The three-spined stickleback - *Schistocephalus solidus* system:

an experimental model for investigating host-parasite interactions in fish

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11   SUMMARY

12   Plerocercoids of the pseudophyllidean cestode *Schistocephalus solidus* infect three-spined

13   stickleback *Gasterosteus aculeatus*, with important consequences for the biology of host fish.

14   Techniques for culturing the parasite *in vitro* and generating infective stages that can be used

15   to experimentally infect sticklebacks have been developed, and the system is increasingly

16   used as a laboratory model for investigating aspects of host-parasite interactions. Recent

17   experimental laboratory studies have focused on the immune responses of hosts to infection,

18   the consequences of infection for the growth and reproductive development of host fish and

19   the effects of infection on host behaviour. Here we introduce the host and the parasite, review

20   the major findings of these recent experimental infection studies and identify further aspects

21   of host parasite interactions that might be investigated using the system.

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24   Key words: threespine, Cestoda, Pseudophyllidea, laboratory model, growth, immunology,

25   *Ligula intestinalis*, behaviour, fitness

26

INTRODUCTION

Plerocercoids of the pseudophyllidean cestode *Schistocephalus solidus* are common parasites of three-spined sticklebacks *Gasterosteus aculeatus* in freshwater and brackish habitats throughout the geographical range of the fish. The three-spined stickleback-*S. solidus* host-parasite system has become an important model in experimental parasitology and is increasingly used to investigate a wide range of questions about host-parasite interactions and co-evolution. Here we present a review of recent studies that have used controlled experimental infections to investigate host-parasite interactions in this system.

We begin our review with background information on the parasite's lifecycle, on the host fish and the 'typical' phenotype of infected sticklebacks in nature, and briefly discuss emerging variation in infection phenotype. We then examine how aspects of the life cycle can be experimentally manipulated in the lab to allow experimental infections of sticklebacks to be undertaken. The remainder of our review focuses on how experimental infection studies have been used to illuminate host-parasite interactions in the stickleback-*S. solidus* system, including the immune responses of the fish host, the energetic consequences of infection and the consequences of infections for fish behaviour and fitness.

Life cycle of S. solidus *in nature*

Schistocephalus solidus is a trophically transmitted pseudophyllidean cestode with a three-host life cycle. The definitive host can be any warm-blooded vertebrate; most typically these are fish-eating birds though other endotherms can harbour adult worms, including otters (Hoberg *et al.*, 1997) and – though presumably only rarely – humans (Coombs and Crompton, 1991). *Schistocephalus solidus* does not grow in the gut of the definitive host but undergoes the final stages of sexual maturation there, reproducing sexually either by selfing (if singly infected) or by cross-fertilization (in multiple infections). Eggs released into the water with

the bird's faeces hatch to produce free-swimming coracidia that are transmitted trophically to a wide range of cyclopoid copepods, the 1st intermediate hosts. Here the parasites develop in the copepod haemocoel into procercoids, becoming infective to three-spined sticklebacks, the obligatory specific 2nd intermediate hosts (Bråten, 1966), with the formation of a hooked cercomer. Sticklebacks acquire infections when they feed on parasitized copepods, and in the stickleback digestive tract infective procercoids shed their outer layer, together with the cercomer, and penetrate the wall of the intestine. The parasite then develops into a plerocercoid, which grows to a large size in the fish host's body cavity. The life cycle is completed when sticklebacks harbouring infective plerocercoids are ingested by a definitive host (Clarke, 1954).

The geographical distribution of the parasite is limited by the distribution of the only obligate host in the life cycle, the three-spined stickleback, which is restricted to the Northern hemisphere and occurs around the margins of the Atlantic and Pacific Oceans (Bell and Foster, 1994). In this geographical region *S. solidus* is a regular parasite of three-spined stickleback populations inhabiting freshwater and brackish ecosystems, and is most common found in those in lacustrine or slow flowing habitats (Kennedy, 1974; Wootton, 1976; Barber, 2007).

Specificity of stickleback host

Three-spined sticklebacks are the only recognised fish host of *S. solidus*, although other *Schistocephalus* spp. infect nine-spined stickleback *Pungitius pungitius* and sculpins (Hyslop and Chubb, 1983; Chubb *et al.*, 2006; Seppala *et al.*, 2007; French and Muzzall, 2008). Experimental exposure of nine-spined sticklebacks (*Pungitius pungitius*) to infective stages of *S. solidus* led to much slower plerocercoid growth and infections were cleared after 14 days, while plerocercoids kept on growing in three-spined sticklebacks (Orr *et al.*, 1969).

77　Heterotransplants of *S. solidus* plerocercoids from *G. aculeatus* to other species of fish

78　(*Cottus gobio*, *Nemacheilus barbatula*, *Phoxinus phoxinus*, *Salmo trutta*, *Coregonus*

79　*clupeoides*, *Perca fluviatilis*, *Rutilus rutilus*, *Esox lucius*), including *P. pungitius*, died within

80　2-10 days after transfer, while homotransplants between *G. aculeatus* survived (Bråten 1966).

81　These observations indicate that in principle *S. solidus* plerocercoids can be cleared by a fish

82　immune system, but obviously *S. solidus* plerocercoids are able to avoid an effective immune

83　response in *G. aculeatus*, their specific second intermediate host.

84

85　*Sticklebacks as experimental model hosts*

86　A major attraction of the stickleback-*S. solidus* host-parasite system for ecological and

87　evolutionary biologists is the rich history of studies investigating the natural history,

88　behaviour and evolutionary biology of the host fish, and a correspondingly substantial

89　literature that has been regularly and thoroughly reviewed (Wootton, 1976; Wootton, 1984;

90　Bell and Foster, 1994; Östlund -Nilsson *et al.*, 2006). This background permits a wide range

91　of ecologically and evolutionarily relevant questions to be addressed. Furthermore, the three-

92　spined stickleback has, in recent years, assumed even more importance as a model species in

93　biology; the publication of linkage and chromosome maps (Peichel *et al.*, 2001; Kingsley *et*

94　*al.*, 2004) and the sequencing of its genome (Kingsley, 2003) has greatly enhanced its utility

95　in molecular studies of evolution and development (McKinnon *et al.*, 2004; Shapiro *et al.*,

96　2004; Colosimo *et al.*, 2005; Gibson, 2005; Shapiro *et al.*, 2006). Sticklebacks are also readily

97　bred in laboratory aquaria (Barber and Arnott, 2000), facilitating the challenge of naïve

98　individuals and thus fulfilling both scientific and local ethical requirements for experimental

99　infection studies.

100

101　*Field studies of* S. solidus *infected sticklebacks*

102   A number of studies have examined *S. solidus* infection prevalence and intensity in natural

103   stickleback populations, and the phenotype (including the appearance, energetic condition,

104   reproductive capacity and behaviour) of naturally infected sticklebacks has been well

105   documented. Observations on the phenotype of infected fish from populations where *S.*

106   *solidus* is endemic are summarised in Table 1. The proportion of fish harbouring infections

107   can be extremely high, in some cases approaching 100% (Dick, 1816; Smyth, 1947; Hopkins

108   and Smyth, 1951), but this varies considerably between populations (MacColl, 2009), and

109   temporally within them (Chappell, 1969). Typical features of classical 'schistocephalosis'

110   include characteristic distension of the fish's abdomen, an altered swimming gait, increased

111   risk-taking behaviour, reduced body condition and functional (if not physiological) castration.

112   However, as more host populations are studied it is becoming clear that there is significant

113   variation in infection phenotype, and there are a number of exceptions to these 'typical'

114   infection phenotypes. Notably, intensive studies of some Alaskan populations suggest that the

115   traditional view of *S. solidus* as an absolute castrator of female sticklebacks may need to be

116   revised (Heins and Baker, 2008), and in a small number of populations infection is

117   additionally associated with almost complete demelanisation (Lobue and Bell, 1993; Ness and

118   Foster, 1999).

119

120   In vitro *culture of* S. solidus

121   Pioneering work by the parasite physiologist J.D. Smyth in the 1940-50s developed protocols

122   for the *in vitro* culture of adult helminths, including *S. solidus* (Smyth, 1946; Smyth and

123   McManus, 1989; Smyth, 1990). In brief, plerocercoids recovered from infected sticklebacks

124   are removed and placed into sterilised culture vessels containing a buffered medium that

125   provides a suitable physico-chemical environment for parasite development (such as horse

126   serum and / or a cell culture medium such as RPMI-1640). Antibiotics may be added to

reduce contamination. The plerocercoid(s) are not placed directly into the medium but are instead constrained within narrow diameter semi-permeable dialysis tubing, which mimics the small intestine of the definitive host and provides the constriction needed for successful fertilisation (Smyth, 1990). Culture vessels are placed into a water bath set to 40ºC and shaken gently to aid the dissolution of metabolic products from developing worms. Due to the progenetic development of pseudophyllidean plerocercoids, sexual maturation occurs rapidly and within 48h the adult worms begin producing eggs. The fact that *S. solidus* attains its final size in the fish body cavity and matures so rapidly to the adult form made it possible for the first time to maintain adult cestodes under sterile experimental conditions and undertake detailed physiological studies. Such studies had been impossible with most other tapeworm species, which typically grow and mature over prolonged time periods in the host intestine, and *S. solidus* became an extremely valuable model in parasite physiology (Smyth and McManus, 1989).

Using Smyth's techniques – or slightly modified versions of them – large numbers of eggs can be generated and hatched to yield coracidia, which in turn can be used to infect lab-bred copepods (Wedekind, 1997). After a period of development to the infective cercomer-bearing stage, procercoids can be fed to sticklebacks inside infected copepods, either by gavage or by natural feeding, to generate experimentally infected fish hosts and allowing detailed experimental studies of fish-parasite interactions.

MECHANISMS OF RESISTING *S. SOLIDUS* INFECTION: HOST BEHAVIOUR, IMMUNE RESPONSES AND HOST MANIPULATION

Animals have evolved three major types of mechanisms to avoid, or reduce the likelihood of developing, debilitating parasite infections; behavioural mechanisms that limit contact with

152   infectious agents, physical barriers to invasive stages and immune systems. The ability to

153   experimentally infect intermediate hosts in large numbers means that it is possible to examine

154   the responses of hosts to controlled, experimental challenge. In recent years, experimental

155   infection studies have been used to examine both behavioural and immunological aspects of

156   stickleback responses to infective stages of *S. solidus*.

157

158   *Behavioural resistance*

159   The strong selection pressure placed on host organisms to avoid debilitating parasite

160   infections, together with the typically high costs of mounting immune responses against

161   invading pathogens, has led to the evolution of a wide range of strategies of behavioural

162   resistance in animals (Hart, 1990; Hart, 1992; Hart, 1997). For parasites that are transmitted

163   trophically between hosts, avoiding infected prey intuitively reduces the level of exposure to

164   infection, but this requires that infected prey are identifiable, and that the benefits of avoiding

165   parasitized prey outweigh the costs of ignoring them. In some cases, feeding on parasitized

166   prey that are easy to catch may benefit potential hosts if the risks of becoming infected, and/or

167   the costs of infection, are low (Lafferty, 1992).

168   Copepods infected with procercoids of *S. solidus* and other related pseudophyllidean cestodes

169   behave differently to those that are non-infected, providing the potential for avoidance by

170   discriminating fish. However, the behaviours that are altered by infection – which include

171   activity patterns, swimming ability and responses to disturbance – make infected copepods

172   more susceptible to human 'predators' armed with pipettes (Pasternak *et al.*, 1995; Urdal *et*

173   *al.*, 1995; Wedekind and Milinski, 1996) and so potentially make them more visible to, and /

174   or more easily caught by, fish predators. Infected copepods have also been reported to

175   actively approach sticklebacks (Jakobsen and Wedekind, 1998). It is therefore likely that

176   behaviour changes in infected copepods are adaptations of the parasite to facilitate

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177　transmission (Parker *et al.*, 2009), and recent evidence examining temporal aspects of their

178　behaviour change supports this (Hammerschmidt *et al.*, 2009; Hammerschmidt and Kurtz,

179　2009). So do sticklebacks avoid eating infected copepods? The results of two critical

180　experimental tests suggest that sticklebacks have no behavioural defence against *S. solidus*,

181　and may even feed on infected copepods preferentially (Urdal *et al.*, 1995; Wedekind and

182　Milinski, 1996). However, there is still further work to be done in this area. One possibility

183　for the apparent non-evolution of avoidance behaviour is that sticklebacks are simply unable

184　to discriminate infected from non-infected copepods, so avoiding infection would mean

185　excluding an important prey type, which is just too costly. Also, because few tests have been

186　carried out, it is not known whether all populations are equally non-discriminating, or whether

187　some populations have evolved to be more selective in their prey choice. Further, individual

188　sticklebacks are known to vary in key personality traits (Bell and Stamps, 2004; Bell, 2005)

189　so are all individuals within populations as likely as others to approach infected copepods, or

190　do fish exhibiting particular personality types suffer increased exposure? Because the

191　probability of acquiring infections after feeding appears to be relatively high (at least in

192　laboratory studies, see below), and the consequences of infections are typically severe, it

193　seems unlikely that feeding on more easily caught infected copepods could be beneficial to

194　sticklebacks. However, it is possible that wild fish develop better immune responses than

195　those reared under sterile laboratory conditions and face a lower risk of infection per infective

196　stage ingested, reducing the pressure to evolve discrimination (see also Hammerschmidt and

197　Kurtz, 2009).

198　The presence of debilitating parasites in the environment can also drive the evolution of mate

199　preferences, either by the avoidance of mates harbouring directly transmissible parasites, or

200　by the selection of individuals with genes that confer parasite resistance on offspring (Keymer

201　and Read, 1991; Andersson, 1994). In sticklebacks, preferences for males with the brightest

202　red nuptial colouration have evolved at least in part as a mechanism for avoiding parasitised

males (Milinski and Bakker, 1990; Bakker and Milinski, 1991). To test the hypothesis that females selecting brightly ornamented males gain indirect genetic benefits by producing more resistant offspring, Barber *et al.* (2001) produced clutches of maternal half-sibling fish that differed only the brightness of the male parent, and exposed them to infective *S. solidus* procercoids. The results showed that male brightness significantly affected the proportion of half-sibships that developed infections, with brighter males producing the most resistant offspring, suggesting indirect benefits of ornamentation based mate choice.

Dynamics of S. solidus *transmission from copepods to sticklebacks*

Interactions of *S. solidus* with its first intermediate copepod host were recently reviewed in detail by Hammerschmidt and Kurtz (2009). With no strong evidence that sticklebacks are capable of adopting behaviours to avoid ingesting infected copepods, those in populations with endemic infection are likely to be exposed regularly to infective *S. solidus* procercoids. After the ingestion of a *S. solidus* infected copepod the prey is digested in the stomach and procercoids are released from copepod tissues. Procercoids retain their outer layer – which is rich in PNA-binding sugars (GalNac, D-galactose) – in the stickleback stomach, and presumably this protects the parasite from enzymatic digestion (Hammerschmidt and Kurtz, 2007). During passage through the stomach, the outer layer is shed (or digested) together with the cercomer and in the intestine the underlying tegument with microtriches is exposed. Typically for vertebrate cell surfaces this cuticle is rich in sialic acid residues, which may help the parasite evade the stickleback's immune system (Hammerschmidt and Kurtz, 2005). From the intestine, the parasites penetrate the gut wall and enter the body cavity.

The period from ingestion to establishment in the stickleback body cavity is critically important in determining infection success, with 50-75% of ingested parasites failing to complete this phase (Hammerschmidt and Kurtz, 2007). In an experimental infection

228   experiment, the majority (>90%) of parasites recovered from sticklebacks were alive in the

229   stomach 16h post-exposure (p-e). After 22h p-e, approximately 40% were still alive in the

230   stomach, 40% had entered the body cavity and about 20% of parasites recovered (by

231   dissection and histological analysis) were dead. By 24h p-e, the majority (>90%) of detectable

232   parasites were alive in the body cavity whereas dead or damaged parasites were no longer

233   detectable, presumably due to progressing degradation in the digestive tract (Hammerschmidt

234   and Kurtz, 2007). This indicates that parasites are vulnerable to the aggressive environment in

235   the digestive tract after losing their outer layer, and progression to the intestine and passage

236   through the gut wall must be achieved quickly to avoid significant losses in viability. In terms

237   of preventing *S. solidus* establishment, the relative contribution of hostile conditions in the

238   fish digestive tract and the host immune system is difficult to estimate; however, no

239   attachment of phagocytic cells to, or encapsulation of, *S. solidus* stages during gut wall

240   penetration or in the body cavity was observed (Hammerschmidt and Kurtz, 2007).

241   There is little evidence yet for a prominent reduction of *S. solidus* viability once the body

242   cavity is reached (i.e. clearance by the immune system). In another infection experiment,

243   relatively few dead parasites (n = 4) were found in the body cavity of infected sticklebacks at

244   7d and 17d p-e, while 78 *S. solidus* plerocercoids were recovered alive after these times

245   (Scharsack *et al.*, 2007). During this experiment, infection rates changed from >60% at 7d and

246   17d p-e to approximately 50% after 27-67d p-e (Scharsack *et al.*, 2007). This suggests that

247   while clearance of *S. solidus* plerocercoids in the body cavity is possible during early stages

248   of infection it occurs less frequently later on.

249   Stimulation of immunity soon after *S. solidus* infection seems to reduce the infection success

250   of *S. solidus*. Wedekind & Little (2004) triggered activation of the host immune system by

251   tissues injury through spine clipping at 7d p-e to *S. solidus*. At 90d p-e, the spine-clipped

252   sticklebacks showed significantly lower infection rates compared to controls without spine

253 clipping (Wedekind and Little, 2004). At which time plerocercoids were cleared was not

254 recorded in this study, but the results might indicate that immune stimulation was most

255 efficient in the early (1-2w) stage of infection, when *S. solidus* is still vulnerable to immune

256 attack.

257

258 *Lymphatic organs and leukocytes during S. solidus infection* Responses from different

259 immunologically active organs have been recorded in sticklebacks infected experimentally

260 with *S. solidus*. The spleens of *S. solidus* infected sticklebacks were enlarged compared to non

261 infected fish (Arnott *et al.*, 2000). Enlargement of the spleen is often observed during parasite

262 infections of fish, e.g. in common carp *Cyprinus carpio* infected with the blood flagellate

263 *Trypanoplasma borreli*, due to proliferation of leukocytes and increased amounts of antigen-

264 antibody immune complexes, which are removed from the blood stream by spleen

265 macrophages (Bunnajirakul *et al.*, 2000). In the stickleback-*S. solidus* system, specific reasons

266 for the enlargement of spleens are to date unclear and await further investigation. In the blood

267 of *S. solidus* infected sticklebacks, distinct changes of leukocyte subsets have been observed.

268 Early in the infection the proportion of granulocytes increased, while the proportion of

269 lymphocytes decreased in the peripheral blood, with both trends levelling out after 60-96d p-e

270 (Scharsack *et al.*, 2004). The offspring of more brightly ornamented male sticklebacks

271 showed elevated white blood cell counts and were less susceptible to *S. solidus* infection

272 (Barber *et al.*, 2001). These observations suggest that peripheral blood leukocytes may indeed

273 play a role in the *S. solidus* infection, but the underlying mechanisms are not yet well

274 understood. Most information about leukocyte responses to *S. solidus* has been generated

275 from experiments with stickleback head kidney leukocytes (see later). To date, limited

276 information is available on the interplay of lymphatic tissues during *S. solidus* infection, in

277 particular the role of (cellular) immune defence at the site of infection, the body cavity.

12

However, because the teleost head kidney is a site of antigen presentation, leukocyte

activation, proliferation and maturation - and consequently interacts closely with

immunological activity in the periphery (Manning, 1994; VanMuiswinkel, 1995) -

information derived from head kidney leukocyte studies can be regarded as representative for

immune activity in the periphery, even if specific interactions at the site of infection might

remain concealed.

Cellular innate immunity

Respiratory burst and monocyte proliferation The respiratory burst activity of head kidney

leukocytes (HKL) is one of the most important mechanisms of cellular innate immunity, so

may be expected to be up-regulated at an early stage in exposed sticklebacks. However, the

HKL respiratory burst from *S. solidus* exposed sticklebacks 7-37d after an experimental

challenge did not differ from that of sham-exposed controls, suggesting the mechanism is not

important in early defence against infection (Scharsack *et al.*, 2007). Interestingly, the

respiratory burst of HKLs was up-regulated from 47-67d p-e, but as neither the survival nor

the growth rates of *S. solidus* plerocercoids were affected during this period this appears to be

an ineffective defence mechanism.

Nevertheless, the proliferation of head kidney monocytes – a component of the cellular innate

immune response – was up-regulated among exposed sticklebacks at 7d p-e, suggesting that

the mobilisation of monocytes could play a role in early defence against *S. solidus*. Among

fish that developed infections, monocyte proliferation dropped then below sham-exposed

controls at 17 d p-e, recovered and dropped again, indicating possible immune-manipulation

by *S. solidus* (Scharsack *et al.*, 2007) (see later). Interestingly the kinetics of monocyte

proliferation in exposed fish that did not develop infections followed a similar pattern,

suggesting an early priming of monocyte responses.

303

Monocyte manipulation? The idea that *S. solidus* is capable of substantially manipulating

stickleback monocyte responses is supported by *in vitro* experiments. Monocytic leucocytes

(granulocytes and macrophages) isolated from the head kidney of experimentally infected

sticklebacks at 45d p-e failed to respond to *S. solidus* antigens *in vitro* (Scharsack *et al.*,

2004). This was not a general anergy, as monocytes from the same sticklebacks responded to

stimulation with a non-specific antigen (poke weed mitogen, PWM) in a comparable manner

as cells from sham-exposed controls (Scharsack *et al.*, 2004). Thus *S. solidus* does not appear

to immune-compromise its stickleback host, but is apparently capable of manipulating

(evading) immune traits that are specifically directed against parasite antigens.

313

Immune priming and susceptibility Priming of the immune system by *S. solidus* does not

induce resistance in *G. aculeatus*, as super infections are possible by sequential exposures (i.e.

there is no 'vaccination effect'). Experimentally infected nine-spined sticklebacks (*Pungitius*

pungitius) have been shown to reject *S. solidus* plerocercoids more rapidly after pre-exposure

to the parasite (Orr *et al.*, 1969). However, *S. solidus* plerocercoids were not able to survive in

P. pungitius longer than 14d (Orr *et al.* 1966). Detailed analysis of infections harboured by

three-spined sticklebacks that had been sequentially exposed to *S. solidus* showed that

plerocercoids from later exposures survived better and grew larger than 'pioneering' worms

(Jäger and Schjørring, 2006). These results include exposures where only the secondary *S.*

solidus survived and may be explained by the first invading worm paying higher costs of

immune manipulation / priming (Jäger and Schjørring, 2006).

These findings, together with the observation that priming of monocytes is detectable in

exposed sticklebacks that do not develop infections (Scharsack *et al.*, 2007) and the loss of

responsiveness of monocytes to (secondary) *in vitro* exposure to *S. solidus* antigens

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328 (Scharsack *et al.*, 2004), suggest that *S. solidus* has a strong impact on the stickleback

329 immune system. Immune priming, initiated to protect the invading parasite from host immune

330 attack, seems to be so efficient that it persists even if the first invader is cleared, facilitating

331 the establishment of subsequent infections.

332 To what extent immune priming by *S. solidus* can influence susceptibility of sticklebacks to

333 other parasites has not yet been experimentally investigated. In populations with endemic *S.*

334 *solidus* infection, fish harbouring plerocercoids tend to be more heavily infected by

335 *Gyrodactylus* sp. parasites than those free from *S. solidus* (M. Kalbe, personal

336 communication). Experimental exposure of *S. solidus* infected sticklebacks to other parasites

337 could reveal the extent to which *S. solidus* can influence susceptibility to additional parasites.

338

339 *Adaptive immunity*

340 Clearance of *S. solidus* infection seems to depend mainly on an early innate immune response,

341 potentially facilitated by previous exposure of the parasite to the aggressive environment in

342 the digestive tract. An adaptive immune response - including the presence of specific

343 antibodies - would need about 2-3 weeks to be fully in place in fish maintained at 18°C

344 (Rijkers *et al.*, 1980). Thus substantial involvement of antibody-mediated immunity in early

345 defence against invading *S. solidus* is unlikely, and since clearance of infection at later stages

346 (beyond 17d p-e) was not observed, antibody-mediated responses to *S. solidus* infection are

347 not expected to make a significant contribution. However, due to the lack of specific tools

348 such responses have not yet been fully investigated.

349

350 *Lymphocyte activation* Nevertheless, in a kinetics study of immune parameters following

351 exposure to *S. solidus*, the proliferation of lymphocytes (B- and T-cells) was measured in

352 head kidney isolates. The clonal expansion and proliferation of lymphocytes forms a

353 significant component of the adaptive immune response and is expected 1-4 weeks after

354 infection. In *S. solidus* infected sticklebacks, significant changes in lymphocyte proliferation,

355 compared to sham exposed controls, were only observed among exposed fish that did not

356 develop infections. Among these fish, lymphocyte proliferation was elevated at 7d p-e,

357 dropped below controls at 17d p-e before returning to control values from 27 to 67d p-e

358 (Scharsack *et al.*, 2004). (A less prominent and statistically non-significant pattern was

359 recorded among sticklebacks that developed infections). The pattern of lymphocyte

360 proliferation among exposed sticklebacks that did not become infected suggests a possible

361 role in defence against *S. solidus*. Since lymphocyte proliferation dipped below controls at

362 17d p-e, B-cell proliferation and production of antibodies (T-helper cell 2 - Th2 mediated

363 humoral immunity) is unlikely. Early lymphocyte proliferation might alternatively be

364 explained by the proliferation of T-cells, maintaining a Th1 response that activates cellular

365 immunity. This corresponds to the observation that monocyte proliferation was regulated

366 contemporarily (see Scharsack *et al.* 2007).

367

368 *Potential role of the Th1-Th2 system?* In mammals, helminth parasites are considered a

369 classical inducer of Th2 responses, which have the potential to damage parasites and clear

370 infections (Maizels and Yazdanbakhsh, 2003; Wang *et al.*, 2008). However, the nature of

371 interactions between helminth parasites and the Th1-Th2 system remains controversial

372 (Maizels and Yazdanbakhsh, 2003); for example, schistosomes appear to have evolved

373 immune evasion strategies in which the Th1-Th2 system is driven towards a Th1 response,

374 thereby avoiding humoral responses (Herve *et al.*, 2003).

375 Information on Th1-Th2 mediated immune function in teleost fish is scarce, but molecular

376 studies indicate that the Th1-Th2 system is at least present (Takizawa *et al.*, 2008a; Takizawa

377 *et al.*, 2008b). The available information does not point towards a typical Th2 response in *S.*

378   *solidus* infected stickleback, as lymphocyte proliferation after an initial weak increase

379   remained unaffected (Scharsack *et al.*, 2007) and degenerative changes at the surface of

380   procercoids (as a result of a Th2 induced humoral response) were not detected by means of

381   electron microscopy (Orr *et al.*, 1969). The extent to which protection against *S. solidus*

382   infections in stickleback hosts might involve a Th1 response (or an abrogated Th2 response)

383   therefore requires further investigation.

384

385   *The MHC and influences on plerocercoid growth* Overall, adaptive immunity seems unlikely

386   to protect three-spined sticklebacks from *S. solidus* infection, but there is evidence that it can

387   restrict parasite growth during ongoing infection. Proteins of the major histocompatibility

388   complex (MHC) play a central role in presenting antigens to the adaptive immune system.

389   Using three-spined sticklebacks that varied in their individual MHC class IIB allelic diversity,

390   Kurtz *et al.* (2004) observed that *S. solidus* grew larger in sticklebacks with low and high

391   MHC diversity compared with those having an intermediate number of MHC alleles. The

392   underlying molecular mechanism is unknown, but these results support observations that

393   sticklebacks with intermediate (optimal) MHC IIB diversity suffered less from parasite

394   infections compared to fish with high and low (suboptimal) MHC IIB diversity (Wegner *et*

395   *al.*, 2003).

396

397   *Summary: innate and adaptive immunity*

398   Clearance of *S. solidus* by the immune system of its specific second intermediate host, the

399   three-spined stickleback, appears to be the exception rather than the rule. Damage to the

400   parasite by the aggressive gut environment might reduce the infection success at least as

401   prominently as attack by the immune system. *S. solidus* does not appear to be very vulnerable

402   to immune attack, but rather appears to be capable of substantial immune evasion and

403 manipulation. The typically rapid death of plerocercoids following experimental transfer to

404 fish species other than three-spined sticklebacks (Bråten, 1966; Orr *et al.*, 1969) strongly

405 suggests that fish immune systems can, in principle, clear *S. solidus* infections. It therefore

406 seems most likely that specific adaptation of *S. solidus* to the immune system of the three-

407 spined stickleback permits its invulnerability to host immune responses. From an evolutionary

408 perspective, adaptation to a host immune system is costly and balancing selection on *S.*

409 *solidus* has resulted in an extremely high degree of specialisation towards a three-spined

410 stickleback host.

411 The results on immune responses of stickleback against *S. solidus* described here are mainly

412 derived from laboratory experiments. In the wild, *S. solidus* infection success and

413 development in sticklebacks might be constrained by factors acting on the host immune

414 system, such as activation of the immune system by pre-exposure to other parasites, and by

415 other environmental stressors, both natural and anthropogenic.

416

417 IMPACTS OF EXPERIMENTAL *S. SOLIDUS* INFECTIONS ON HOST ENERGETICS

418 AND BEHAVIOUR

419 *Schistocephalus solidus* infections are expected to impact host energetics and behaviour of

420 host sticklebacks for two main reasons. First, plerocercoids grow to a large size and – because

421 the nutrients to fuel this growth are entirely host-derived – this incurs a considerable energetic

422 burden on host fish (Walkey and Meakins, 1970; Lester, 1971). Second, the parasite relies on

423 the ingestion of the stickleback host to complete its life cycle, facilitating the evolution of

424 parasite adaptations that increase the predation risk of host sticklebacks. A number of studies

425 have quantified infection-associated variation in stickleback energetics and behaviour among

426 fish from naturally infected populations. Experimental infection studies allow a number of

427 fitness correlates to be measured under standardised conditions.

428

Effects of experimental infections on host energetics, growth and sexual development

Laboratory investigations of the impact of *S. solidus* on the growth and development of stickleback hosts have been the subject of a recent review (Barber *et al.*, 2008), so here we provide an overview of the major findings of these studies and outline future approaches and potentially research questions.

In naturally infected populations, fish infected with *S. solidus* typically exhibit low growth and poor body condition, and as a result – in most studied populations at least – they suffer reduced sexual development and are unlikely to participate successfully in spawning (Arme and Owen, 1967; Pennycuick, 1971; Tierney *et al.*, 1996; Bagamian *et al.*, 2004; Heins and Baker, 2008). However, when naturally or experimentally-infected fish are maintained under laboratory conditions such effects are less frequently observed, often because ethical guidance on animal husbandry requires fish to experience benign environments, with access to abundant, high quality food. The growth and energetic condition of infected fish can even exceed that of non-infected individuals under certain types of lab housing.

443

Experimental studies of fish held under benign conditions Barber & Svensson (2003) experimentally exposed lab-bred juvenile sticklebacks to single infective procercoids and held them under a constant host ration of 8% body mass per day. Following, the length of infected sticklebacks followed approximately the same trajectory of non-exposed, control fish over the 16-week p-e period. The mass of infected fish (including plerocercoid mass) also followed a similar trajectory to controls, with infected fish actually showing elevated growth rates during weeks 5-7 p-e. However, when the mass contributed by developing plerocercoids was removed, the trajectory of mass increase of infected fish clearly differed from that of controls, and infected fish weighed significantly less at the end of the experiment. On dissection,

19

453   infected females were found to have equivalent liver mass, but lower perivisceral fat reserves

454   and, surprisingly, larger ovaries than non-exposed control fish. One explanation for the

455   counterintuitive investment in gonad development is that it may reflect a life history change

456   that could compensate for the likely reduction in survival associated with infection

457   (Minchella, 1985).

458   Other studies under similarly benign conditions have recorded a similar lack of detectable

459   impact of the parasite on host growth. In a recent infection experiment sticklebacks were fed

460   *ad libitum* with frozen chironomids 3 times a week. Here, the mass of infected stickleback

461   including parasite mass was significantly higher at 57 and 67d p-e compared to controls, but

462   equally high with parasite mass subtracted (Koch, Scharsack, Hammerschmidt, unpublished

463   data). In a study by Arnott *et al.* (2000), experimentally infected fish were held individually

464   and fed *ad libitum* to excess each day. Under these conditions, infected sticklebacks outgrew

465   non-infected fish, weighing significantly more than the latter at the end of the study even

466   when correcting for plerocercoid mass. Infected female fish (though not males) held under

467   these conditions also developed significantly larger livers relative to their body size, and they

468   had an equivalent amount of perivisceral fat to fish that did not develop infections after

469   exposure.

470

471   *Experimental studies of fish held under more naturalistic conditions* The results describesd

472   above suggest that the feeding regime experienced by hosts has considerable influence on the

473   energetic costs of infection experience by host fish, and hence the phenotype exhibited by *S.*

474   *solidus* infection. Synthesising the results from a number of lab studies, Barber *et al.* (2008)

475   showed that infection phenotypes more closely reflecting those found in natural populations,

476   were more commonly found when experimentally infected fish are reared under less benign

477   conditions in the lab. For example, when housing exposed and non-exposed sticklebacks

together in groups, effectively forcing competition between infection classes, Barber (2005) found the relative liver mass (hepatosomatic index, HSI) to be significantly reduced among experimentally-infected, compared to sham-exposed, females. Wright *et al* (2007) examined the effect of temporary food restriction on the growth and energetics of sticklebacks experimentally infected with *S. solidus*. In contrast to sham-exposed sticklebacks – which undertook rapid compensatory growth on commencement of *ad libitum* feeding to catch up to the mass of continually-fed fish after only three weeks of re-feeding – experimentally infected sticklebacks showed only partial compensation, reaching just 80% of the mass of continually-fed infected fish after six weeks of re-feeding. Infected fish reared under the compensatory regime also developed smaller livers than sham-exposed 'compensatory' fish, whereas infection status did not affect liver size among fish held under a continual feeding regime. Analysis of the food intake of individual fish revealed that the likely cause of the inability of infected fish to compensate was their failure to mount significant hyperphagic responses post-deprivation (Wright *et al.*, 2007). A subsequent study confirmed that the maximum voluntary meal size of infected fish was reduced in infected sticklebacks (Wright *et al.*, 2006). Because fish in natural environments, with temporally unpredictable food availability, are expected to rely heavily on compensatory growth responses, the inability to undertake such responses may exacerbate the growth effects of *S. solidus* and represent a hitherto 'hidden cost' of infection.

The goal of laboratory investigations of the stickleback-*S. solidus* system is generally to better understand the selective role that parasites play in nature, so it is becoming increasingly clear that investigating the growth and development effects of *S. solidus* in lab studies presents certain challenges. However, at the same time there is an urgent need to better understand how parasites and hosts interact under altered environments. A possible way in which laboratory studies of the stickleback-*S. solidus* system could contribute considerably to our understanding of host-parasite interactions in nature is to investigate the role of variation in

504   the rearing environment experienced. To date few studies have systematically investigated

505   such effects, but the effects of factors such as temperature, food availability and other

506   environmental stressors (including pollutants) could readily be examined in an experimental

507   framework.

508

509   *Behavioural effects of infection*

510   *Infection associated behavioural variation among wild-caught fish* A number of authors have

511   compared the behaviour of wild caught sticklebacks naturally infected with *S. solidus* with

512   non-infected fish from the same population. These studies have identified a wide range of

513   behaviours in which individual variation is associated with infection status, including shoaling

514   behaviour (Barber and Huntingford, 1995; Barber *et al.*, 1995; Barber *et al.*, 1998)

515   antipredator and risk-taking behaviour (Giles, 1983; Milinski, 1985; Giles, 1987b; Giles,

516   1987a; Godin and Sproul, 1988; Tierney *et al.*, 1993; Ness and Foster, 1999), prey choice

517   (Milinski, 1984; Ranta, 1995) and competitive ability (Barber and Ruxton, 1998). In many

518   cases behaviour studies are carried out to investigate hypotheses about the basis of altered

519   behaviour, and specifically whether they may constitute examples of host 'manipulation' by

520   parasites (Poulin, 1994). However although the results of these studies often suggest adaptive

521   manipulation by parasites, such an approach can only ever produce correlational data, as

522   infection status is not imposed experimentally. Alternative explanations, including the

523   possibility that pre-existing behavioural variation influences exposure to infections, or

524   underlying 'quality' factors that impact both susceptibility to infection and behaviour, mean

525   that experimental infection studies are needed to unambiguously assign causality.

526

527   *Behaviour change in experimentally infected fish* In contrast to studies of wild-caught,

528   naturally infected sticklebacks, relatively few have examined the behaviour in experimentally

529   infected sticklebacks. Aeschlimann *et al.* (2000) tested the risk taking behaviour of

530   experimentally infected sticklebacks under threat of predation by pike *Esox lucius* during the

531   early phase of infection before the parasite was infective to the definitive host. The aim of the

532   study was to examine whether experimentally infected fish increased their risk-taking

533   behaviour in order to maximise food intake, to reach sexual maturity early in an attempt to

534   reduce the fitness impacts of infection. The results showed that during these early stages of

535   infection, when host behaviour was predicted to reflect host responses to infection rather than

536   being influenced by 'manipulative' parasites, there was no effect of infection status on the

537   propensity to taking risks whilst foraging. This suggests that infected fish do not respond to

538   infection by exploiting risky yet available prey, and is consistent with the finding that

539   increased food intake actually appears to benefit parasites as well as hosts (Barber, 2005).

540   Studies of naturally and experimentally infected sticklebacks suggest that reduced predator

541   avoidance behaviour is typically shown when fish harbour either a high burden (parasite

542   index > 25%) of *S. solidus* (Milinski, 1984; Milinski, 1985) or when parasite mass exceeds

543   50mg (Tierney *et al.*, 1993), but not during early stages of infection (Aeschlimann *et al.*,

544   2000). Given the fact that 50mg appears to be the threshold mass for successful production of

545   fertile eggs in the avian host (Tierney and Crompton, 1992), these observations are consistent

546   with adaptive manipulation of behaviour. To examine this more closely, Barber *et al.* (2004)

547   experimentally infected juvenile sticklebacks and used image analysis to track parasite growth

548   alongside behavioural analysis of host escape responses over a 16 week p-e period. Reduced

549   antipredator behaviour responses to a heron model were only observed in experimentally

550   infected fish once plerocercoids had reached an estimated mass of 50mg, corroborating

551   observational studies of the behaviour of naturally infected stickleback. This was the first

552   study to use experimentally infected sticklebacks to demonstrate that *S. solidus* was

553   responsible for the observed changes in behaviour.

554

555 *Potential for laboratory artefacts in behavioural studies* However, extrapolating results from

556 experimental lab studies to the field situation may again be difficult, as benign laboratory

557 conditions can also affect the behaviours exhibited by *S. solidus* infected sticklebacks.

558 Candolin and Voigt (2001) captured nest-holding males from a population in which 26% of

559 males harboured *S. solidus* and showed that nest holders were almost exclusively (33/35) non-

560 infected. They then transferred naturally infected fish to the lab, and found that after a 7d

561 period of *ad libitum* feeding, with access to nesting territory and materials, most infected fish

562 readily built nests and courted females. *Schistocephalus solidus* therefore appears to have

563 influenced reproductive performance of males in this population indirectly, by reducing the

564 ability of host sticklebacks to gain access to resources (food, territory, nesting material etc)

565 essential for successful reproduction. There also appears to be population variation in the

566 capacity to reverse the effects of infection under lab housing, with naturally infected males

567 from different populations being differentially capable of reproductive behaviour following a

568 period of benign housing (Rushbrook and Barber, 2006; Macnab *et al.*, in press).

569

570 *Manipulation of host behaviour: potential role of the immune system*

571 Reduced predator avoidance behaviour in the stickleback-*S. solidus* system is thought to be

572 caused by increased concentrations of monoamine neurotransmitters in neuronal tissues of the

573 brain in *S. solidus* infected sticklebacks (Øverli *et al.*, 2001). Whether the neuronal changes

574 are a consequence of changes in energy or endocrine status of the fish or are induced directly

575 by the parasite, for instance by the release of a neuroactive substance is unclear. However,

576 changes in neuroendocrine status are consistent with a chronic stress response in infected fish,

577 which could be, among other stressors, the result of an immune response (Øverli *et al.*, 2001).

Investigations of immune kinetics in *S. solidus* infected sticklebacks, detailed in section 2.3

above, reveal distinct changes in immune parameters during the period of parasite growth that

corresponds with host behaviour change, when the parasites had passed the 50mg threshold

weight between 40-60d p-e. Nonetheless, effects on parasite survival and fitness were not

observed and plerocercoids kept on growing to attain 150mg at 67d p-e (Scharsack *et al.*,

2007) and about 200mg by 98d p-e (Scharsack *et al.*, 2004). As immune responses are

presumably very costly for the host, but do not appear to have any effects on the parasite

survival at this late stage of infection, one possible explanation for the observed pattern is that

S. solidus – on attaining an infective size – triggers the stickleback immune system (in a

'controlled' manner that is not harmful for the parasite) to interfere with the crosstalk between

neuro-endocrine system and immune system to induce reduced predation avoidance behaviour

of its stickleback host (Scharsack *et al.*, 2007). A second explanation could be that *S. solidus*

interacts directly with the neuro-endocrine system and that the observed changes in immune

parameters are side effects of a host stress response. However, stress responses generally

result in a broad (ubiquitous) activation of immunity, to which *S. solidus* is apparently

vulnerable (Wedekind and Little, 2004; see above). The survival and growth of *S. solidus*

plerocercoids relies on their ability to control the stickleback immune system, which would be

more costly to maintain in a stress-induced activation of several immune traits, instead of

single immune traits manipulated specifically by the parasite.

Due to the complex interactions between immunity and nervous system, it is difficult to

distinguish whether a parasite directly or indirectly manipulates its host behaviour (Milinski,

1990; Adamo, 2002; Thomas *et al.*, 2005). In mammals the crosstalk between the immune

response and brain is known as acute sickness behaviour, where behavioural changes that are

associated with acute infections are typically immunologically mediated (Vollmer-Conna,

2001). For the *S. solidus* infection of sticklebacks, the exact mechanism responsible for

translating the immune signal into a neural signal is still unclear, but it is well established in

teleosts and mammals that activation of innate immunity interferes with the neuro-endocrine

system (Engelsma *et al.*, 2002; Dantzer, 2004). The observed changes in leukocyte responses

during late infection with *S. solidus* could thus lead to the neuronal changes, which induce

behavioural modifications of the stickleback and so elegantly enhance parasite transmission to

the final host (Scharsack *et al*., 2007)

STUDIES OF *IN VIVO* PARASITE GROWTH AND FITNESS

The large size of *S. solidus* plerocercoids relative to their stickleback hosts, and the

subsequent distension of the host body cavity that is associated with infection, provides a

useful tool to examine the growth of plerocercoids *in vivo.* The degree of distension caused by

such parasites can be accurately measured using digital photography and image analysis

software and converted into an estimate of plerocercoid mass (Barber, 1997; Loot *et al.*, 2002;

Barber and Svensson, 2003), enabling plerocercoid growth to be examined non-invasively

over the post-infection period. If fish are fed singly-infected copepods then the mass of

individual plerocercoids can be tracked. This approach enabled Barber & Svensson (2003) to

construct the growth curve for plerocercoids infecting stickleback hosts fed on a fixed ration

of 8%bw.d-1 and could be of considerable value in future studies designed to establish the

impacts of host environmental factors on plerocercoid performance.

Furthermore, the ability to sexually mature the worms recovered from sticklebacks and collect

data on adult fecundity makes it possible to examine the effects of host rearing environments

and plerocercoid growth history on the fecundity of adult parasites (Dörücü *et al.*, 2007) as

well as providing useful models for investigating egg production strategies, mate choice and

the 'hermaphrodite's dilemma' (Luscher and Wedekind, 2002).

CONCLUSIONS

629   In many ways *S. solidus* plerocercoids are unusual parasites; their typically extreme body size

630   relative to the stickleback host is a dominant feature of infection that is not often observed in

631   other host-parasite systems. It is likely that some of the effects of the parasites on stickleback

632   hosts will be rather specific to this and a limited number of other systems that involve large

633   bodied parasites, such as *Ligula intestinalis* infections of cyprinid fish, *Spirometra*

634   *mansonoides* infections of mammals and some invertebrate-parasitoid systems. On the other

635   hand, *S. solidus* exhibits features common to many ecologically important parasites, such as

636   an indirect life cycle with trophic transmission and the potential to affect host growth,

637   reproduction and survival. The great utility of the system lies in three key attributes; the

638   typically important consequences that infections have for host performance, the ready

639   availability of experimental infection techniques and the fact that the host fish is an extremely

640   well characterised model organism. These attributes combine to facilitate experimental

641   investigations into the role of parasites as agents of selection in host populations.

642

643

Table I. Selected field studies documenting traits associated with *Schistocephalus solidus* infection in natural stickleback populations

Aspect of stickleback biology studied	Infection-associated trait	Reference
Nutritional condition	Infected fish had reduced body condition in the spring and autumn	(Tierney *et al*., 1996)
	Infected fish had reduced stomach fullness and fed on smaller prey	(Tierney *et al.*, 1996)
	Seasonal differences in stomach fullness and diet composition of infected and non-infected fish	(Bergersen, 1996)
	Infected fish had reduced body condition and liver energy reserves	(Tierney, 1994)
Sexual development	Infected females showed reduced gonad development	(Arme and Owen, 1967)
	Infected females were less likely to be gravid	(Heins *et al.*, 1999)
	Infected males developed less red nuptial coloration	(McPhail and Peacock, 1983)
Behaviour	Infected males were less likely to hold nests	(Folstad *et al.*, 1994)
	Infected fish found further from cover in autumn	(Candolin and Voigt, 2001)
Morphology	Infected fish had less symmetric lateral plate counts	(Jakobsen *et al.*, 1988)
	Adults with asymmetric pelvis had increased incidence of infection (pattern reversed in 0+ fish).	(Reimchen and Nosil, 2001)
	Skin of infected fish was demelanised	(Reimchen, 1997)

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648   **References**

649

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