

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

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17 Immunology

## 18 **1.0 Abstract**

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

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

## 35 **2.0 Introduction**

36 Aquaculture is currently the fastest growing animal food-producing sector, increasing by 6%  
37 annually in the 2000s (The World Bank, 2013a). In 2014, 73.8 million tonnes of fish were  
38 farmed, rising from 55.7 million tonnes in 2009 (FAO, 2016). In order to maintain the current  
39 level of consumption, whilst compensating for shortfalls from fisheries that have reached  
40 their maximum potential output, global aquaculture production will have to reach 93 million  
41 tonnes by 2030 (The World Bank, 2013b). As with agriculture, fish production can be  
42 increased via two main approaches: increasing the area turned over to the industry or  
43 improving yields. With the use of terrestrial and aquatic environments reaching their  
44 sustainable maximum, the focus of aquaculture is now firmly set on yield improvement via  
45 selective breeding, genetic modification and feed conversion efficiency (Myhr and Dalmo,  
46 2005; FAO, 2016; Janssen et al., 2016). These goals, however, must be coupled with a better  
47 understanding of host-parasite interactions and improved disease prevention, since a major  
48 inhibitory factor to fisheries' yield improvement are losses to infectious diseases, many of  
49 which are caused by parasitic organisms (Meyer, 1991).

50

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

62

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

66 Gibson, 2005; Barber and Nettleship, 2010; Jones et al., 2012; Barber, 2013). This ancestrally  
67 marine fish occurs in coastal marine, brackish and freshwater environments north of 30°N  
68 latitude. Sticklebacks have been utilised as a model of adaptive radiation due to their  
69 remarkable morphological diversity, including variation in size, shape and protective armour,  
70 which has arisen following the post-glacial colonisation of innumerable freshwaters from  
71 marine refugia (Schluter, 1993; Reimchen, 1994; Walker, 1997; Colosimo et al., 2005; Jones  
72 et al., 2012). The reproductive isolation of populations inhabiting a wide variety of habitat  
73 types and exploiting diverse resources are generally thought to be the primary causes of  
74 stickleback adaptive radiation (Schluter, 1993; Lackey and Boughman, 2016); with  
75 phenologic differences among morphotypes being linked to idiosyncratic genome variation  
76 (Jones et al., 2012; Feulner et al., 2015; Marques et al., 2016; reviewed in Lackey and  
77 Boughman, 2016) and at least partially controlled by the epigenome (Smith et al., 2015a). Of  
78 particular interest are the Canadian limnetic-benthic ‘species pairs’ (that inhabit the pelagic  
79 and littoral zones respectively) and the river-lake morphs of sticklebacks which, despite that  
80 fact that hybridization is possible both in nature and the laboratory, display high levels of  
81 reproductive isolation (McPhail, 1992; Gow et al., 2006; Berner et al., 2009; Eizaguirre et al.,  
82 2011). In the case of the limnetic-benthic pairs, both forms are thought to have evolved from  
83 independent marine ancestors (McPhail, 1992), while a mixed pattern of morphotypes is  
84 likely the cause of the river-lake differentiation (Reusch et al., 2001a; Berner et al., 2008).  
85 Supporting predictions of adaptive radiation, the limnetic and benthic stickleback forms each  
86 have growth advantages in their native habitats, which are lost in the alternative habitat, while  
87 hybrids are intermediate; the efficiency of this exploitation matches the observed  
88 morphological differences (Schluter, 1993, 1995). The same holds true for river-lake fish  
89 ecotypes, which are locally adapted and suffer from translocations in non-native habitats  
90 (Eizaguirre et al., 2012a; Räsänen and Hendry, 2014; Stutz et al., 2015).

91

92 In addition to their wide geographic range and diverse morphology, the stickleback has many  
93 amenable features that make it ideal for experimental studies of host-parasite interactions.  
94 First, sticklebacks are easily maintained and bred in the laboratory as a result of their general  
95 hardiness, small size and low maintenance cost. Second, within their habitat range,  
96 sticklebacks can be collected easily from the wild. Third, unlike many vertebrates, there is  
97 comprehensive knowledge of stickleback parasitology (Arme and Owen, 1967; Kalbe et al.,  
98 2002; Barber and Scharsack, 2010; MacNab and Barber, 2012), natural history and ecology  
99 (Wootton, 1976, 1984a; Östlund-Nilsson et al., 2006), evolutionary history (Schluter, 1996;

100 Taylor and McPhail, 1999; Mckinnon and Rundle, 2002; MacColl, 2009), physiology (Taylor  
101 and McPhail, 1986; Pottinger et al., 2002) and behaviour (Tinbergen and van Iersel, 1947;  
102 Giles, 1983; Milinski, 1985, 1987; Milinski and Bakker, 1990; Reusch et al., 2001b; Barber  
103 et al., 2004). Fourth, publication of the stickleback genome (Kingsley, 2003; Hubbard et al.,  
104 2007; Jones et al., 2012) coupled with advanced post-genomic techniques makes this fish an  
105 ideal model for molecular study, including host immunology (Kurtz et al., 2004; Hibbeler et  
106 al., 2008; Brown et al., 2016; Hablützel et al., 2016). All of this allows one to focus, not on a  
107 single aspect of the system, but to take a holistic systems approach to studying host-parasite  
108 interactions.

109

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

126

127 The impact of infection on host behaviour is well documented (Giles, 1983; Milinski, 1985,  
128 1990; Milinski and Bakker, 1990; Poulin, 1995; Urdal et al., 1995; Barber et al., 2004;  
129 Spagnoli et al., 2016) but uncontrolled parasitic infections may confound results (as recently  
130 demonstrated in zebrafish; Spagnoli et al., 2016). Parasitic contamination applies not only to  
131 behavioural studies but to all research (immunological, parasitological, molecular etc.) where  
132 uncontrolled parasite infections other than those under investigation may have confounding  
133 effects, via stimulation of the immune system or interactions with co-infecting parasites.

134 While pharmaceutical treatments may be useful to control or limit confounding parasitic  
135 factors, their use is a double-edged sword bringing other problems linked to the severity of  
136 the treatment (Buchmann et al., 2004; Srivastava et al., 2004) and it can never be assumed  
137 that such treatments have 100% efficacy (Schelkle et al., 2009). It is also increasingly  
138 important that infection models can conform to a ‘wild’ or ‘uncaged’ state (Leslie, 2010) in  
139 order to understand the complex interaction of parasites, host immunological responses and  
140 ecological variation that are the prevailing state. The immune systems of wild animals and  
141 humans are rarely naïve and co-infection is the norm (e.g. Lello et al., 2004; Behnke et al.,  
142 2005, 2009; Benesh and Kalbe, 2016), partly explaining the many inconsistencies between  
143 laboratory models and wild animals.

144

145 The difficulty and importance of maintaining parasite populations in the laboratory is often  
146 underestimated and partly hampered by the lack of published practical information on  
147 establishing and maintaining host-parasite systems. In addition, molecular (drug) and  
148 immunological (vaccine) based approaches are increasingly needed for mitigating the impacts  
149 of disease. Effective models of aquaculture fish species are limited: the zebrafish, although  
150 ideal for molecular studies, is unrepresentative in terms of habitat, evolutionary history and  
151 parasitology. In this respect the stickleback provides a useful study species, being susceptible  
152 to a range of problematic aquaculture diseases, including those caused by the oomycete  
153 *Saprolegnia parasitica*, *Diplostomum* trematodes and *Gyrodactylus monogeneans*, as well  
154 other parasites closely related to aquaculture-relevant species. This review first covers the  
155 basic husbandry of the three-spined stickleback and then focuses on the parasites that are  
156 most frequently used in research projects: *Argulus* spp., *Camallanus lacustris*, *Diplostomum*  
157 spp., *Gyrodactylus* spp., *Saprolegnia parasitica* and *Schistocephalus solidus*. For each taxon,  
158 culture methods, experimental infection techniques and host immune responses are outlined.  
159 *Glugea anomala*, although not widely used experimentally, is a common infection of  
160 sticklebacks and is included in this review to stimulate future research. Whilst all of these  
161 parasites are common, until now there has been no single resource that summarizes all  
162 available culture methods. We also provide an overview of the host’s immunological  
163 responses to these parasites, and to put these studies in a wider context we recommend  
164 reviews of vertebrate (Murphy 2012; Owen et al., 2013) and teleost immunology (see Miller,  
165 1998; Morvan et al., 1998; Press and Evensen, 1999; Claire et al., 2002; Watts et al., 2008;  
166 Takano et al., 2011; Forn-Cuni et al., 2014). Overall, we aim to provide a comprehensive and  
167 standardised approach to support new research utilising the three-spined stickleback as a

168 model for experimental parasitology and immunology, while increasing awareness of the  
169 impact of any infections for non-parasitological studies.

### 170 **3.0 Stickleback husbandry**

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

#### 174 *3.1 Ethics*

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

#### 184 *3.2 Collection*

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

195

196 Sticklebacks may be acquired from other researchers actively breeding these fish, possibly  
197 holding multiple inbred and/or outbred lines (e.g. Mazzi et al., 2002; Aeschlimann et al.,  
198 2003; Frommen and Bakker, 2006). Alternatively, they may be purchased from a commercial  
199 fish supplier (e.g. Katsiadaki et al., 2002a). Given the diversity and abundance of stickleback  
200 parasites, the principal of ‘buyer beware’ must apply, as rarely can a supplier guarantee

201 ‘parasite-free’ fish and most fish will have been treated chemically (e.g. Giles, 1983; Poulin,  
202 1995; Urdal et al., 1995; Barber et al., 2004; Spagnoli et al., 2016). Fish suppliers or  
203 researchers may be willing to provide infected sticklebacks for research or teaching,  
204 particularly in the case of overt infections, such as *Glugea anomala* or *Schistocephalus*  
205 *solidus*. A third option is to collect wild fish and use them directly (e.g. Bakker, 1993; Cresko  
206 et al., 2004; Bernhardt et al., 2006) after treating for infections (e.g. Soleng and Bakke, 1998;  
207 Ernst and Whittington, 2001; Cable et al., 2002a; Morrell et al., 2012; Anaya-Rojas et al.,  
208 2016; Hablützel et al., 2016) or breeding from these wild fish (e.g. Mazzi et al., 2002;  
209 Aeschlimann et al., 2003; Wegner et al., 2003a; Frommen and Bakker, 2006; Eizaguirre et  
210 al., 2012b).

211

212 Most institutions in Europe and continental North America neighbour a water body  
213 containing sticklebacks, particularly around coastal regions. Sticklebacks can be captured in  
214 commercial (e.g. Hendry et al., 2002; Gow et al., 2007; MacColl et al., 2013) or hand-made  
215 minnow traps constructed from 2-3 L soft drinks bottles. Each bottle, with holes in the sides,  
216 is cut such that the spout may be inverted and reattached using cable ties to resemble a  
217 minnow trap and partially filled with pebbles so it remains immersed. Typically, the traps are  
218 placed into water with one end secured by string to a concealed marker. Bait is not normally  
219 required as sticklebacks are inquisitive and catching one fish entices others. The trap is left  
220 for a maximum of 24 h to prevent fish becoming overly stressed. Dip-netting, using a hand  
221 net, is also effective (e.g. Gow et al., 2007; Brown et al., 2016), especially targeting areas of  
222 vegetation along the bank or under bridges where sticklebacks shoal and hide (Wootton,  
223 1976). Permission should be sought from the landowner and appropriate regulatory authority  
224 before using traps or nets and these should be of a design so as not to endanger other aquatic  
225 organisms. Most wild sticklebacks will be infected with parasites (Barber, 2007) and  
226 appropriate measures must be taken to limit mortality (see Section 5). Importantly, ‘trapping’  
227 stresses fish and compromises the immune system, but ‘netting’ can be used to sample fish in  
228 their natural state if euthanized immediately (e.g. Brown et al., 2016).

### 229 3.3 Maintenance

230 Sticklebacks are normally kept at densities not exceeding 1 fish/L to reduce fish stress (e.g.  
231 Mazzi et al., 2002; Aeschlimann et al., 2003; Barber, 2005; de Roij et al., 2011).  
232 Dechlorinated water is always used: 0.1-0.3 parts per million (ppm) of chlorine is lethal to the  
233 majority of fish (Wedemeyer, 1996), although brief exposure to chlorinated water (1-2 h) can

234 be beneficial in removing some parasites (Johnson et al., 2003; Ferguson et al., 2007).  
235 Dechlorinated water is typically obtained either through an activated charcoal filter,  
236 commercially available dechlorinating and water conditioning solutions (follow  
237 manufacturer's instructions) or vigorous aeration of tap water for 24 h before use.  
238 Dechlorinated water should not be fed through copper pipes as high concentrations of copper  
239 ions can kill fish (Cardeilhac and Whitaker, 1988; Sellin et al., 2005; Grosell et al., 2007).  
240 Although sticklebacks are normally kept in fresh water, routine addition of 0.5-1 % salt water  
241 (aquarium or marine grade) inhibits some infections (e.g. Cresko et al., 2004; Bernhardt et al.,  
242 2006; Schluter, 2016). Freshwater captured sticklebacks are exceptionally salt tolerant, even  
243 tolerating sea water levels (3% salt), by means of differential gene expression; particularly  
244 those associated with hypertension including MAP3K15 (Wang et al., 2014). Care should be  
245 taken to adjust salinity levels gradually over a period of several days to avoid osmotic shock.  
246 Aeration to each tank is often provided by means of an air stone or filter. The physiological  
247 temperature range of sticklebacks is 0-34.6°C (Jordan and Garside, 1972; Wootton, 1984b);  
248 fish in our laboratories are typically maintained between 10-20°C, 15-18°C being optimal  
249 (e.g. Cresko et al., 2004; Barber, 2005; Scharsack and Kalbe, 2014; Kalbe et al., 2016).  
250 Lower (5-7°C) and warmer (18-20°C) temperatures are often used to induce a winter- or  
251 summer-like state (Bakker and Milinski, 1991; Barber and Arnott, 2000; Katsiadaki et al.,  
252 2002b; Kalbe and Kurtz, 2006; Hopkins et al., 2011; Eizaguirre et al., 2012b). Fish exposed  
253 to lower temperatures display growth rates that can be up to 60% slower (Lefébure et al.,  
254 2011), whereas those at temperatures above 20°C are subject to higher stochastic mortality.  
255 Sticklebacks are typically kept on a summer 14-16 h light: 8-10 h dark cycle (e.g. Barber,  
256 2005; MacNab and Barber, 2012; Scharsack and Kalbe, 2014), which is altered to induce  
257 breeding (see Section 3.4).

258  
259 Adult sticklebacks are most commonly fed on live, frozen or freeze-dried bloodworm (larvae  
260 of the non-biting midge in the Family Chironomidae), *Tubifex* spp. (also commercially  
261 referred to as bloodworm) or *Daphnia* spp. The preferred laboratory food is frozen  
262 bloodworm, which is easily stored and the most nutrient dense (Wouters et al., 2001), but  
263 should be defrosted and rinsed in a strainer before use to maintain water quality. Due to  
264 dietary conservatism (Thomas et al., 2010), wild fish prefer live food and may not feed  
265 immediately after capture but will begin eating defrosted bloodworm after 48 h. Commercial  
266 flake food can be used to supplement the diet, particularly if used during fish rearing (e.g.



267 Katsiadaki et al., 2002a). Optimal diets for stickleback fry are outlined in Table 1.  
268 Precautions should be taken with live food that may contain parasites (e.g. copepods are the  
269 intermediate host for *Schistocephalus solidus* and *Camallanus lacustris*), although laboratory  
270 culture and gamma irradiated food will remove many of these risks. For experimental  
271 protocols, sticklebacks can be isolated in tanks at 1 fish/L, with 90% water changes at least  
272 every 48 h to prevent increased ammonia and nitrite levels (e.g. de Roij et al., 2011).  
273 Chemical cleaning products, particularly those containing chlorine, should be avoided or  
274 chosen carefully as they may impact parasite infections and fish health (Brungs, 1973; Finlay,  
275 1978).

276 **[Insert table 1 here]**

#### 277 *3.4 Breeding sticklebacks in vivo and in vitro*

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

284 Photoperiod is considered an important stimulus in stickleback breeding, although this is  
285 dependent on the latitudinal origin of each fish population (Yeates-Burghart et al., 2009).  
286 Sticklebacks are typically exposed to a winter light cycle (8 h light: 16 h dark) for 2-3  
287 months, before the length of daylight is increased to a summer light cycle (15-16 h light: 7-8  
288 h dark) (Wootton, 1976; Bakker and Milinski, 1991; Barber and Arnott, 2000; Katsiadaki et  
289 al., 2002b; Kalbe and Kurtz, 2006; Hopkins et al., 2011); although Wootton (1984c)  
290 describes additional light cycles to induce reproduction. Temperature is also a major factor in  
291 inducing breeding condition (Borg, 1982; Sokołowska and Kulczykowska, 2009). We  
292 suggest a summer light cycle (see above) and a temperature of 18-20°C to be the most  
293 conducive for bringing fish into breeding condition. For both *in vivo* and *in vitro* breeding in  
294 the laboratory, a male and low density of females can be initially separated by sex in a tank  
295 divided with a mesh net, thus allowing reciprocal visual and chemical stimulation without  
296 direct contact. If males and females are housed in the same tank for *in vitro* breeding, the  
297 most gravid individuals are selected for fertilisation, and/or any males that become aggressive  
298 separated or euthanised for fertilisation. Alternatively, a female enclosed in a water filled

299 transparent container can be placed into a tank containing males twice daily for  
300 approximately 30 min (e.g. Barber and Arnott, 2000). The fish should be fed at least 2-3  
301 times a day on bloodworm; unrestricted feeding will also allow the sticklebacks to  
302 compensate for infection (Barber et al., 2008). Extra care should be taken to clean these tanks  
303 regularly, as a result of extra food waste and faeces.

304

305 Breeding *in vivo* is a common practice that does not require euthanasia of fish: eggs and fry  
306 are often raised in hatcheries to inhibit parasite transmission (e.g. Aeschlimann et al., 2003;  
307 Frommen and Bakker, 2006; Kalbe and Kurtz, 2006; Kim and Velando, 2015). All aquaria  
308 should be equipped with environmental enrichment, such as gravel, rocks and pipes or plant  
309 pots for refugia. Males must be provided with a submerged Petri dish containing aquarium-  
310 grade sand or gravel and 50-100 cotton threads (5 cm long), which they use for nest building  
311 (e.g. Kalbe and Kurtz, 2006; Little et al., 2008; Hopkins et al., 2011; Morrell et al., 2012).  
312 Alternatively, pondweed and other natural nest building material can be provided (see  
313 Jakobsson et al., 1999; Katsiadaki et al., 2002b; Östlund-Nilsson and Holmlund, 2003), but  
314 this may introduce unwanted pathogens or plant growth into the tank. Once the nest is built,  
315 once or twice a day the most egg bound female is introduced into the male tank for 30 min; if  
316 breeding does not occur within this period it is unlikely to do so. Stickleback courtship goes  
317 through a series of stages (see Wootton, 1984c; Östlund-Nilsson et al., 2006), then after the  
318 female has laid eggs she will swim out of the nest and the male will immediately enter,  
319 fertilise the eggs and proceed to chase away the female. At this stage, the female is removed  
320 from the tank and the male left to raise the clutch of eggs until they hatch or the eggs are  
321 removed into a hatchery (e.g. Barber and Arnott, 2000; Kalbe and Kurtz, 2006; Pike et al.,  
322 2009). The use of a hatchery reduces the likelihood of pathogen transmission between the  
323 parent and offspring. The male may be used again for breeding by supplying it with more  
324 nest building material allowing generation of half-siblings.

325

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

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

340

341 Sperm may be stored by shredding the testes into multiple pieces using forceps, releasing the  
342 sperm into a small dish of HBSS or adjusted Ginsburg's ringer solution and transferring it to  
343 an Eppendorf microtube containing HBSS. The sperm can then be stored at 4°C for 2-3 days  
344 with HBSS or 2 weeks in Ginsburg's solution if it is refreshed after 7 days (see Schluter,  
345 2016 for Ginsberg's). Large testes can be cut into 2-3 sections using a sterile blade and the  
346 egg mass divided using artists' fine paint brushes in order to perform multiple fertilisations  
347 and produce half siblings (Barber and Arnott, 2000). Similarly, sperm from different males  
348 can be combined for sperm competition assays (Kaufmann et al., 2015; Mehlis et al., 2015).  
349 Fertilisations are carried out by stirring the shredded testes around the egg mass or adding a  
350 portion of the stored sperm; the testes are then removed after a few minutes replacing the lid  
351 of the Petri dish. Testes may also be macerated in 300 µl of HBSS and 50 µl added to a 'dry'  
352 Petri dish containing eggs for fertilisation; maceration can be conducted using a 40 µm cell  
353 strainer to avoid contamination with the tissue (Kaufmann et al., 2014). After 30 min at 15°C,  
354 the eggs can be checked for successful fertilisation, as indicated by separation of the inner  
355 and outer membranes, using a low power microscope (x10-60). Cell division should begin  
356 within 45-60 min, after which the egg mass is transferred to a hatchery (described below).  
357 Breeding *in vitro* is more reliable than *in vivo* breeding, requiring less time, and allows  
358 generation of maternal half siblings (e.g. Barber and Arnott, 2000; Pike et al., 2009; de Roij  
359 et al., 2011; MacNab and Barber, 2012).

360 **[Insert Figure 1]**

361 **[Insert table 2 here]**

### 362 *3.5 Hatchery*

363 For the hatchery, a small tank is used (20-30 x 40-50 x 10-20 cm deep) containing Hatchery  
364 Water (Table 2), which inhibits bacterial, fungal and oomycete growth, particularly  
365 *Saprolegnia declina* (e.g. Barber and Arnott, 2000; Pike et al., 2009). Methylene blue fades

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

387 [Insert Figure 2 here.]

## 388 **4.0 Common Stickleback Parasite Cultures**

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

### 393 **4.1 *Argulus foliaceus***

#### 394 *4.1.1 Introduction*

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

399 (*Oncorhynchus mykiss*) and roach (*Rutilus rutilus*) in addition to sticklebacks (*Gasterosteus*  
400 spp.) (see Bower-Shore, 1940). According to Kearn (2004), *Argulus foliaceus* may parasitise  
401 any freshwater British fish species. At high infection intensities, major fish stock losses have  
402 resulted in the closure of some fisheries (Northcott et al., 1997; Gault et al., 2002). When  
403 attaching to the host *A. foliaceus* makes use of circular sucking disks (see Figures 3 and 4),  
404 with contraction of disk muscles resulting in adhesion (Møller et al., 2008). Alternate  
405 relaxation and contraction of these two disks allows the parasite to move around the host's  
406 surface. Further support is provided by a series of spines on the underside and edges of the  
407 carapace (Figure 4A). Individual *A. foliaceus* have two compound eyes for vision alongside  
408 olfaction and mechanoreceptors used for ambush detection of the host in light conditions  
409 (Mikheev et al., 2000). This behaviour switches in the dark to a 'cruising search strategy'  
410 accompanied by increased swimming speed, allowing the parasite to cover an area 3-4 times  
411 greater (Mikheev et al., 2000). Argulids feed using a stylet (Figure 4A) and proboscis (Figure  
412 4B), the latter possessing serrated mandibles surrounding the mouth. During feeding, the  
413 spine-like stylet is inserted into the host's skin. Whilst the role of the stylet is still unclear, it  
414 is thought to involve injection of cytolytic substances that aid breakdown of tissues  
415 (Hoffman, 1977; Walker et al., 2011; Møller, 2012). This action with the rasping mouthparts  
416 and grazing behaviour of the parasite can inflict considerable damage to the skin of infected  
417 fish, particularly during heavy infection. Partly because of its feeding mechanism, *A.*  
418 *foliaceus* may act as a vector for viruses, bacteria and flagellates, including Spring Viremia  
419 Carp Virus (Ahne, 1985; Ahne et al., 2002). Depending on fish species, argulids will detach  
420 from their host and spend some time in the water column (Mikheev et al., 2015).

421 **[Insert Figure 3 & 4]**

422 Egg-laying of argulids is seasonal in the wild, being most active between July and August,  
423 but can occur all year round in the laboratory (Pasternak et al., 2000; Harrison et al., 2006).  
424 The first life stage is the nauplius, which depending on *Argulus* spp., develops to the  
425 metanauplius or first pre-adult stage prior to hatching (some authors refer to these stages as a  
426 'copepodids' because of the historical inclusion of the *Argulus* genus in the Copepoda  
427 subclass). After hatching, 7 pre-adult stages occur before adulthood (Hoffman, 1977). Males  
428 are generally smaller than females and both moult frequently once sexually mature. Once  
429 adult, sexes can be easily distinguished through examination of the abdominal lobes (Fryer,  
430 1982).

431 4.1.2 Source, culture and infection

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

435

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

446

447 Infections with all *A. foliaceus* life stages can be performed by anaesthetising a stickleback in  
448 0.02% MS222, transferring the fish to 100 ml of dechlorinated water and adding argulids.  
449 Alternatively, argulids can be allowed to infect fish naturally (e.g. Ruane et al., 1999;  
450 Forlenza et al., 2008; Kar et al., 2015); although we suggest placing the fish in the dark and  
451 adding refugia to reduce predation, which works well with metanauplii and pre-adults. To  
452 improve attachment, argulids can be starved for up to 24 h before exposure to a potential  
453 host.

454

455 For *A. foliaceus* breeding, infected fish are kept at 15-25°C (optimally 20°C), with one adult  
456 male and female *Argulus* per host; temperatures below 8-10°C cause egg laying to cease  
457 (Hoffman, 1977; Pasternak et al., 2000; Gault et al., 2002; Harrison et al., 2006; Taylor et al.,  
458 2009). Mating occurs on the host and then the female detaches to lay eggs, often in shaded  
459 areas on a hard substrate, such as the underside of rocks, stones or wood (Pasternak et al.,  
460 2000; Taylor et al., 2009; Sahoo et al., 2013). The eggs are laid in 2-4 rows with between 20  
461 and 300 eggs per string (Figure 5A). Each egg is 0.3-0.6 mm in length and coated in cement,  
462 which anchors it firmly to the substrate. Tanks should be regularly checked for eggs to  
463 prevent unwanted infections when nauplii hatch. Eggs laid directly on the walls or bottom of  
464 the tank can be collected, but it is easier to transfer the infected fish to a new 1 L pot, as the

465 eggs can be damaged even if carefully removed using a cell scraper. Alternatively, fertilised  
466 female argulids can be removed from the fish when they develop large ovaries and placed  
467 into a Petri dish (90 mm dia.) containing dechlorinated water for 24 h allowing them to lay  
468 their eggs.

469

470 Egg hatching time varies with parasite species and temperature (Table 3). *Argulus* spp. eggs  
471 can be stored at 4-5°C, which arrests embryo development, causing the nauplii to go into an  
472 ‘over winter’ state (Shimura, 1983; Gault et al., 2002; Harrison et al., 2006; Taylor et al.,  
473 2009). Photoperiod may also alter hatching in *A. siamensis* (see Bai, 1981), but has not been  
474 fully explored in other species. As a result of the temperature range and potential photoperiod  
475 required for hatching, a domestic fridge (4°C) provides ideal storage conditions. It is  
476 unknown how long eggs can be maintained in an arrested state, but successful hatching of  
477 eggs up to 4 months old has been achieved in our Cardiff aquarium. To induce hatching, eggs  
478 are transferred to a 1 L container of freshwater with aeration (Table 3). Egg development can  
479 be monitored by examining the egg string under a low power microscope (x10-40) the  
480 conspicuous eye spots of the developing metanauplii are easily seen, along with increased  
481 movement prior to hatching. Once hatched the metanauplii (Figure 5B) can survive off the  
482 host for 2-3 days. The metanauplii and pre-adults can be kept on sticklebacks (maximum of  
483 5) or carp (20 max. on a 20 g fish). Infected fish should be maintained at 15-20°C; warmer  
484 temperatures will increase *A. foliaceus* growth rate but also stochastic fish mortality. To  
485 reduce pathology when argulids reach the later pre-adult and adult stages, all but two argulids  
486 should be removed, by gently encouraging them off the fish with a pipette tip or blunt  
487 forceps, and then excess detached argulids can be used to infect other fish.

488 **[Insert Figure 5 here]**

489 **[Insert table 3 here]**

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

### 497 4.1.3 Immunology

498 Argulids induce a consistent innate response with the addition of an adaptive response  
499 approximately 7-10 days post-infection. The immunology of *A. foliaceus* infection has been  
500 little studied; there are however some closely related species for which the host immune  
501 phenotype has been documented. The majority of these studies have focused on sea lice of  
502 the genus *Lepeophtheirus* which, despite belonging to a different sub-class of the Copepoda,  
503 exhibit a similar life cycle to argulids. Typically, these studies have found constant increases  
504 in expression of *il-1 $\beta$* , *tnf- $\alpha$*  and MHC II throughout the course of the experiment (9-40 days  
505 post-infection) (Fast et al., 2006a, b). Over a 6 day period *A. japonicus*, which infects  
506 common carp, produces a similar response to that of sea lice including up-regulation of *tnf- $\alpha$*   
507 and the chemokines *CXC $\alpha$*  and *CXCR1* in the skin (Forlenza et al., 2008). Infections of rohu  
508 (*Labeo rohita*) with *A. siamensis* also demonstrate increased expression in the skin,  
509 particularly of innate responses, including *tnf- $\alpha$*  (although later at 15 days-post infection),  
510 lysozyme and natural killer cell enhancing factor (Saurabh et al., 2011; Kar et al., 2015). Kar  
511 et al. (2015) demonstrated a further role for adaptive immunity as IgM and  $\beta_2$ M also appear  
512 to be upregulated in the head kidney, although not consistently, from 0.5 to 15 days post-  
513 infection. Of further interest is the downregulation of TLR22 early in infection, complement  
514 and  $\alpha_2$ M more or less consistently across experiments, demonstrating that *A. siamensis* has  
515 the ability to modulate the immune system and other biological responses (Saurabh et al.,  
516 2010, 2011; Shailesh and Sahoo, 2010; Kar et al., 2015). Downregulation of the coagulation  
517 inhibitor  $\alpha_2$ M suggests a strategy that allows the argulid to inhibit clotting, making feeding  
518 easier. A key problem interpreting these studies is the harvesting of different organs and  
519 tissues, (skin, head kidney, kidney, serum and/or liver) for extraction of genetic material or  
520 immunological assays. While harvesting of the skin was performed in the majority of these  
521 studies, the range of other tissues taken and differences in methodology makes correlations  
522 between studies difficult to assess.

## 523 4.2 *Camallanus lacustris*

### 524 4.2.1 Introduction

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



530 Nie and Kennedy, 1991). Gravid female nematodes may contain several thousand active L1  
531 larvae, which are free moving, visibly coiling and uncoiling in the parental uterus. These  
532 larvae are shed from the vulva into the environment within fish faeces. Free-living L1s are  
533 viable in water for 12 days at 22°C and 80 days at 7°C (Campana-Rouget, 1961). They are  
534 ingested by a range of Cyclopidae copepods that act as intermediate hosts in which the larvae  
535 develop into L2s after 3 days at 25°C or 5 days at 20°C. For *C. lacustris* the second moult  
536 into the L3 stage occurs after 6 days at 25°C or 10-12 days at 20°C (Campana-Rouget, 1961).  
537 This is similar for other species within the genus, with *C. oxycephalus* reaching the L3 nine  
538 days post-infection at 25°C (Stromberg and Crites, 1974, 1975). Only at the L3 stage, coiled  
539 in the haemocoel of the copepod after migration from the digestive tract (De, 1999), is the  
540 camallanid larva infective to the definitive host on ingestion of the intermediate host  
541 (Moravec, 1969). These L3 larvae are relatively large within the haemocoel and at high  
542 intensities (>3 worms per copepod) copepod survival is reduced in a sex dependant manner  
543 (Benesh, 2011); smaller copepod species likely suffer reduced survival at lower infection  
544 intensities. Infected copepods are at a greater risk of predation upon attainment of *C. lacustris*  
545 infectivity (Wedekind and Milinski, 1996; Hafer and Milinski, 2016). Direct transmission  
546 from the copepod to the definitive host may occur by ingestion (Chubb, 1982), although more  
547 likely the copepods are first eaten by planktivorous fish, such as sticklebacks. When these  
548 paratenic hosts are predated, the camallanid reaches adulthood, producing *in utero* L1s within  
549 69 days (Chubb, 1982).

#### 550 4.2.2 Source, culture and infection

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

557

558 The characteristic red adult *Camallanus* worms (Figure 6A) survive for 1-2 weeks *in vitro* at  
559 4°C in 50% PBS. L1s can be removed from the adult worm (Figure 6C), held in a watch glass  
560 with 50% PBS, by puncturing the uterus with watchmakers forceps and allowing uterine  
561 contractions to force out the larvae. The L1s are visible using a dissection microscope (x10-  
562 60) and are conspicuous due to their high motility (Figure 6D), which is likely an adaptation to

563 increase predation. L1s survive for a minimum of 2-3 days *in vitro* at 4°C in tank water. They  
564 can be transferred using a *Caenorhabditis elegans* worm pick or P2 pipette to a non-treated  
565 culture dish or watch glass with lid containing copepods from the Family Cyclopidae. For  
566 larger infections 100 copepods are kept in beakers (250-500 ml) with 500 L1 larvae for ~10  
567 days, changing the water 3 days post-infection. Larvae within the copepod should be counted  
568 before infection (see below). Previous experiments have used many copepod species as hosts  
569 for camallanids, including *Mesocyclops*, *Thermocyclops* (see Bashirullah and Ahmed, 1976),  
570 *Macrocyclus* (see Krobbach et al., 2007), *Acanthocyclops* (see Chubb, 1982) and *Cyclops*  
571 spp. The larger of the *Macrocyclus* spp. have been used as a host for up to six larvae of  
572 *Camallanus lacustris* (see Krobbach et al., 2007). Smaller copepod species may be less able  
573 to survive such a high infection. Female copepods are also subject to increased mortality at  
574 high infection intensities in comparison to males (Benesh, 2011).

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

576 *Macrocyclus* spp. should be fed on *Artemia* spp. (see Krobbach et al., 2007) although  
577 species such as *Cyclops strenuus* survive well on a daily mixture of *Spirulina* and yeast  
578 (approximately 1 ml per 10 L tank of copepods; see Table 2). For copepods kept in culture  
579 dishes, half their water should be removed and replaced with a dilute feed mixture (100 µl in  
580 100 ml) every 2-3 days.

581

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

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

#### 597 4.2.3 Immunology

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

607

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

## 627 **4.3 *Diplostomum* spp.**

### 628 4.3.1 Introduction

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

639

640 *Diplostomum* utilises a complex, three stage life cycle comprising freshwater snails (Family  
641 Lymnaeidae) as the first intermediate host, fish as second intermediate hosts and a range of  
642 piscivorous birds as definitive hosts (e.g. common gulls *Larus canus*; see Karvonen et al.,  
643 2006a). Sticklebacks obtain *Diplostomum* infections by encountering free-swimming  
644 cercariae (Figure 7A) shed from infected snails, commonly of the genera *Lymnaea* or *Radix*.  
645 Whilst *Diplostomum* are typically described as eye flukes in the fish host, forming  
646 metacercariae (Figure 7B) in the lens, vitreous humour, and/or retina; specific lineages may  
647 also be present in brain tissue (see Blasco-Costa et al., 2014; Faltýnková et al., 2014).  
648 Although not covered here, Rieger et al. (2013) provide details for maintaining the parasite  
649 through its complete life cycle including the intermediate and definitive hosts *Lymnaea*  
650 *stagnalis* and the herring gulls (*Larus argentatus*) respectively.

651 **[Insert figures 7 A&B here]**

### 652 4.3.2 Source, culture and infection

653 If an infection of *Diplostomum* has been identified in a stickleback population, it is highly  
654 likely that *Lymnaea* or *Radix* snails from the same habitat will be infected. The prevalence of  
655 *Diplostomum*, however, varies considerably between seasons, localities and snail species (e.g.  
656 Karvonen et al., 2006b, c; Rieger et al., 2013; Faltýnková et al., 2014). To optimise  
657 *Diplostomum* collection, individual snails of larger size classes (e.g. *Lymnaea stagnalis* shell  
658 length > 40 mm) should be selected during late summer/early autumn to coincide with high  
659 prevalence and fully developed cercarial infections (Karvonen et al., 2006b). Infected snail  
660 populations can be maintained in laboratory aquaria containing continuously aerated water

661 (dechlorinated tap or filtered from source locality), fed *ad libitum* on washed lettuce in  
662 controlled climate facilities (reflecting source environment or 18 h light: 6 h dark cycle, ca.  
663 15°C). Light stress is commonly used to stimulate cercarial release, by placing snails  
664 individually into beakers of water (ca. 100 ml) at 10-20°C under a light source (e.g.  
665 Scharsack and Kalbe, 2014). Cercariae will be shed within 2-4 h, provided that fully  
666 developed *Diplostomum* cercarial infections are present, at a rate of 400-2400 cercariae/ h  
667 depending on temperature (Lyholt and Buchmann, 1996).

668

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

676

677 Sticklebacks can be infected individually in ~ 1 L water containing freshly emerged  
678 cercariae; typical exposure doses range from 20-220 cercariae per fish (Brassard et al., 1982;  
679 Lyholt and Buchmann, 1996; Kalbe and Kurtz, 2006; Scharsack and Kalbe, 2014; Haase et  
680 al., 2016) to 5,000-10,000 for other fish species (Sweeting, 1974; Rintamäki-Kinnunen et al.,  
681 2004). Whilst the parasite rapidly reaches the ocular tissues (within 24 h post-infection;  
682 Chappell et al. 1994), *D. pseudospathaceum* metacercariae establishment is best assessed  
683 after 1 week, since low numbers of early infections may be overlooked (Rauch et al., 2006).  
684 Kalbe and Kurtz (2006) have, however, demonstrated that 2 day and 8 week old  
685 metacercariae may be identified when sticklebacks are exposed to repeated cercarial  
686 infections. *Diplostomum* spp. infections are determined by counting the number of  
687 metacercariae in the eye tissues but this necessarily involves destructive sampling (e.g. Bortz  
688 et al., 1984; Lyholt and Buchmann, 1996; Kalbe and Kurtz, 2006; Locke et al., 2010;  
689 Scharsack and Kalbe, 2014).

#### 690 4.3.3 Immunology

691 The eyes of teleosts are assumed to have the same immune privileged status of mammals (i.e.  
692 no localised immune response; Niederkorn, 2006; Sitjà-Bobadilla, 2008), thus for parasites  
693 invading the eye such as *Diplostomum*, we assume the immune response is limited to the

694 migratory period between epidermal penetration of the cercariae and their arrival in the eye.  
695 Given this short window of vulnerability, it is generally acknowledged that the classical  
696 adaptive response plays no role in resistance against a primary parasite infection (Rauch *et*  
697 *al.*, 2006). Instead, oxidative burst and reactive oxygen species are thought to be the key  
698 components of the innate immune response against these pathogens. Head kidney lymphocyte  
699 respiratory burst activity is upregulated in fish 1.5 days post-infection but not from 5 days  
700 post-infection (Kalbe & Kurtz, 2006; Scharsack & Kalbe, 2014), while macrophages produce  
701 reactive oxygen species that are capable of killing larval *Diplostomum* (see Whyte *et al.*,  
702 1989). The phagocytic activity of granulocytes and monocytes has also been cited as  
703 inhibiting *Diplostomum* migration into the eye (Erasmus, 1959; Ratanarat-Brockelman,  
704 1974). Despite this apparent bias towards the innate response against this parasite, a recent  
705 transcriptomic study identified antibody mediated responses and increased MHC and *il-4r*  
706 expression (a gene in mammals associated with adaptive helminth resistance) in response to  
707 infection (Haase *et al.*, 2016). Such results support the notion that the innate and adaptive  
708 immune systems cannot be considered in isolation but must be viewed as a fluid and versatile  
709 network (Magnadóttir, 2006). There is also a level of concomitant immunity as sticklebacks  
710 that receive a primary infection of *D. pseudospathaceum* acquire lower levels of  
711 metacercariae in a secondary infection in contrast to the primary infection (Scharsack &  
712 Kalbe, 2014). In addition, sonicated metacercariae injected into sticklebacks induce antibody  
713 responses capable of providing immunity to subsequent infection (Bortz *et al.*, 1984; Whyte  
714 *et al.*, 1987); suggesting that the adaptive response may play a role in concomitant immunity  
715 if not the primary immune response.

716

717 While the host genotype, particularly that of the MHC, is cited as a major factor in resistance  
718 and susceptibility, the parasite's genotype is also involved in determining infection outcome,  
719 with differential gene expression in different *Diplostomum* clones (Haase *et al.*, 2014). As  
720 with MHC experiments that find homozygous individuals to be more susceptible to infection  
721 (see Wegner *et al.*, 2003a, b), infections using a single clone of *Diplostomum* were less  
722 successful than mixed infections (Haase *et al.*, 2014). Lake ecotype sticklebacks carry heavier  
723 and more diverse infections than their riverine ecotype counterparts (Kalbe *et al.*, 2002;  
724 Scharsack *et al.*, 2007a), with lake fish demonstrating a heightened level of resistance to  
725 *Diplostomum* infection (Scharsack *et al.*, 2007a; Scharsack and Kalbe, 2014), in part due to  
726 selection within the MHC (Kalbe and Kurtz, 2006; Eizaguirre *et al.*, 2011). In addition, lakes  
727 typically harbour a greater diversity of snails making the presence of the intermediate host

728 more likely, but also making a greater range of parasite genotypes available, which may  
729 account for some of the ecotype variation (Karvonen et al., 2012).

#### 730 **4.4 *Glugea anomala***

##### 731 *4.4.1 Introduction*

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

743 **[Insert figure 8 here]**

##### 744 *4.4.2 Source, culture and infection*

745 There are multiple published methods for infection of fish with *G. anomala* and other  
746 microsporidians (Olson, 1976; Shaw and Kent, 1999; Kurtz et al., 2004; Lom and Dyková,  
747 2005), including *Tetramicra brevifilum* (see Figueras et al., 1992). It is assumed that *G.*  
748 *anomala* is transmitted orally during cohabitation of infected and uninfected fish (Lom and  
749 Dyková, 2005). In theory infection can be achieved experimentally by exposing fish to a  
750 spore suspension produced from infected fish (Kurtz et al., 2004), intraperitoneal,  
751 intramuscular or intravascular injection, and anal or oral gavage (Shaw and Kent, 1999).  
752 Crustaceans, including *Artemia salina* (brine shrimp) and *Corophium spinocorne*  
753 (amphipod), may also act as intermediate hosts for *G. stephani* (see Olson, 1976). However,  
754 preliminary testing of several infection methods in our Cardiff laboratory (oral transmission  
755 of extracted spores in the water column, oral gavage, intramuscular injection, co-habitation of  
756 infected and uninfected fish and exposure of putative intermediate hosts (*Artemia salina*,  
757 *Cyclops strenuous* and *Daphnia magna* to *Glugea* spores for 48 h) to date, has not resulted in  
758 parasite transmission 90 days post-treatment, despite xenomas reportedly developing 3-4  
759 weeks post-infection (Lom and Dyková, 2005). The intensity of *G. anomala* can be measured  
760 by the number and size of xenoma visible externally (e.g. Schmahl et al., 1990; Lom et al.,

761 1995; Dezfuli et al., 2004; Kurtz et al., 2004), internal xenomas may occur and these can be  
762 identified during dissection (e.g. Dezfuli et al., 2004).

#### 763 4.4.3 Immunology

764 To date, there is only preliminary data on the immune response to *Glugea*. There is little or  
765 no detectable host response to the microsporidian until the xenoma is fully developed.  
766 Macrophage aggregates occur around the outside of the xenoma wall with eosinophils and  
767 neutrophils being recruited to reduce the mass of spores within the xenoma (Dezfuli et al.,  
768 2004; Lom and Dyková, 2005). Intermediate levels of individual allelic diversity in the MHC  
769 class *IIB* have been linked with increased *G. anomala* resistance (Kurtz et al., 2004).

### 770 4.5 *Gyrodactylus* spp.

#### 771 4.5.1 Introduction

772 *Gyrodactylus* species are ubiquitous monogenean parasites of teleosts with over 400  
773 described species (Harris et al., 2008). Identification of species is commonly conducted by  
774 rDNA internal transcribed spacer (ITS) region sequencing supplemented by the  
775 morphological characteristics of the marginal hooks and hamuli (Shinn et al., 2010), although  
776 mtDNA gene sequencing may also be necessary to reveal cryptic species (Xavier et al.,  
777 2015). The viviparous nature of their reproductive life cycle means that they are capable of  
778 uncontrolled infrapopulation growth that at high densities become pathogenic (e.g. Scott and  
779 Anderson, 1984; Bakke et al., 1990), although this is limited in most species by thermally-  
780 dependent host immune responses (e.g. Bakke et al., 1992; Harris et al., 1998; Lindenstrøm et  
781 al., 2004; Lindenstrøm et al., 2006; Kania et al., 2010) and hosts may seek elevated  
782 temperatures to 'self-medicate' (Mohammed et al. 2016).

783

784 *Gyrodactylus salaris* (Malmberg, 1957) is of particular economic importance as it infects  
785 salmonids and has been the focus of intensive eradication schemes particularly in Norway  
786 since the 1980s (Linaker et al., 2012). As such, *G. salaris* has a published genome (Hahn et  
787 al., 2014). Studies on salmon are often costly and their fry are particularly sensitive to  
788 stressors (Barton et al., 1986). Therefore, many studies have used model fish, including the  
789 guppy and stickleback (reviews by Cable, 2011; Barber, 2013, respectively) to assess  
790 potential ecological, pathological or immunological effects of these parasites on tropical and  
791 temperate fish species (Bakke et al., 2007). Because the parasites infect the gills, body and/or  
792 fins of the host, and most detached parasites have no swimming ability (a notable exception  
793 being *G. rysavji* Ergens, 1973 see El-Naggar et al., 2004), transmission typically occurs



794 during host contact. Some parasite species, though, may drift or hang in the water column or  
795 attach to the substrate if detached from the host (Bakke et al., 1992; Soleng et al., 1999;  
796 Cable et al., 2002b), adopting a ‘sit-and-wait’ re-infection strategy. In high host density  
797 aquaculture systems, gyrodactylid infections can spread quickly with devastating  
798 consequences.

#### 799 4.5.2 Source, culture and infection

800 Stickleback *Gyrodactylus* spp. may be obtained from research institutions or the wild. The  
801 two common species found infecting sticklebacks are: *G. gasterostei* (Glaser, 1974) and *G.*  
802 *arcuatus* (Bychowsky, 1933); *G. alexanderi* (Mizelle & Kritsky, 1967) and *G. branchicus*  
803 (Malmberg, 1964) are rare, whereas other species such as *G. salaris* or *G. pungitii*  
804 (Malmberg, 1964) may infect the three-spined stickleback but are not specialists; for a full  
805 list see Harris et al. (2008). Using a dissection microscope with fibre optic illumination,  
806 sticklebacks can be experimentally infected by anesthetizing a donor and recipient fish in  
807 0.02% MS222 and allowing *Gyrodactylus* worms to cross from one fish to another by  
808 overlapping the stickleback caudal fins. Infections can also be performed by removing  
809 parasites on a fin clip or scale, or gently dislodging the worms from donors using an insect  
810 pin (Buchmann and Bresciani, 1997; Buchmann and Uldal, 1997), and then bringing a known  
811 number of parasites into close contact with a recipient fish. Alternatively, infections can be  
812 performed by co-habitation of recipient and donor fish (e.g. Lindenstrøm et al., 2006; Kania  
813 et al., 2010; Ramírez et al., 2015), but this results in inconsistent starting infection intensities.  
814 For controlled infections, typically one or two worms are added to the caudal fin to initiate an  
815 infection (e.g. Cable et al., 2000; van Oosterhout et al., 2003; Cable and van Oosterhout,  
816 2007; de Roij et al., 2011; Konijnendijk et al., 2013; Smallbone et al., 2016a), but up to four  
817 have been used (Anaya-Rojas et al., 2016).

818

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

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

836

837 Measuring the infection intensity of some gyrodactylid species is remarkably simple given its  
838 ectoparasitic nature. It is, however, important to note that some gyrodactylid species of the  
839 three-spined stickleback, e.g. *G. arcuatus*, infect the gills and therefore cannot be counted  
840 without autopsy (Harris, 1982; Raeymaekers et al., 2008). When using a species such as *G.*  
841 *gasterostei*, which is predominantly found on the skin and fins (Harris, 1982), the infection  
842 trajectory can be monitored non-invasively (e.g. Buchmann and Uldal, 1997; Cable et al.,  
843 2000; Kania et al., 2010; Raeymaekers et al., 2011; Ramírez et al., 2015).

#### 844 4.5.3 Immunology

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

858

859 Immunity to *Gyrodactylus* spp. is primarily mediated by a ‘scorched earth strategy’, whereby  
860 parasites are starved of nutrients and exposed to increased expression of host complement  
861 (Buchmann, 1998; Harris et al., 1998; Kania et al., 2010). As such, resistant salmon show no  
862 increase in the mucus secretagogue *il-1 $\beta$*  while susceptible salmon show a marked increase in  
863 *il-1 $\beta$*  24 h post-infection (Lindenstrøm et al., 2006; Kania et al., 2010). Likewise rainbow  
864 trout (*Oncorhynchus mykiss*), exposed to primary *G. derjavini* infections and then a  
865 secondary infection 35 days after parasite clearance, demonstrated susceptibility in the  
866 primary infections linked with increased *il-1 $\beta$*  transcript in the skin while resistant  
867 secondarily infected fish showed no increase in *il-1 $\beta$*  (Lindenstrøm et al., 2003).  
868 Gyrodactylids feeding on the mucus and epithelium will therefore be at a disadvantage on  
869 any host able to suppress the increase in *il-1 $\beta$*  production. Indeed, a reduction in the density  
870 of mucous cells is also associated with infection (Buchmann and Uldal, 1997; Dalgaard et al.,  
871 2003), however, this relationship may reverse later in infection as the mucous begins to  
872 contain higher concentrations of anthelmintic effectors (Buchmann and Bresciani, 1997).  
873 The major effector associated with resistance is alternatively activated complement present in  
874 both the serum and mucus (Buchmann, 1998; Harris et al., 1998). Immuno-cytochemical  
875 assays demonstrated binding of C3 to the cephalic gland opening, body and hamulus sheath  
876 of the parasite but found no immunoglobulin binding (Buchmann, 1998). Resistant salmon  
877 also have increased *il-10*, *mhc II* and *serum amyloid A* transcript 3-6 weeks post-infection in  
878 the epidermis of infected fins (Kania et al., 2010). The immune response to gyrodactylids can  
879 therefore be separated into two distinct stages: the passive stage where mucus production is  
880 inhibited to restrict parasite population growth and the immunologically active stage where  
881 complement and other effectors reduce the intensity of infection allowing host recovery. In  
882 infections with *Gyrodactylus* spp. it is therefore possible to infer the point at which the  
883 immune system is most active by virtue of the declining parasite population. For example, on  
884 *G. salaris* infected Baltic salmon and *G. gasterostei* infected sticklebacks, population  
885 reduction occurs at 2-3 weeks post infection at 12°C (see Bakke et al., 2002; de Roij et al.,  
886 2011; Raeymaekers et al., 2011), although such data may be confounded by the death of  
887 heavily infected fish during this time period.

## 888 **4.6 *Saprolegnia parasitica***

### 889 **4.6.1 Introduction**

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

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

902

903 The *Saprolegnia* lifecycle, as with other oomycetes, has an asexual stage including the  
904 development of sporangia and zoospores, and a sexual stage resulting in the production of  
905 oospores (see van West, 2006). The asexual stage is the primary method of infecting new  
906 hosts as free-swimming zoospores are released into the environment (Hatai and Hoshiai,  
907 1994; Willoughby, 1994; Bruno and Wood, 1999). The sexual production of oospores is  
908 thought to enhance survival under acute stress conditions, such as temperature extremes or  
909 desiccation, until conditions become more favourable. Some *Saprolegnia* species (including  
910 most strains of *S. parasitica* Coker 1923), however, seem to lack a sexual cycle and do not  
911 produce oospores, at least under laboratory conditions.

912

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

922

923 During infection, *S. parasitica* secretes a SpHtp1 protein, which is able to translocate  
924 independently into fish cells via an interaction with a host cell surface tyrosine-O-sulphated  
925 molecule (van West *et al.*, 2010; Wawra *et al.*, 2012). The precise function of SpHtp1 is

926 unknown, but it likely plays a role in the infection process. This finding and the  
927 immunomodulation capabilities of *S. parasitica* (see Belmonte et al., 2014) suggest that the  
928 interaction is more complex than previously considered. It is now becoming clear that *S.*  
929 *parasitica* is a primary pathogen rather than a secondary opportunistic pathogen as has often  
930 been assumed (e.g. Hoole et al., 2001).

#### 931 4.6.2 Source, culture and infection

932 Cultivated strains of *S. parasitica* are held at various institutions but the parasite can also be  
933 isolated from wild fish. The mycelia can be maintained on potato dextrose agar (PDA) (e.g.  
934 van West et al., 2010; Belmonte et al., 2014; Sun et al., 2014; Parra-Laca et al., 2015) (Table  
935 2) in 140 mm Petri dishes indefinitely at 15-25°C (light cycle and humidity unimportant).  
936 Cultures should be re-plated every month, to protect against bacterial and fungal  
937 contamination, by transferring a 5 mm dia. plug of healthy (white/grey in colour with no  
938 yellowing or other fungal growth) mycelium from one Petri dish to another. Cultures held on  
939 PDA should also be passaged through fish or cell lines every few generations in order to  
940 maintain virulence (Songe et al., 2014). To isolate a wild strain, mycelia are scrapped off an  
941 infected fish and inoculated onto a potato dextrose agar plate containing chloramphenicol at  
942 50mg/ml to inhibit contamination (e.g. Songe et al., 2014; Kalatehjari et al., 2015; Thoen et  
943 al., 2015); chloramphenicol should not be used to maintain the culture as it is fungistatic  
944 (Rooke and Shattock, 1983). The *Saprolegnia* mycelium should then be re-plated (typically  
945 2-5 times), taking 5 mm dia. plugs from the leading edge until a pure culture is obtained  
946 devoid of bacteria and fungi. The *Saprolegnia* mycelium is cotton-like and white/grey in  
947 colour, all other growth should be avoided when taking the plug for culture.

948

949 To infect sticklebacks from a stock PDA culture, three mycelium plugs (5 mm dia.) should be  
950 taken from the PDA stock and placed on a 140 mm Petri dish with 70 ml of pea broth (Table  
951 2) for 72 h at 25°C. Following incubation, agar plugs are removed using sterile forceps and  
952 the pea broth withdrawn using a sterile syringe or pipette. The mycelium is then washed three  
953 times with 70 ml of a 50/50 mixture of distilled and tank water in the Petri dish. During each  
954 wash, after the addition of the water mix, the mycelium should be agitated before the water  
955 mix is removed. Finally, 30 ml of the 50/50 distilled and tank water mixture is added to the  
956 Petri dish and before it is incubated for a further 24-48 h at 15°C (Powell et al., 1972;  
957 Riberio, 1983). Alternatively, cleaned mycelium can be dispensed from one Petri dish into  
958 500 ml of 50/50 distilled and tank water, incubating for 24-48 h at 15°C. The cultures should

959 be checked for spore production under a microscope (x100), and the spores isolated by  
960 straining the *Saprolegnia* through a 40 µm cell strainer using a cell scraper to remove encysted  
961 spores from the Petri dish. Spore density is calculated using a haemocytometer, if necessary  
962 concentrating the sample by centrifuging at 3000 g for 5 min at room temperature, removing  
963 the excess supernatant and re-suspending the spores in distilled water. Fish are infected using  
964 the ami-momi technique, in which salmonids are typically shaken in a net for 2 min (Hatai  
965 and Hoshiai, 1994), this duration of shaking is excessive for sticklebacks instead we  
966 recommend 30 sec. Shaken fish are then exposed, ideally individually, to  $3 \times 10^5$  spores per  
967 litre (e.g. Belmonte et al., 2014), consistent with spore concentrations found in fish farms  
968 (Thoen et al., 2010).

969

970 The infection intensity of *S. parasitica* can be crudely analysed by photographing an infected  
971 fish and calculating the total body coverage of erupted hyphae (e.g. Fregeneda Grandes et al.,  
972 2001), but qPCR methods are being developed (van West et al. unpublished). Given the rapid  
973 time to mortality for infected fish, morbidity and prevalence of infection can also be used as a  
974 measure of *S. parasitica* virulence (e.g. Pickering and Duston, 1983; Hussein and Hatai,  
975 2002; Gieseke et al., 2006).

#### 976 4.6.3 Immunology

977 With true fungal infections it is generally accepted that cellular mediated immunity,  
978 particularly T-helper cell type 1 ( $T_H1$ ) responses, are required for clearance of an infection  
979 (Blanco and Garcia, 2008). In general, hosts infected with oomycetes induce innate immune  
980 responses to infection, but some aspects of humoral immunity have also been found (see  
981 Roberge et al., 2007; Blanco and Garcia, 2008; Belmonte et al., 2014; Minor et al., 2014). Of  
982 particular interest is the humoral response towards the protein SpSsp1, which may provide a  
983 novel target for vaccine development (Minor et al., 2014). Given the rapid and destructive  
984 progression of *S. parasitica* infections, immune responses must likewise be fast acting and  
985 avid. Upon infection with *S. parasitica*, fish undergo a rapid acute response including  
986 upregulation of genes transcripts involved in all three complement pathways (classical,  
987 alternative and lectin) (Roberge et al., 2007). Upregulation of C1r, C2, mannose-binding  
988 lectin (MBL) indicate involvement of the alternative and lectin pathways, while substantial  
989 up regulation of C3 and C6, beyond what might be expected from just classical and MBL  
990 pathway activation, is postulated as the main reason for involvement of the alternative  
991 pathway (Roberge et al., 2007). Other immune related genes including *ATP-binding cassette*

992 *transporter* (required for MHC class I antigen presentation), and the cytokine receptors  
993 *CXCR4* (chemokine of importance in humoral immunity) and *cd63* (cell development and  
994 growth of multiple immune cells) are upregulated (Roberge et al., 2007). Fish also produce a  
995 response to tissue damage caused by *S. parasitica*, including induction of proinflammatory  
996 genes such as *il-1 $\beta$* , *il-6*, *tnf- $\alpha$*  and *cox2* (Kales et al., 2007; de Bruijn et al., 2012; Belmonte  
997 et al., 2014). In addition to upregulation of inflammatory genes, the parasite is capable of  
998 immunomodulation by means of prostaglandin E<sub>2</sub> causing suppression of cellular immunity,  
999 including a reduction in *cd8a* and *ifn- $\gamma$*  transcripts (Belmonte et al., 2014). Proinflammatory  
1000 genes are also upregulated by prostaglandin E<sub>2</sub> (IL-6, IL-8, IL-17) (Belmonte et al., 2014); an  
1001 expression profile that in fungal infections is permissive to infection (Traynor and Huffnagle,  
1002 2001). Similar immune evasion strategies are employed by true fungi, which are capable of  
1003 driving anti-inflammatory response and a shift towards a T<sub>H</sub>2 profile, through TLR2 (Netea et  
1004 al., 2003; Netea et al., 2004).

## 1005 **4.7 *Schistocephalus solidus***

### 1006 *4.7.1 Introduction*

1007 Plerocercoid larvae of the diphylobothriidean cestode *Schistocephalus solidus* (Müller, 1776)  
1008 (Figure 9) commonly infect sticklebacks in ponds, lakes and slow flowing rivers (Wootton,  
1009 1976; Barber, 2007). *S. solidus* is one of the most studied stickleback parasites, and was the  
1010 first parasite for which a complex, multi-host life cycle was demonstrated experimentally  
1011 (Abildgaard, 1790) (Figure 10). Experimental culture techniques, which permit physiological  
1012 and developmental studies of the maturing plerocercoid, have been in existence for decades  
1013 (Hopkins and Smyth, 1951; Clarke, 1954; Smyth, 1954, 1959, 1962; Arme and Owen, 1967)  
1014 and are well-established (Jakobsen et al., 2012). The stickleback-*Schistocephalus* host-  
1015 parasite model has been widely used for studying the impacts of infection on host energetics  
1016 (Barber et al., 2008), growth and reproductive development (Heins and Baker, 2008) as well  
1017 as on host behaviour (Milinski, 1985, 1990; Barber and Scharsack, 2010; Hafer and Milinski,  
1018 2016). Recently, experimental infection studies have been used to investigate evolutionary  
1019 aspects of host-parasite interactions (MacColl, 2009; Barber, 2013) and host immune  
1020 responses (Scharsack et al., 2004, 2007b; Barber and Scharsack, 2010), as well as the impacts  
1021 of changing environments on patterns of infection (MacNab and Barber, 2012; Dittmar et al.,  
1022 2014; MacNab et al., 2016).

1023 **[Insert figures 9 and 10 here]**

1024 4.7.2 Source, culture and infection

1025 Naturally infected sticklebacks, which are readily identifiable by their swollen profile  
1026 (Barber, 1997) can be collected from the wild and used as a source of infective parasites for  
1027 experimental culture (e.g. Arnott et al., 2000; Barber and Svensson, 2003; Scharsack et al.,  
1028 2007b). Whilst sticklebacks can harbour multiple *S. solidus* plerocercoids, infected fish often  
1029 support a low number of large plerocercoids (Arme and Owen, 1967; Heins et al., 2002). The  
1030 total mass of plerocercoids can approach that of the host fish (Arme and Owen, 1967).  
1031 Plerocercoids can be successfully cultured *in vitro* from sizes of 20 mg (Tierney and  
1032 Crompton, 1992; Dörücü et al., 2007) but they are only reliably infective to avian hosts at a  
1033 body size of  $\geq 50$  mg (Tierney and Crompton, 1992).

1034

1035 Infective *S. solidus* plerocercoids are readily recovered from the body cavity of euthanised,  
1036 naturally-infected sticklebacks following ventral incision. Complete, whole plerocercoids  
1037 should be transferred using sterilised laboratory forceps to a pre-autoclaved culture vessel  
1038 containing a loop of narrow-diameter semi-permeable membrane suspended in *S. solidus*  
1039 culture media (see Table 2). As they are hermaphroditic, worms can be cultured individually  
1040 (i.e. ‘selfed’) or in pairs (i.e. outcrossed) (Milinski, 2006). Compression of the worms by the  
1041 cellulose tubing simulates conditions in the intestine of the bird definitive host and  
1042 encourages fertilisation (Smyth, 1990). The worms, suspended in this ‘model gut’ inside the  
1043 culture vessel, are incubated at 40°C in darkness, ideally in a water bath with lateral shaking  
1044 at a frequency of 80 cycles per minute, which dissipates metabolic products. To reduce  
1045 bacterial and fungal infections, antibiotics and anti-fungal chemicals can be added to the  
1046 culture medium (Jakobsen et al., 2012). Plerocercoids are progenetic (i.e. exhibit advanced  
1047 sexual development in the larval stage) and the morphological transition to the adult worm is  
1048 rapid, with fertilised eggs being produced from day two onwards *in vitro*. Egg production  
1049 continues for several days, after which the adult worm dies (Dörücü et al., 2007).

1050

1051 The eggs, along with the senescent or dead adult worm(s), should be flushed with dH<sub>2</sub>O from  
1052 the cellulose tubing into a Petri dish (12 cm dia.). To clean the egg solution, excess dH<sub>2</sub>O is  
1053 added to the dish and a gentle swirling movement used to concentrate the eggs; this is best  
1054 achieved whilst viewing under low power using a dissecting microscope with cold light  
1055 illumination. Because the eggs are negatively buoyant, they readily aggregate in the centre of  
1056 the Petri dish. A pipette can then be used to remove detritus, including tegument of the adult  
1057 worm, from the egg solution. Repeated iterations of this process, interspersed with dispersing



1058 the egg mass, generate a sufficiently clean egg solution for subsequent incubation. Eggs can  
1059 then be split between multiple sterile Petri dishes, filled to a depth of 5 mm with dH<sub>2</sub>O,  
1060 sealed with Parafilm and wrapped in aluminium foil to restrict premature exposure to light.

1061  
1062 Eggs are incubated for 21 d at 20°C in the dark before being exposed to natural daylight to  
1063 induce hatching (Scharsack et al., 2007b). Pre-exposure to a short (ca. 2 h) period of light, the  
1064 evening before desired hatching, may improve subsequent hatch rates (Dubinina, 1966).  
1065 Hatched eggs release coracidia, which are spherical, ciliated, free-swimming first stage  
1066 larvae. Coracidia move actively for ca. 12-24 h after hatching at normal laboratory  
1067 temperatures, but apparently senescent (i.e. motionless) coracidia can establish infections in  
1068 copepod hosts (unpublished data). Coracidia are collected using a Pasteur pipette and  
1069 transferred to a drop of dH<sub>2</sub>O on a watch glass, Petri dish, microscope slide, or in a well of a  
1070 96-well microtitre plate. An individual cyclopoid copepod (typically *Cyclops strenuus*  
1071 *abyssorum* or *Macrocyclus albinus*) is then added to the water drop containing the hatched  
1072 coracidium (coracidia) to allow trophic transmission. It is important to cover the water  
1073 droplet to prevent evaporation. The water droplet is visually inspected under a dissection  
1074 microscope to check that the coracidium has been ingested, after which the exposed copepod  
1075 can be transferred to a larger volume of water and fed under normal culture conditions for 7  
1076 d, fed either newly-hatched *Artemia* spp. nauplii or a few drops of *Spirulina* feed (Table 2).  
1077 Copepods are then screened at 7 d post-exposure for infection status. The procercoid stage  
1078 that develops within the copepod is infective to sticklebacks (Dubinina, 1966) when it  
1079 develops a hooked cercomer - a caudal appendage used by the parasite during invasion of the  
1080 fish host (Barber and Scharsack, 2010; Benesh and Hafer, 2012; Benesh, 2013).

1081  
1082 Infection of sticklebacks in the laboratory can be achieved by gavage feeding or allowing free  
1083 feeding by isolated sticklebacks (e.g. Barber and Svensson, 2003; Hammerschmidt and  
1084 Kurtz, 2005; Scharsack et al., 2007b; MacNab and Barber, 2012). Individual sticklebacks can  
1085 be held in a crystallising dish (15 cm dia.) filled to 3 cm with aquarium water, illuminated  
1086 from above using a cold light source and surrounded by black paper to improve contrast.  
1087 Feeding can be encouraged by moving an infected (i.e. cercomer-bearing procercoid)  
1088 copepod up and down within the neck of a long-form Pasteur pipette immediately in front of  
1089 a stickleback that has been starved for 24 h, before releasing it into the water. Alternatively,  
1090 fish can be left to forage for 6 h in a small (1 L) plastic aquarium containing a few newly-  
1091 hatched *Artemia* spp. nauplii and an infected copepod. Exposure can be confirmed by direct

1092 observation of the ingestion event or by sieving the water to confirm ingestion of the  
1093 copepod.

1094

1095 Infections of sticklebacks with *S. solidus* most commonly use the parasite mass as an  
1096 endpoint measurement to determine the intensity of infection. The mass of both the  
1097 stickleback and parasites in this infection system can vary dramatically and, as such, the

1098  $parasite\ index = \frac{Total\ parasite\ mass}{Total\ fish\ \&\ parasite\ mass} \times 100$  (Arme and Owen, 1967) is often used as a

1099 measure of intensity (e.g. Giles, 1983; Tierney et al., 1996; Kurtz et al., 2004; Barber, 2005).

1100 Alternatively, a measure of volume can be produced for plerocercoids whose mass is too  
1101 small to be measured directly (e.g. Wedekind et al., 2000; Scharsack et al., 2007b): the

1102 plerocercoid is photographed under a microscope and taking the maximal area of the  
1103 longitudinal section of its body and applying the following formula  $volume\ (mm^3) =$

1104  $e^{0.279} \times area\ (\mu m^2) \times 10^{-9}$  (see Wedekind et al., 2000).

1105

1106 The growth of the plerocercoid stage *in vivo* can be estimated non-invasively using image  
1107 analysis based on the infection-induced swelling (Barber, 2007), facilitating longitudinal  
1108 studies of infection and parasite growth. Individual coracidia can be stained using persistent  
1109 fluorescent dyes (Kurtz et al., 2002), allowing differentiation of individual parasites in mixed  
1110 infections. Finally, there are now microsatellite markers and other ecological, genomic and  
1111 transcriptomic resources that facilitate taxonomic studies (Binz et al., 2000; Nishimura et al.,  
1112 2011; Sprehn et al., 2015; Hébert et al., 2016).

#### 1113 4.7.3 Immunology

1114 A rapid host immune response is thought to be crucial for host resistance against *S. solidus*,  
1115 preventing establishment within the body cavity. Infection prevalence drops from 60% in the  
1116 first week to 54-52% one month post-infection, but with no further decline thereafter  
1117 (Scharsack et al., 2007b; Benesh, 2013). In addition, no dead *S. solidus* are detected in the  
1118 body cavity after 17 days post-infection, suggesting that this is the effective limit of the  
1119 immune response against the parasite (Scharsack et al., 2007b). Resistance to *S. solidus* is  
1120 associated with early proliferation of head kidney monocytes and lymphocyte proliferation 7  
1121 days post-infection (Barber and Scharsack, 2010), the rate of lymphocyte production then  
1122 drops drastically in both resistant and susceptible fish 17 days post-infection (Scharsack et  
1123 al., 2007b). Monocyte production also undergoes changes during infection, being elevated in  
1124 susceptible fish at 7 and 27 days post-infection but reduced at 17 days post-infection

1125 compared to controls (Scharsack et al., 2007b). There is no obvious involvement of the  
1126 adaptive response in resistance to a primary *S. solidus* infection, as this would take 2-3 weeks  
1127 to be active in fish at 18°C, by which time plerocercoids are already established (Barber and  
1128 Scharsack, 2010). There is, however, evidence that at some levels the adaptive response is  
1129 involved at least in tolerating an infection. Intermediate MHC class *IIB* diversity has been  
1130 linked to a reduction in the parasite index and an increase in the respiratory burst response;  
1131 the prevalence of infection was unaffected by this diversity (Kurtz et al., 2004).

1132

1133 The stickleback immune response to *S. solidus* also involves upregulation of responses,  
1134 including adaptive immunity, from 47 days post-infection that are not linked to resistance in a  
1135 primary infection as the plerocercoid is already well established. Head kidney lymphocyte  
1136 respiratory burst is upregulated 47-67 days post-infection (Barber and Scharsack, 2010) and  
1137 granulocytes increase in proportion until 63 days post-infection (Scharsack et al., 2004).  
1138 Further transcriptomic analysis found upregulation of innate toll-like receptor, complement  
1139 and macrophage genes as well as upregulation of adaptive MHC genes 50 days post-infection  
1140 (Haase et al., 2016).

1141

1142 An active adaptive response late in infection may support a role for immunological tolerance  
1143 of *S. solidus* infections (Jackson et al., 2014), or concomitant immunity, though we are  
1144 unaware of any direct tests of this hypothesis. In addition, sticklebacks with high or low  
1145 diversity in the MHC class *IIB*, which is correlated with MHC expression (Wegner et al.,  
1146 2006), harboured larger parasites while those with intermediate diversity had smaller worms  
1147 (Kurtz et al., 2004). This supports the notion of hosts with intermediate (optimal) MHC  
1148 diversity suffering less from infection (Wegner et al., 2003a, b). Such a result may also  
1149 support a role for tolerance, as the immune system shifts (~47 days post-infection) to focus  
1150 less on resistance and more on restricting plerocercoid growth rate and perhaps improving  
1151 fish condition. This late immune response, which is known to last from 45-67 days post-  
1152 infection, correlates with plerocercoids reaching infective weight for the definitive host at  
1153 approximately 47 days post-infection (Scharsack et al., 2007b). Concomitant immunity may  
1154 therefore also be a viable hypothesis as this would inhibit secondary infections from  
1155 acquiring vital nutrients at this crucial life history stage (and *S. solidus* is known to alter the  
1156 susceptibility of the host to infection by other species; Benesh and Kalbe, 2016). In addition,  
1157 head kidney lymphocytes exposed to the excretory products of mature *S. solidus* (>50 mg) in

1158 conditioned culture media expressed higher respiratory burst activity, associated with  
1159 granulocyte viability, which may also manipulate host behaviour via the immune-  
1160 neuroendocrine axis and aid transmission to the definitive host (Scharsack et al., 2013).

## 1161 **5.0 Treating common infections**

1162 Not all parasitic infections of sticklebacks can be eliminated, and the decision to treat fish,  
1163 and the nature of treatment chosen, will be dependent both on infection history and the nature  
1164 of the experiment as well as a cost benefit trade-off. A list of common treatments for  
1165 common parasite infections of fish is provided in Table 4.

1166 **[Insert table 4 here]**

1167 The most common endemic infections to occur in laboratory studies of sticklebacks are  
1168 microparasites, commonly *Aeromonas* spp., *Flavobacterium* spp., *Pseudomonas* spp.,  
1169 *Ichthyophthirius multifiliis* and *Saprolegnia parasitica*. These infections often establish when  
1170 fish are physiologically stressed, for example by experimental procedures, altered  
1171 environmental conditions or following capture and/or transportation. These pathogens are  
1172 ubiquitous, present in most water bodies and therefore are difficult to eliminate from aquatic  
1173 systems. Additionally, *Gyrodactylus* spp. and *Trichodina* spp. (Figures 11A & B) are easily  
1174 introduced into tanks with other fish or as a result of imperfect net hygiene. Most *Trichodina*  
1175 spp. and other ecto-commensals including *Epistylis* spp. and *Apiosoma* spp. are asymptomatic  
1176 at low numbers but may become pathogenic at high intensities (Collymore et al., 2013). Even  
1177 low level endemic *Gyrodactylus* infections can result in epidemics after several weeks in  
1178 captivity if not treated immediately, and even mild infections probably affect host behaviour  
1179 and physiology. Wild sticklebacks may be infected with heteroxenous parasites such as  
1180 *Schistocephalus solidus*, *Diplostomum* spp. and *Camallanus lacustris*, but these parasites  
1181 cannot be transmitted without the presence of their intermediate hosts. Although *Glugea*  
1182 *anomala* may be transmitted directly, the details of transmission are unclear. Transfer of  
1183 water between tanks should be avoided in all cases. Nets are a common source of water  
1184 transfer and should be sterilised in Virkon or sodium metabisulfite (in accordance with  
1185 manufacturer's instructions), rinsed and fully dried before reuse. Infected fish should be  
1186 isolated and treated as indicated in Table 4; early detection and rapid treatment is key for the  
1187 majority of infections.

1188 **[Insert Figure 11]**

1189 *Aeromonas* spp. and *P. fluorescens* cause red ulcers, small white/grey marks on the fins and  
1190 head, fin rot and ultimately death. Because it is often difficult to distinguish these two  
1191 infections without biochemical or molecular techniques, a broad-spectrum antibiotic should  
1192 be used following consultation with a veterinarian; if severe damage occurs the fish should be  
1193 euthanized using a procedure approved by the relevant regulatory authority.

1194

1195 The highly contagious protozoan parasite *I. multifiliis* causes small white spots on the fins  
1196 and skin of the fish. The simplest method of treatment is increasing water salinity (Selosse  
1197 and Rowland, 1990; Miron et al., 2003; Garcia et al., 2007) and adding methylene blue  
1198 (Tieman and Goodwin, 2001) (see Table 4). A low concentration formalin or malachite green  
1199 treatment may also be used (e.g. Leteux and Meyer, 1972; Tieman and Goodwin, 2001)  
1200 following the low and prolonged immersion dose (Table 4) or an off-the-shelf formulation  
1201 used following manufacturer's instructions. Given the complexity of the life cycle, and the  
1202 fact that resistance is common, multiple treatment doses are likely to be required.

1203

1204 For *Saprolegnia* infections, prevention (0.5% saline water) is definitely better than cure (Ali,  
1205 2005; van West, 2006); once a fish is symptomatic it may survive no more than a few days,  
1206 occasionally even hours, or be irreparably damaged and must be euthanized using an  
1207 approved procedure. If *Saprolegnia* infection does occur the most effective treatment is a  
1208 high dose malachite green in formalin treatment (Table 4), or a low concentration formalin  
1209 treatment (see van West, 2006). To aid recovery and prevent reinfection following formalin  
1210 exposure, the fish should be transferred to 0.5-1% salt solution, with the possible addition of  
1211 methylene blue (Table 4).

1212

1213 Gyrodactylid treatments are problematic because 100% efficacy is required and transmission  
1214 can easily occur between adjacent tanks by water or net transfer. The only tested treatment  
1215 that works consistently for stickleback gyrodactylids in our laboratory at Cardiff University is  
1216 a high concentration formalin bath (Table 4) (Buchmann and Kristensson, 2003). Other less  
1217 damaging pharmaceutical treatments for the fish, such as Praziquantil and Levamisole, are of  
1218 variable efficacy that may depend on the exact conditions of exposure, at least for this fish  
1219 species (Schelkle et al., 2009). After treatment, screening for the parasite should be  
1220 performed three times, no more than once per day, to ensure the parasite has been removed  
1221 effectively from the entire host population (see Schelkle et al., 2009).

1222

1223 Ciliated *Trichodina* spp. protists are only visible under a low powered (x10-60 mag.)  
1224 microscope (Figure 11). They appear as ‘flying-saucer’ shaped disks gliding over the body,  
1225 fins and gills of the fish. Changing tank water regularly to keep the water crystal clear  
1226 effectively eliminates most *Trichodina* spp., which feed on bacteria (Lom, 1973). If the clean  
1227 water treatment fails, which is rare, low dose malachite green treatment is usually successful  
1228 after 2-3 doses (Table 4) (Leteux and Meyer, 1972). Other infections, *G. anomala*,  
1229 *Diplostomum* spp. and the macroparasitic internal parasites are either difficult to treat, cannot  
1230 be treated or may not need treatment. *Diplostomum* spp. found in the lens and vitreous  
1231 humour may be treated with Praziquantel, although efficacy is variable and depends on  
1232 undetermined factors. *S. solidus* worms that have migrated through the intestine and into the  
1233 body cavity cannot be treated. *Glugea anomala* also cannot be cured, although some success  
1234 has been achieved in reducing spore survival using benzimidazole treatments (Schmahl and  
1235 Benini, 1998).

## 1236 **6.0 Co-infecting parasites**

1237 Despite the overwhelming tendency for wild and even commercially bred sticklebacks to be  
1238 co-infected, there is relatively little knowledge about interspecific parasite competition in  
1239 sticklebacks (Benesh and Kalbe, 2016). Parasites occupying similar niches are in direct  
1240 physical and chemical competition for resources such as nutrients and habitat (Knowles et al.,  
1241 2013). Such parasites are likely to be antagonistic and may alter their distribution on the host  
1242 in order to avoid direct competition; as is the case with co-infecting gyrodactylid species  
1243 (Harris, 1982) and co-infecting *Proteocephalus filicolis* and *Neoechinorhynchus rutili* (see  
1244 Chappell, 1969). On the other hand, parasites separated by niche may interact indirectly via  
1245 the immune system whilst simultaneously competing for host resources (Pedersen and  
1246 Fenton, 2007). Suppression or enhancement of the immune response by a parasite will then  
1247 alter the outcome of subsequent infections; changing host susceptibility and pathology,  
1248 parasite virulence and infection duration (Correa-Oliveira et al., 2002; Lively, 2005; Fleming  
1249 et al., 2006; Benesh and Kalbe, 2016). Such responses, particularly those mediated by the  
1250 immune system, may even be synergistic as immunosuppression by one parasite increases  
1251 prevalence or intensity of another (Su et al., 2005; Fleming et al., 2006; Benesh and Kalbe,  
1252 2016). There is a general lack of information on *Glugea anomala* infections and associated  
1253 immune responses and so this will not be covered here; however, given the site of infection  
1254 and the occasional severity of infection it is highly likely that this species does impact co-  
1255 infecting parasites.

1256

1257 Some parasites may be used as a ‘marker of other infections’ (where a change in prevalence,  
1258 intensity or distribution indicates an interaction between co-infecting parasites); such  
1259 relationships may be synergistic or antagonistic. The ability to track viviparous gyrodactylid  
1260 population trajectories over time, directly and non-invasively, makes them particularly useful  
1261 as a marker for the consequences of co-infection. Modulation of the immune system (Section  
1262 4.5.3) and resource competition by co-infecting parasites will alter the population trajectory,  
1263 allowing the effects of co-infection to be tracked over time. In addition, the migration of  
1264 gyrodactylids across the exterior surfaces of hosts (Harris, 1982) allows population  
1265 distribution patterns to be utilised as a method of assessing the outcome of competition  
1266 among co-infecting parasites. Such spatial positioning assessments may also be made with  
1267 other ectoparasites, such as argulids, and endoparasites, for example by considering position  
1268 in the gut (e.g. Chappell, 1969). The terminal nature of this approach with endoparasites,  
1269 however, means that such studies cannot produce the repeated measures that make  
1270 gyrodactylids so useful. Changes in the prevalence of secondary infections will also be linked  
1271 to high levels of stress or immune modulation associated with the primary infection (e.g.  
1272 Shoemaker et al., 2008; Roon et al., 2015). As such, secondary *S. parasitica* infections as a  
1273 ‘marker’ might also prove possible in the absence of the ami-momi infection technique,  
1274 particularly if the strain is virulent and the primary infection induces stress.

1275

1276 For co-infection studies where only a short period of immune regulation or infection is  
1277 required, *Diplostomum* spp. and *Argulus* spp. provide ideal models. As *Diplostomum*  
1278 migrates to the immune privileged eye it generates a short lived spike in the innate response  
1279 between 1.5 and 5 days post-infection (Kalbe and Kurtz, 2006; Scharsack and Kalbe, 2014),  
1280 after which it will no longer modulate the immune system and will not be in direct  
1281 competition with other parasite genera. Short-medium term competition and innate immune  
1282 responses can be induced by *Argulus* spp. with the period of co-infection dictated by  
1283 removing the infected individuals from the fish (see Section 4.1). The immunomodulatory  
1284 effects of *Argulus* also provide an opportunity to study the consequences of immune  
1285 suppression (Saurabh et al., 2010; Kar et al., 2015). Short-term co-infections with  
1286 *Saprolegnia parasitica* are also possible, but the usefulness of this pathogen is hindered by its  
1287 virulence and infection method.

1288

1289 Long term infections can usually be achieved with endoparasites, which – because of their  
1290 life cycles – will often provide long periods of competition with concurrently infecting  
1291 parasites and the host’s immune response. The major drawback with endoparasitic species is  
1292 an inability to accurately determine prevalence, intensity and distribution without destructive  
1293 sampling. Gastrointestinal parasites (e.g. *C. lacustris*) typically provide a sustained long-term  
1294 infection that will be in direct competition with other gastrointestinal parasites. Such  
1295 infections typically provide a long term immune response either as a result of host resistance,  
1296 tolerance or parasite induced immunomodulation (e.g. *C. lacustris*; Section 4.2.3). Being the  
1297 only species to inhabit the peritoneal cavity of the stickleback, the plerocercoid cestode *S.*  
1298 *solidus* is unique, and likely subject only to direct intraspecific competition. Once established  
1299 in the peritoneal cavity, at a mass of 50 mg, it is not possible for the fish to clear an infection.  
1300 The timing of the immunological response is therefore quite specific (Section 4.7.3); giving a  
1301 clear period of time in which the immune response could affect concurrent infections  
1302 (Scharsack et al., 2007b; Barber and Scharsack, 2010). The utility *S. solidus* is therefore  
1303 specific to its ability to induce long term competition for resources, a short term immune  
1304 resistance phenotype and a delayed response; the purpose of the delayed response is not yet  
1305 fully elucidated (Section 4.7.3).

## 1306 **7.0 Summary**

1307 With an increasing threat of disease in aquaculture and with climate change altering host-  
1308 parasite interactions a reliable model for studying these impacts has been found in the  
1309 stickleback. The stickleback provides a particularly useful model as it shares many  
1310 characteristics with economically important fish species such as salmon and trout including  
1311 its temperate habitat, omnivorous nature and evolutionary history. In depth knowledge of the  
1312 stickleback’s evolutionary history, ecology, parasitology and genetic architecture has put this  
1313 species at the pinnacle of aquatic vertebrate research. Despite this, much of the knowledge of  
1314 parasite culture techniques and treatments along with basic stickleback husbandry was  
1315 confined to older and sometimes inaccessible literature, with methods that had been updated  
1316 sporadically or that varied between different research groups. This article has brought  
1317 together expertise in the culture of sticklebacks and parasites to generate a single text that  
1318 lays out a framework of techniques for new or established laboratories that wish to begin  
1319 investigating stickleback host-parasite interactions in the laboratory, or to expand their  
1320 repertoire of available parasite models.

1321



1322 While the number of studies on the three-spined stickleback immune system is increasing,  
1323 different laboratories have focussed on different aspects: direct measurements of *ex vivo* or *in*  
1324 *vivo* phenotypic responses, MHC genetics, or gene expression measurements employing real  
1325 time PCR or RNAseq, in response to different pathogens. As a result it can be difficult to  
1326 reconcile the different approaches. For example, while we know that MHC constitution plays  
1327 a part in parasite resistance, we know little about how that translates into the active immune  
1328 phenotype that actually combats infection. Certain alleles may stimulate specific immune  
1329 phenotypes or more simply allelic diversity may lead to an overall more active immune  
1330 response. At a functional level, greater diversity of MHC alleles means different repertoires  
1331 of peptides may be presented during an immune response, leading to expansion of T- and B-  
1332 cell receptor specificities that affect the success of the adaptive response. When we begin to  
1333 take a more holistic approach to such problems it is likely that we will lift the shroud on  
1334 previously unknown aspects of the teleost immune system.

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