynor and Huffnagle, 2001). Similar immune evasion strategies are employed by true fungi, which are capable of driving anti-inflammatory response and a shift towards a T_H2 profile, through TLR2 (Netea et al., 2003; Netea et al., 2004).

4.7 *Schistocephalus solidus*

4.7.1 Introduction

Plerocercoid larvae of the diphyllobothriidean cestode *Schistocephalus solidus* (Müller, 1776) (Figure 9) commonly infect sticklebacks in ponds, lakes and slow flowing rivers (Wootton, 1976; Barber, 2007). *S. solidus* is one of the most studied stickleback parasites, and was the first parasite for which a complex, multi-host life cycle was demonstrated experimentally (Abildgaard, 1790) (Figure 10). Experimental culture techniques, which permit physiological and developmental studies of the maturing plerocercoid, have been in existence for decades (Hopkins and Smyth, 1951; Clarke, 1954; Smyth, 1954, 1959, 1962; Arme and Owen, 1967) and are well-established (Jakobsen et al., 2012). The stickleback-*Schistocephalus* host-parasite model has been widely used for studying the impacts of infection on host energetics (Barber et al., 2008), growth and reproductive development (Heins and Baker, 2008) as well as on host behaviour (Milinski, 1985, 1990; Barber and Scharsack, 2010; Hafer and Milinski, 2016). Recently, experimental infection studies have been used to investigate evolutionary aspects of host-parasite interactions (MacColl, 2009; Barber, 2013) and host immune responses (Scharsack et al., 2004, 2007b; Barber and Scharsack, 2010), as well as the impacts of changing environments on patterns of infection (MacNab and Barber, 2012; Dittmar et al., 2014; MacNab et al., 2016).

[**Insert figures 9 and 10 here**]

4.7.2 Source, culture and infection

Naturally infected sticklebacks, which are readily identifiable by their swollen profile (Barber, 1997) can be collected from the wild and used as a source of infective parasites for experimental culture (e.g. Arnott et al., 2000; Barber and Svensson, 2003; Scharsack et al., 2007b). Whilst sticklebacks can harbour multiple *S. solidus* plerocercoids, infected fish often support a low number of large plerocercoids (Arme and Owen, 1967; Heins et al., 2002). The total mass of plerocercoids can approach that of the host fish (Arme and Owen, 1967). Plerocercoids can be successfully cultured *in vitro* from sizes of 20 mg (Tierney and Crompton, 1992; Dörücü et al., 2007) but they are only reliably infective to avian hosts at a body size of ≥ 50 mg (Tierney and Crompton, 1992).

Infective *S. solidus* plerocercoids are readily recovered from the body cavity of euthanised, naturally-infected sticklebacks following ventral incision. Complete, whole plerocercoids should be transferred using sterilised laboratory forceps to a pre-autoclaved culture vessel containing a loop of narrow-diameter semi-permeable membrane suspended in *S. solidus* culture media (see Table 2). As they are hermaphroditic, worms can be cultured individually (i.e. 'selfed') or in pairs (i.e. outcrossed) (Milinski, 2006). Compression of the worms by the cellulose tubing simulates conditions in the intestine of the bird definitive host and encourages fertilisation (Smyth, 1990). The worms, suspended in this 'model gut' inside the culture vessel, are incubated at 40°C in darkness, ideally in a water bath with lateral shaking at a frequency of 80 cycles per minute, which dissipates metabolic products. To reduce bacterial and fungal infections, antibiotics and anti-fungal chemicals can be added to the culture medium (Jakobsen et al., 2012). Plerocercoids are progenetic (i.e. exhibit advanced sexual development in the larval stage) and the morphological transition to the adult worm is rapid, with fertilised eggs being produced from day two onwards *in vitro*. Egg production continues for several days, after which the adult worm dies (Dörücü et al., 2007).

The eggs, along with the senescent or dead adult worm(s), should be flushed with dH_2O from the cellulose tubing into a Petri dish (12 cm dia.). To clean the egg solution, excess dH_2O is added to the dish and a gentle swirling movement used to concentrate the eggs; this is best achieved whilst viewing under low power using a dissecting microscope with cold light illumination. Because the eggs are negatively buoyant, they readily aggregate in the centre of the Petri dish. A pipette can then be used to remove detritus, including tegument of the adult worm, from the egg solution. Repeated iterations of this process, interspersed with dispersing the egg mass, generate a sufficiently clean egg solution for subsequent incubation. Eggs can then be split between multiple sterile Petri dishes, filled to a depth of 5 mm with dH_2O, sealed with Parafilm and wrapped in aluminium foil to restrict premature exposure to light.

Eggs are incubated for 21 d at 20°C in the dark before being exposed to natural daylight to induce hatching (Scharsack et al., 2007b). Pre-exposure to a short (ca. 2 h) period of light, the evening before desired hatching, may improve subsequent hatch rates (Dubinina, 1966). Hatched eggs release coracidia, which are spherical, ciliated, free-swimming first stage larvae. Coracidia move actively for ca. 12-24 h after hatching at normal laboratory temperatures, but apparently senescent (i.e. motionless) coracidia can establish infections in copepod hosts (unpublished data). Coracidia are collected using a Pasteur pipette and transferred to a drop of dH_2O on a watch glass, Petri dish, microscope slide, or in a well of a 96-well microtitre plate. An individual cyclopoid copepod (typically *Cyclops strenuus abyssorum* or *Macrocyclops albinus*) is then added to the water drop containing the hatched coracidium (coracidia) to allow trophic transmission. It is important to cover the water droplet to prevent evaporation. The water droplet is visually inspected under a dissection microscope to check that the coracidium has been ingested, after which the exposed copepod can be transferred to a larger volume of water and fed under normal culture conditions for 7 d, fed either newly-hatched *Artemia* spp. nauplii or a few drops of *Spirulina* feed (Table 2). Copepods are then screened at 7 d post-exposure for infection status. The procercoid stage that develops within the copepod is infective to sticklebacks (Dubinina, 1966) when it develops a hooked cercomer - a caudal appendage used by the parasite during invasion of the fish host (Barber and Scharsack, 2010; Benesh and Hafer, 2012; Benesh, 2013).

Infection of sticklebacks in the laboratory can be achieved by gavage feeding or allowing free feeding by isolated sticklebacks (e.g. Barber and Svensson, 2003; Hammerschmidt and Kurtz, 2005; Scharsack et al., 2007b; MacNab and Barber, 2012). Individual sticklebacks can be held in a crystallising dish (15 cm dia.) filled to 3 cm with aquarium water, illuminated from above using a cold light source and surrounded by black paper to improve contrast. Feeding can be encouraged by moving an infected (i.e. cercomer-bearing procercoid) copepod up and down within the neck of a long-form Pasteur pipette immediately in front of a stickleback that has been starved for 24 h, before releasing it into the water. Alternatively, fish can be left to forage for 6 h in a small (1 L) plastic aquarium containing a few newly-hatched *Artemia* spp. nauplii and an infected copepod. Exposure can be confirmed by direct observation of the ingestion event or by sieving the water to confirm ingestion of the copepod.

Infections of sticklebacks with *S. solidus* most commonly use the parasite mass as an endpoint measurement to determine the intensity of infection. The mass of both the stickleback and parasites in this infection system can vary dramatically and, as such, the $parasite\ index = \frac{Total\ parasite\ mass}{Total\ fish\ \&\ parasite\ mass}\ x100$ (Arme and Owen, 1967) is often used as a measure of intensity (e.g. Giles, 1983; Tierney et al., 1996; Kurtz et al., 2004; Barber, 2005). Alternatively, a measure of volume can be produced for plerocercoids whose mass is too small to be measured directly (e.g. Wedekind et al., 2000; Scharsack et al., 2007b): the plerocercoid is photographed under a microscope and taking the maximal area of the longitudinal section of its body and applying the following formula $volume\ (mm^3) = e^{0.279}\ \mathrm{X}\ area\ (\mu m^2)\ \mathrm{X}\ 10^{-9}$ (see Wedekind et al., 2000).

The growth of the plerocercoid stage *in vivo* can be estimated non-invasively using image analysis based on the infection-induced swelling (Barber, 2007), facilitating longitudinal studies of infection and parasite growth. Individual coracidia can be stained using persistent fluorescent dyes (Kurtz et al., 2002), allowing differentiation of individual parasites in mixed infections. Finally, there are now microsatellite markers and other ecological, genomic and transcriptomic resources that facilitate taxonomic studies (Binz et al., 2000; Nishimura et al., 2011; Sprehn et al., 2015; Hébert et al., 2016).

4.7.3 Immunology

A rapid host immune response is thought to be crucial for host resistance against *S. solidus*, preventing establishment within the body cavity. Infection prevalence drops from 60% in the first week to 54-52% one month post-infection, but with no further decline thereafter (Scharsack et al., 2007b; Benesh, 2013). In addition, no dead *S. solidus* are detected in the body cavity after 17 days post-infection, suggesting that this is the effective limit of the immune response against the parasite (Scharsack et al., 2007b). Resistance to *S. solidus* is associated with early proliferation of head kidney monocytes and lymphocyte proliferation 7 days post-infection (Barber and Scharsack, 2010), the rate of lymphocyte production then drops drastically in both resistant and susceptible fish 17 days post-infection (Scharsack et al., 2007b). Monocyte production also undergoes changes during infection, being elevated in susceptible fish at 7 and 27 days post-infection but reduced at 17 days post-infection compared to controls (Scharsack et al., 2007b). There is no obvious involvement of the adaptive response in resistance to a primary *S. solidus* infection, as this would take 2-3 weeks to be active in fish at 18°C, by which time plerocercoids are already established (Barber and Scharsack, 2010). There is, however, evidence that at some levels the adaptive response is involved at least in tolerating an infection. Intermediate MHC class *IIB* diversity has been linked to a reduction in the parasite index and an increase in the respiratory burst response; the prevalence of infection was unaffected by this diversity (Kurtz et al., 2004).

The stickleback immune response to *S. solidus* also involves upregulation of responses, including adaptive immunity, from 47 days post-infection that are not linked to resistance in a primary infection as the pleroceroid is already well established. Head kidney lymphocyte respiratory burst is upregulated 47-67 days post-infection (Barber and Scharsack, 2010) and granulocytes increase in proportion until 63 days post-infection (Scharsack et al., 2004). Further transcriptomic analysis found upregulation of innate toll-like receptor, complement and macrophage genes as well as upregulation of adaptive MHC genes 50 days post-infection (Haase et al., 2016).

An active adaptive response late in infection may support a role for immunological tolerance of *S. solidus* infections (Jackson et al., 2014), or concomitant immunity, though we are unaware of any direct tests of this hypothesis. In addition, sticklebacks with high or low diversity in the MHC class *IIB*, which is correlated with MHC expression (Wegner et al., 2006), harboured larger parasites while those with intermediate diversity had smaller worms (Kurtz et al., 2004). This supports the notion of hosts with intermediate (optimal) MHC diversity suffering less from infection (Wegner et al., 2003a, b). Such a result may also support a role for tolerance, as the immune system shifts (~47 days post-infection) to focus less on resistance and more on restricting plerocercoid growth rate and perhaps improving fish condition. This late immune response, which is known to last from 45-67 days post-infection, correlates with plerocercoids reaching infective weight for the definitive host at approximately 47 days post-infection (Scharsack et al., 2007b). Concomitant immunity may therefore also be a viable hypothesis as this would inhibit secondary infections from acquiring vital nutrients at this crucial life history stage (and *S. solidus* is known to alter the susceptibility of the host to infection by other species; Benesh and Kalbe, 2016). In addition, head kidney lymphocytes exposed to the excretory products of mature *S. solidus* (>50 mg) in conditioned culture media expressed higher respiratory burst activity, associated with granulocyte viability, which may also manipulate host behaviour via the immune-neuroendocrine axis and aid transmission to the definitive host (Scharsack et al., 2013).

5.0 Treating common infections

Not all parasitic infections of sticklebacks can be eliminated, and the decision to treat fish, and the nature of treatment chosen, will be dependent both on infection history and the nature of the experiment as well as a cost benefit trade-off. A list of common treatments for common parasite infections of fish is provided in Table 4.

[**Insert table 4 here**]

The most common endemic infections to occur in laboratory studies of sticklebacks are microparasites, commonly *Aeromonas* spp., *Flavobacterium* spp., *Pseudomonas* spp., *Ichthyophthirius multifiliis* and *Saprolegnia parasitica*. These infections often establish when fish are physiologically stressed, for example by experimental procedures, altered environmental conditions or following capture and/or transportation. These pathogens are ubiquitous, present in most water bodies and therefore are difficult to eliminate from aquatic systems. Additionally, *Gyrodactylus* spp. and *Trichodina* spp. (Figures 11A & B) are easily introduced into tanks with other fish or as a result of imperfect net hygiene. Most *Trichodina* spp. and other ecto-commensals including *Epistylis* spp. and *Apiosoma* spp. are asymptomatic at low numbers but may become pathogenic at high intensities (Collymore et al., 2013). Even low level endemic *Gyrodactylus* infections can result in epidemics after several weeks in captivity if not treated immediately, and even mild infections probably affect host behaviour and physiology. Wild sticklebacks may be infected with heteroxenous parasites such as *Schistocephalus solidus*, *Diplostomum* spp. and *Camallanus lacustris,* but these parasites cannot be transmitted without the presence of their intermediate hosts. Although *Glugea anomala* may be transmitted directly, the details of transmission are unclear. Transfer of water between tanks should be avoided in all cases. Nets are a common source of water transfer and should be sterilised in Virkon or sodium metabisulfite (in accordance with manufacturer's instructions), rinsed and fully dried before reuse. Infected fish should be isolated and treated as indicated in Table 4; early detection and rapid treatment is key for the majority of infections.

[**Insert Figure 11**]

Aeromonas spp. and *P. fluorescens* cause red ulcers, small white/grey marks on the fins and head, fin rot and ultimately death. Because it is often difficult to distinguish these two infections without biochemical or molecular techniques, a broad-spectrum antibiotic should be used following consultation with a veterinarian; if severe damage occurs the fish should be euthanized using a procedure approved by the relevant regulatory authority.

The highly contagious protozoan parasite *I. multifiliis* causes small white spots on the fins and skin of the fish. The simplest method of treatment is increasing water salinity (Selosse and Rowland, 1990; Miron et al., 2003; Garcia et al., 2007) and adding methylene blue (Tieman and Goodwin, 2001) (see Table 4). A low concentration formalin or malachite green treatment may also be used (e.g. Leteux and Meyer, 1972; Tieman and Goodwin, 2001) following the low and prolonged immersion dose (Table 4) or an off-the-shelf formulation used following manufacturer's instructions. Given the complexity of the life cycle, and the fact that resistance is common, multiple treatment doses are likely to be required.

For *Saprolegnia* infections, prevention (0.5% saline water) is definitely better than cure (Ali, 2005; van West, 2006); once a fish is symptomatic it may survive no more than a few days, occasionally even hours, or be irreparably damaged and must be euthanized using an approved procedure. If *Saprolegnia* infection does occur the most effective treatment is a high dose malachite green in formalin treatment (Table 4), or a low concentration formalin treatment (see van West, 2006). To aid recovery and prevent reinfection following formalin exposure, the fish should be transferred to 0.5-1% salt solution, with the possible addition of methylene blue (Table 4).

Gyrodactylid treatments are problematic because 100% efficacy is required and transmission can easily occur between adjacent tanks by water or net transfer. The only tested treatment that works consistently for stickleback gyrodactylids in our laboratory at Cardiff University is a high concentration formalin bath (Table 4) (Buchmann and Kristensson, 2003). Other less damaging pharmaceutical treatments for the fish, such as Praziquantil and Levamisole, are of variable efficacy that may depend on the exact conditions of exposure, at least for this fish species (Schelkle et al., 2009). After treatment, screening for the parasite should be performed three times, no more than once per day, to ensure the parasite has been removed effectively from the entire host population (see Schelkle et al., 2009).

Ciliated *Trichodina* spp. protists are only visible under a low powered (x10-60 mag.) microscope (Figure 11). They appear as ‘flying-saucer’ shaped disks gliding over the body, fins and gills of the fish. Changing tank water regularly to keep the water crystal clear effectively eliminates most *Trichodina* spp., which feed on bacteria (Lom, 1973). If the clean water treatment fails, which is rare, low dose malachite green treatment is usually successful after 2-3 doses (Table 4) (Leteux and Meyer, 1972). Other infections, *G. anomala*, *Diplostomum* spp. and the macroparasitic internal parasites are either difficult to treat, cannot be treated or may not need treatment. *Diplostomum* spp. found in the lens and vitreous humour may be treated with Praziquantel, although efficacy is variable and depends on undetermined factors. *S. solidus* worms that have migrated through the intestine and into the body cavity cannot be treated. *Glugea anomala* also cannot be cured, although some success has been achieved in reducing spore survival using benzimidazole treatments (Schmahl and Benini, 1998).

6.0 Co-infecting parasites

Despite the overwhelming tendency for wild and even commercially bred sticklebacks to be co-infected, there is relatively little knowledge about interspecific parasite competition in sticklebacks (Benesh and Kalbe, 2016). Parasites occupying similar niches are in direct physical and chemical competition for resources such as nutrients and habitat (Knowles et al., 2013). Such parasites are likely to be antagonistic and may alter their distribution on the host in order to avoid direct competition; as is the case with co-infecting gyrodactylid species (Harris, 1982) and co-infecting *Proteocephalus filicolis* and *Neoechinorhynchus rutili* (see Chappell, 1969). On the other hand, parasites separated by niche may interact indirectly via the immune system whilst simultaneously competing for host resources (Pedersen and Fenton, 2007). Suppression or enhancement of the immune response by a parasite will then alter the outcome of subsequent infections; changing host susceptibility and pathology, parasite virulence and infection duration (Correa-Oliveira et al., 2002; Lively, 2005; Fleming et al., 2006; Benesh and Kalbe, 2016). Such responses, particularly those mediated by the immune system, may even be synergistic as immunosuppression by one parasite increases prevalence or intensity of another (Su et al., 2005; Fleming et al., 2006; Benesh and Kalbe, 2016). There is a general lack of information on *Glugea anomala* infections and associated immune responses and so this will not be covered here; however, given the site of infection and the occasional severity of infection it is highly likely that this species does impact co-infecting parasites.

Some parasites may be used as a ‘marker of other infections’ (where a change in prevalence, intensity or distribution indicates an interaction between co-infecting parasites); such relationships may be synergistic or antagonistic. The ability to track viviparous gyrodactylid population trajectories over time, directly and non-invasively, makes them particularly useful as a marker for the consequences of co-infection. Modulation of the immune system (Section 4.5.3) and resource competition by co-infecting parasites will alter the population trajectory, allowing the effects of co-infection to be tracked over time. In addition, the migration of gyrodactylids across the exterior surfaces of hosts (Harris, 1982) allows population distribution patterns to be utilised as a method of assessing the outcome of competition among co-infecting parasites. Such spatial positioning assessments may also be made with other ectoparasites, such as argulids, and endoparasites, for example by considering position in the gut (e.g. Chappell, 1969). The terminal nature of this approach with endoparasites, however, means that such studies cannot produce the repeated measures that make gyrodactylids so useful. Changes in the prevalence of secondary infections will also be linked to high levels of stress or immune modulation associated with the primary infection (e.g. Shoemaker et al., 2008; Roon et al., 2015). As such, secondary *S. parasitica* infections as a ’marker’ might also prove possible in the absence of the ami-momi infection technique, particularly if the strain is virulent and the primary infection induces stress.

For co-infection studies where only a short period of immune regulation or infection is required, *Diplostomum* spp. and *Argulus* spp. provide ideal models. As *Diplostomum* migrates to the immune privileged eye it generates a short lived spike in the innate response between 1.5 and 5 days post-infection (Kalbe and Kurtz, 2006; Scharsack and Kalbe, 2014), after which it will no longer modulate the immune system and will not be in direct competition with other parasite genera. Short-medium term competition and innate immune responses can be induced by *Argulus* spp. with the period of co-infection dictated by removing the infected individuals from the fish (see Section 4.1). The immunomodulatory effects of *Argulus* also provide an opportunity to study the consequences of immune suppression (Saurabh et al., 2010; Kar et al., 2015). Short-term co-infections with *Saprolegnia parasitica* are also possible, but the usefulness of this pathogen is hindered by its virulence and infection method.

Long term infections can usually be achieved with endoparasites, which – because of their life cycles – will often provide long periods of competition with concurrently infecting parasites and the host's immune response. The major drawback with endoparasitic species is an inability to accurately determine prevalence, intensity and distribution without destructive sampling. Gastrointestinal parasites (e.g. *C. lacustris*) typically provide a sustained long-term infection that will be in direct competition with other gastrointestinal parasites. Such infections typically provide a long term immune response either as a result of host resistance, tolerance or parasite induced immunomodulation (e.g. *C. lacustris*; Section 4.2.3). Being the only species to inhabit the peritoneal cavity of the stickleback, the plerocercoid cestode *S. solidus* is unique, and likely subject only to direct intraspecific competition. Once established in the peritoneal cavity, at a mass of 50 mg, it is not possible for the fish to clear an infection. The timing of the immunological response is therefore quite specific (Section 4.7.3); giving a clear period of time in which the immune response could affect concurrent infections (Scharsack et al., 2007b; Barber and Scharsack, 2010). The utility *S. solidus* is therefore specific to its ability to induce long term competition for resources, a short term immune resistance phenotype and a delayed response; the purpose of the delayed response is not yet fully elucidated (Section 4.7.3).

7.0 Summary

With an increasing threat of disease in aquaculture and with climate change altering host-parasite interactions a reliable model for studying these impacts has been found in the stickleback. The stickleback provides a particularly useful model as it shares many characteristics with economically important fish species such as salmon and trout including its temperate habitat, omnivorous nature and evolutionary history. In depth knowledge of the stickleback's evolutionary history, ecology, parasitology and genetic architecture has put this species at the pinnacle of aquatic vertebrate research. Despite this, much of the knowledge of parasite culture techniques and treatments along with basic stickleback husbandry was confined to older and sometimes inaccessible literature, with methods that had been updated sporadically or that varied between different research groups. This article has brought together expertise in the culture of sticklebacks and parasites to generate a single text that lays out a framework of techniques for new or established laboratories that wish to begin investigating stickleback host-parasite interactions in the laboratory, or to expand their repertoire of available parasite models.

While the number of studies on the three-spined stickleback immune system is increasing, different laboratories have focussed on different aspects: direct measurements of *ex vivo* or *in vivo* phenotypic responses, MHC genetics, or gene expression measurements employing real time PCR or RNAseq, in response to different pathogens. As a result it can be difficult to reconcile the different approaches. For example, while we know that MHC constitution plays a part in parasite resistance, we know little about how that translates into the active immune phenotype that actually combats infection. Certain alleles may stimulate specific immune phenotypes or more simply allelic diversity may lead to an overall more active immune response. At a functional level, greater diversity of MHC alleles means different repertoires of peptides may be presented during an immune response, leading to expansion of T- and B-cell receptor specificities that affect the success of the adaptive response. When we begin to take a more holistic approach to such problems it is likely that we will lift the shroud on previously unknown aspects of the teleost immune system.

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