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1    Shoaling with infected conspecifics does not improve resistance to trematode infection

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10   running title: Socially triggered resistance

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15

16    Abstract

18    Group living animals can gain protection against parasitic infections through social contacts with

19    previously infected conspecifics (social immunisation). Recent research suggests that such

20    protective effects can be induced through visual or chemical cues released by infected

21    individuals, resulting in anticipatory immune upregulation among group members. Here, we

22    study cue-induced social resistance in rainbow trout *Oncorhynchus mykiss* exposed to a

23    trematode parasite, the eye-fluke *Diplostomum pseudospathaceum*. We established groups of

24    naïve individuals (receivers) that were paired with previously infected individuals (donors) at

25    different ratios of donors to receivers and at different time points since donor exposure to capture

26    varying concentrations of the anticipated cues. While the pre-infection elevated resistance among

27    the donors, there was no evidence of social transfer of resistance, regardless of the ratio of

28    donors and receivers in a group or the time since the pre-infection. The results suggest that

29    resistance through social signalling may be system-specific and requires further study into the

30    generality of the phenomenon as well as the nature of the cues involved.

33    Key words: group living, social immunisation, parasite, cue, rainbow trout, *Diplostomum*

34    *pseudospathaceum*

37    Introduction

38

39    Parasites are expected to play an important role in the evolution of sociality as group living is

40    typically accompanied by both health-related costs and benefits (reviewed in Côté & Poulin

41    1995; Kappeler et al. 2015). For example, social interactions between group members increase

42    the risk of contracting contagious parasites (Alexander 1974; Côté & Poulin 1995; Rifkin et al.

43    2012; Patterson & Ruckstuhl 2013; Kappeler et al. 2015). On the other hand, the risk of infection

44    with non-contagious parasites acquired from the environment can be lower in groups due to a

45    decreased *per capita* attack rate with increasing group size (dilution effect, Poulin & FitzGerald

46    1989; Mooring & Hart 1992), or due to improved parasite avoidance, possibly through increased

47    vigilance and information sharing (Stumbo et al. 2012; Mikheev et al. 2013).

48           Recent research has revealed that group-living can also confer protection against

49    contagious parasites through social immunisation, where naïve group members show improved

50    resistance to parasites after social contacts with previously exposed group mates (reviewed in

51    Masri & Cremer 2014). This could result from immune priming following a low-dose parasite

52    transfer from infected individuals to naïve group members (Konrad et al. 2012) or from a direct

53    transfer of antimicrobial compounds between individuals (Hamilton et al. 2010). Moreover, two

54    recent studies suggest that social immunisation can also be induced through cues perceived by

55    naïve individuals in infected group mates that cause anticipatory immune upregulation. For

56    example, naïve rainbow trout (*Oncorhynchus mykiss*) shoaling with conspecifics that have

57    recovered from a recent nonlethal bacterial infection show improved survival after a challenge

58    with more lethal doses of the same pathogen (Mothersill et al. 2015). The nature of the cue

59    aiding in transfer of resistance is unknown, but it appears to be released as response to

60  pathogenic stress (Mothersill et al. 2015). Further, humans visually perceiving symptoms of

61  infectious disease upregulate their immune system compared to individuals that perceive non-

62  disease-related threats (Schaller et al. 2010). In fact, many animals show clear signs of infection,

63  such as altered behaviour or changes in appearance or olfactory identity, that are perceived by

64  conspecifics (reviewed in Hart 1990; Kavaliers et al. 2004; Curtis 2014) and could be used to

65  assess infection risk. Given the general nature of these cues, we propose that social immunisation

66  may not only protect gregarious animals against contact-transmitted diseases, but also against

67  infectious parasitic stages prevailing in the environment. For example, although group members

68  carrying non-contagious parasites do not pose a direct infection risk, they may signal the risk of

69  acquiring such parasites from the environment, making anticipatory defence reactions beneficial.

70      As immune functions incur a number of costs (Sheldon & Verhulst 1996; Lochmiller &

71  Deerenberg 2000; Graham et al. 2005), the cues used for preventive upregulation must be

72  reliable. The risk of exposure to free-living parasitic stages can be variable and unpredictable,

73  particularly for mobile hosts that move in and out of infection areas. Here, the proportion of

74  infected individuals within a group and/or the intensity of infection in individual group members

75  may indicate parasite prevalence in the environment and consequently, general infection risk.

76  This could be mediated by the strength of cue emission in the group with a certain threshold at

77  which immune upregulation becomes cost effective for naïve conspecifics. Further, the reliability

78  of cues indicating infection with parasitic stages acquired in the environment may decrease with

79  the age of infection, as older infections do not necessarily coincide with the presence of parasites

80  in the environment. However, if cue emission is induced by pathogenic stress (see Mothersill et

81  al. 2015), its occurrence or strength may vary from the initial exposure to the appearance of

82  symptoms and possibly clearance, depending on the specifics of each host-parasite interaction.

83    Thus, conspecifics may be able to assess the stage of infection through the quantity or quality of

84    cues emitted.

85        Here, we study cue-induced immunisation against the trematode *Diplostomum*

86    *pseudospathaceum*, in relation to infection prevalence within host groups and the time from the

87    initial parasite exposure of the host group members. The parasite's life-cycle involves asexual

88    reproduction in the first intermediate snail host, a second intermediate fish host that serves as

89    transmission vehicle (no reproduction) and finally sexual reproduction in the final bird host

90    (Chappell et al. 1994). Free-living stages (cercariae) emerge in large numbers from the snail

91    hosts and upon encounter penetrate the skin or gills of the fish. Then, they move towards its eye

92    lenses, causing damage to body tissues and blood vessels during migration (Erasmus 1959;

93    Ratanara-Brockelman 1974). Fish hosts have been shown to suffer from pathogenic stress caused

94    directly by the acute invasion of the parasite, as heart rates can increase for several days

95    following exposure (Laitinen et al. 1996), and activity decreases (Gopko et al. 2015). In the

96    host's eye lens, the parasites develop to metacercariae (the bird-infecting stage) within 4-8 weeks

97    that induce eye cataracts (Chappell et al. 1994; Karvonen 2012). These cataracts impair host

98    vision (Shariff et al. 1980) and consequently affect fish physiology and behaviour (Seppälä et al.

99    2005b;a; Karvonen & Seppälä 2008; Seppänen et al. 2008; Voutilainen et al. 2008), but in a

100   different manner than acute invasion. Although, this eye-fluke is one of the most common fish

101   parasites in both natural populations (Chappell et al. 1994; Valtonen & Gibson 1997) and

102   aquaculture conditions (Chappell et al. 1994; Karvonen et al. 2006), the prevalence of infection

103   varies greatly among host species and populations (Chappell et al. 1994; Valtonen & Gibson

104   1997; Rellstab et al. 2011; Karvonen 2012), leading to variation in the ratio of infected to

105   uninfected individuals. As infection prevalence among snail hosts is also variable among

106 populations, but generally low (Louhi et al. 2013) and cercarial shedding is seasonal (Karvonen

107 2012), fish hosts experience variable encounter rates in space and time.

108       Many fish species, including rainbow trout, aggregate in groups that include extensive

109 social interactions (shoals; Pitcher & Parrish 1993; Delcourt & Poncin 2012), mediated by

110 visual, chemical, mechanical, electrical and acoustic communication (Rosenthal & Lobel 2006).

111 Whether infections with *D. pseudospathaceum* can be communicated among group members is

112 unknown, but there is evidence suggesting that fish are able to recognize infections with other

113 non-contagious parasites (Barber et al. 1998; Tobler & Schlupp 2008) and that they can transfer

114 stress via chemical cues (Toa et al. 2004; Vavrek & Brown 2009; Barcellos et al. 2011;

115 Giacomini et al. 2015). We established experimental shoals of rainbow trout that were composed

116 of naïve individuals (receivers) and already infected individuals (donors) in different ratios

117 (30:10 and 10:30) and exposed them to *D. pseudospathaceum* five days after shoal

118 establishment. We repeated this setup at different time points after the original infection of the

119 donors. Based on the anticipated stress-related emission of cues indicating infection risk, we

120 predict that (i) receivers show socially induced resistance to *D. pseudospathaceum* and such

121 effects are stronger when cues most likely coincide with the presence of the parasite in the

122 environment, i.e. (ii) in donor-biased compared to receiver-biased groups and (iii) when

123 infections are recent.

124

125

126 Methods

127

128 Experimental animals

6

129      Juvenile rainbow trout (size selected, average body mass 2.9 g) were obtained from a fish farm in

130      Finland on 23 June 2015. The farm is supplied with groundwater, which ensured that all

131      individuals were free of *D. pseudospathaceum* infection. During the experiment, the fish were

132      kept in aerated ground water (17°C) and fed daily with commercial fish pellets.

133      Cercariae of *D. pseudospathaceum* were obtained from naturally infected snail hosts

134      *Lymnaea stagnalis*, collected from Lake Vuojärvi (Central Finland, 62° N, 25° E) during 22 – 28

135      June 2015. The snails were maintained individually at 4 °C and were fed with lettuce *ad libitum*.

136      Before each experimental infection (see below), 12-14 snails were transferred to room

137      temperature and allowed to shed cercariae for a maximum of 3 hours. The cercarial suspensions

138      of all snails were combined and parasite density was estimated by counting the number of

139      parasites in ten 1 ml samples.

140      All procedures performed in this study were in accordance with the ethical standards of

141      the Finnish Regional State Administrative Agency (License code: ESAVI/4415/04.10.07/2014).

142

143      Experimental setup

144      Rainbow trout were haphazardly divided into six randomly assigned tanks (500 l), two of which

145      housed 290 individuals each (receivers) while the remaining four housed 230 individuals each

146      (donors). On 27 June, receivers were marked by clipping the adipose fin under anaesthesia (MS-

147      222) so that they could be separated from donors in the experimental groupings. The donors were

148      also anaesthetised, but returned to their tanks without fin clipping.

149      On 28 June, the water volume in all six tanks was lowered to 100 l. To produce the

150      donors, fish in two randomly selected tanks were each exposed to an estimated number of 2300

151      parasite cercariae (10 cercariae per fish; 'infected donors') while the two other donor groups

152 were sham exposed with ground water without parasites ('control donors'). Receiver groups

153 were not exposed. After 30 minutes, the water volume in all tanks was brought back to 500 l.

154       Fish groups consisting of donors and receivers were established at three different time

155 points after the exposure of donor individuals: 2 days post-exposure (p.e.) capturing the initial

156 stress effects of the infection, 21 days p.e., when parasites were still developing, and 34 days

157 p.e., when parasites were fully developed and inducing cataracts. Each time, 80 'infected

158 donors', 80 'control donors' and 160 'receivers' were haphazardly selected from the holding

159 tanks and distributed among eight other tanks, each containing 40 individuals in 180 l of water.

160 The groups were formed so that four tanks had combinations of 'infected donors' and 'receivers'

161 in proportions 30:10 (2 tanks) and 10:30 (2 tanks), while the other four tanks had combinations

162 of 'control donors' and 'receivers' in equal proportions and replication.

163       After five days of social contact within a tank, all group members were exposed

164 individually to *D. pseudospathaceum* in small containers with 500 ml of water and 100 cercariae.

165 After 30 minutes of exposure, all fish were returned to their groups for 24 hours to allow parasite

166 establishment in the eye lenses. Subsequently, all fish groups were euthanized with an overdose

167 of MS-222 anaesthetic, measured for length and dissected for parasite numbers. Dissection was

168 conducted blind to the treatment applied to each group. Metacercariae established during the pre-

169 exposure and the re-exposure of 'infected donors' could be differentiated by their clear size

170 difference (Sweeting 1974). Fish length increased with time (GLM, $X^2 = 772.8$, $P < 0.001$), but

171 did not differ between infected donors, control donors and receivers grouped with infected and

172 control donors ($X^2 = 4.8$, $P = 0.184$, interaction not significant).

173

174 Statistics

175 Parasite load (both eyes combined) was analysed using generalized linear mixed models

176 (GLMM) with Laplace approximation and negative binomial probability distribution. Parasite

177 load (excluding parasites from the pre-exposure of 'infected donors') was entered as dependent

178 variable and treatment ('infected donors', 'control donors', 'receivers' and 'control receivers'),

179 ratio of donors to receivers (30:10 and 10:30), time since pre-exposure (7, 26 and 39 days) and

180 all interactions were entered as fixed factors, and fish length as covariate. Each fish group was

181 labelled with an individual ID, which was included as random factor (N = 24) to account for

182 potential group effects. The analysis revealed a negative effect of fin-clipping on parasite

183 resistance, as fin-clipped receiver fish had significantly higher parasite loads than both control

184 donors and infected donors ($P < 0.019$ for all pairwise *Bonferroni* corrected comparisons of

185 donor and receiver fish). This was unexpected as adipose fin clipping in salmonid fish is

186 considered to be non-invasive with negligible effects (Use of Fishes in Research Committee

187 2014). To exclude this effect, two separate models, one including the two donor groups

188 ('infected donors' and 'control donors') and the other the two receiver groups (grouped with

189 'infected donors' and with 'control donors') were used. Further, due to a miscalculation, one

190 group (round 2, 30 control donors : 10 receivers) consisted only of 29 individuals. However, as

191 the ratio of donors to receivers was comparable to the original setup (21 control donors : 8

192 receivers), the group was included into the statistical analyses. Thus, the final sample sizes were

193 240 for 'infected donors', 231 for 'control donors', 240 for 'receivers' and 238 for 'control

194 receivers'. All analyses were conducted using SAS (v. 9.4).

195

196

197 Results

198

199 All 'infected donors' harboured fully developed metacercariae following the pre-exposure with

200 an average of ($\bar{x} \pm$ SE) 8.1 ± 0.2 parasites per fish (range 2-17). These infections activated host

201 resistance and resulted in a reduced parasite infection success among the 'infected donors'

202 compared to 'control donors' (Table 1, Figure 1a). The reduction in parasite load among the

203 'infected donors' also increased with time from 2.7% one week post exposure, to 10.4 % four

204 weeks post exposure and 13.6 % six weeks post exposure (Figure 1 a), but this change was not

205 statistically significant (Table 1). Parasite load also decreased with time since pre-exposure in

206 both 'infected donors' and 'control donors' (Table 1, Figure 1a), which was most likely caused

207 by a decreasing parasite infectivity with time. The ratio of donors to receivers in a group did not

208 affect parasite load among donor individuals (Table 1). Finally, parasite load was negatively

209 related to fish length (Table 1), a pattern that is commonly observed in this system.

210      In contrast, parasite load did not differ between receivers grouped either with 'infected

211 donors' or 'control donors' (Table 2, Figure 1b) suggesting absence of transfer of infection

212 resistance. Parasite load decreased again with time (Table 2, Figure 1b), but there was no

213 interaction with treatment, indicating that the time since pre-exposure in donors had no effect on

214 the result. There was also no effect of the ratio of donors to receivers in a group (Table 2).

215

216

217 Discussion

218

219 Recent research suggests that infection resistance of fish to bacteria can be induced in naïve

220 individuals without an actual contact with the pathogen through cues emitted by previously

221 infected conspecifics (Mothersill et al. 2015). Here, we did not find such an effect in rainbow

222 trout exposed to the trematode *D. pseudospathaceum*. Resistance was comparable for individuals

223 that had been grouped either with infected conspecifics or with uninfected control individuals.

224 Resistance was also not affected by the ratio of infected individuals in a group or the time from

225 their initial exposure. Overall, this suggests that the occurrence of cue-induced resistance may be

226 system-specific and requires more study as to the exact mechanisms.

227       In the rainbow trout - *Vibrio* system, the cue perceived by receiver individuals caused an

228 increase in cellular calcium (Mothersill et al. 2015). A similar response was observed by receiver

229 fish paired with conspecifics that had been exposed to physical stressors, such as radiation (Lyng

230 et al. 2000; Mothersill et al. 2006). Other studies have also shown that chemical cues emitted by

231 infected individuals and individuals experiencing other forms of stress can induce similar

232 responses in conspecifics. For example, female mice exposed to urine of both, males infected

233 with a sporozoan or nematode parasite and physically stressed males, show decreased sensitivity

234 to pain mediated through increased opioid levels (Kavaliers & Colwell 1993; Kavaliers et al.

235 2006). Although, the main explanation for this response is facilitation of behavioural infection

236 avoidance (Kavaliers et al. 2004), opioids also play a role in immune signalling and may thus be

237 involved in anticipatory immune upregulation (Penn & Potts 1998 and references therein).

238 Generally, fish can perceive stress induced by various sources in conspecifics and consequently

239 produce a physiological stress response (Toa et al. 2004; Vavrek & Brown 2009; Barcellos et al.

240 2011; Giacomini et al. 2015). Thus, cues released in consequence of stress could be good

241 candidates for immunisation through social signalling. However, our findings suggest that this

242 needs to be verified in different systems.

243       The perception of a cue inducing protection against infection likely depends on the

244 strength of its emission within a social group. This may vary not only with the ratio of donors to

11

245  receivers, but also with exposure doses experienced by the donors and the resulting infection

246  intensities. For example, in some fish species, physiological responses associated with exposure

247  to *D. pseudospathaceum* have been observed only at high exposure doses (Laitinen et al. 1996).

248  Although exposure doses and the resulting parasite loads in the present study were in the range

249  of those expected (dose) or observed (load) under natural conditions (e.g. Valtonen & Gibson

250  1997), they were on the lower end of the range, as donor individuals harboured on average four

251  parasites per eye after the pre-exposure. Consequently, pathogenic stress levels of donors may

252  have been too low to induce socially triggered resistance in receivers. However, a resistance

253  response was elicited earlier in rainbow trout grouped with conspecifics that likely experienced

254  only moderate pathogenic stress, as these had been exposed to a nonlethal dose of bacteria and

255  had already recovered from the infection (Mothersill et al. 2015).

256          Using cues emitted by infected conspecifics to upregulate personal immune responses

257  can offset the increased risk of contracting contagious parasites in groups and may thus be seen

258  as an adaption to compensate health related costs of sociality (Masri & Cremer 2014). If so,

259  selection pressures on social immunisation against non-contagious parasites may be low. First,

260  infections with non-socially transmitted parasites may not reliably signal infection risk to

261  conspecifics, as they do not necessarily coincide with the presence of infective stages in the

262  environment. For example, in the present system with infection hotspots and highly mobile hosts,

263  infection risk may be too variable and unpredictable to make cue-induced immunisation cost-

264  effective. However, infection risk also varies seasonally, as cercarial production in the snail hosts

265  is temperature regulated (Karvonen 2012). In northern latitudes, for example, infection risk

266  prevails only during 3-4 months each year (Karvonen et al. 2004). Thus, infection in others,

267  particularly if recent, may signal the onset of cercarial shedding and thus, an overall risk of

268    infection. Second, grouping is expected to provide other benefits against free-living parasitic

269    stages. In our study system, infection intensities with *D. pseudospathaceum* decrease with group

270    size, possibly due to a dilution effect (Karvonen et al. 2005). Other experiments have also

271    demonstrated a decreased exposure risk to trematode parasites in shoaling versus solitary fish

272    (Stumbo et al. 2012). Additionally, groups are also more efficient in behaviourally avoiding *D.*

273    *pseudospathaceum* compared to solitary individuals (Mikheev et al. 2013), possibly due to an

274    enhanced potential for parasite detection ("many eyes" theory; Treherne & Foster 1980; Lima

275    1995). However, as sociality enhances the possibility of acquiring information from others, it

276    may provide additional protection against virulent non-contagious parasites present in a group's

277    environment.

278        In conclusion, our results do not support the prediction that group living induces social

279    resistance to a trematode infection. Social immunisation is an emerging field of research that

280    may have important implications for disease dynamics and, owing to a natural vaccine effect, for

281    the management of natural and captive populations. However, the mechanisms of cue-induced

282    social immunisation are not well understood. More studies are needed to gain insights into the

283    generality of the phenomenon in different host-parasite systems and the nature of the cues

284    involved in protective immune stimulation.

285

286

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428

429 Table 1 General linear mixed model (GLMM) analysis of parasite load in 'infected donors' and

430 'control donors', explained by treatment (pre-exposure and sham exposure), ratio of donors to

431 receivers in a group (30:10 and 10:30), time since pre-exposure (7, 26 and 39 days) and fish

432 length. Fish group is included in the model as a random factor.

| factors | *df* denominator | *df* numerator | *F* | *P* |
|---|---|---|---|---|
| treatment | 1 | 442 | 4.41 | 0.041 |
| ratio | 1 | 442 | 0.19 | 0.667 |
| time | 2 | 12 | 249.70 | <0.001 |
| treatment*ratio | 1 | 442 | 1.285 | 0.258 |
| treatment*time | 2 | 442 | 0.58 | 0.562 |
| time*ratio | 2 | 442 | 0.26 | 0.772 |
| treatment*time*ratio | 2 | 442 | 0.26 | 0.772 |
| length | 1 | 441 | 8.61 | 0.004 |

433

434

435

436    Table 2 General linear mixed model (GLMM) analysis of parasite load in receivers, explained by

437    treatment (grouped with 'infected donors' and with 'control donors'), ratio of donors to receivers

438    in a group (30:10 and 10:30), time since pre-exposure of donors (7, 26 and 39 days) and fish

439    length. Fish group is included in the model as a random factor.
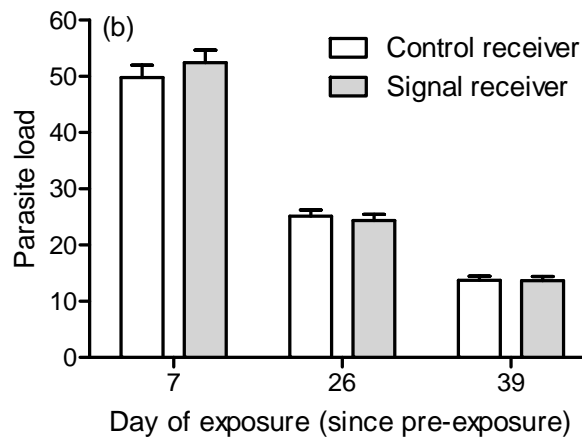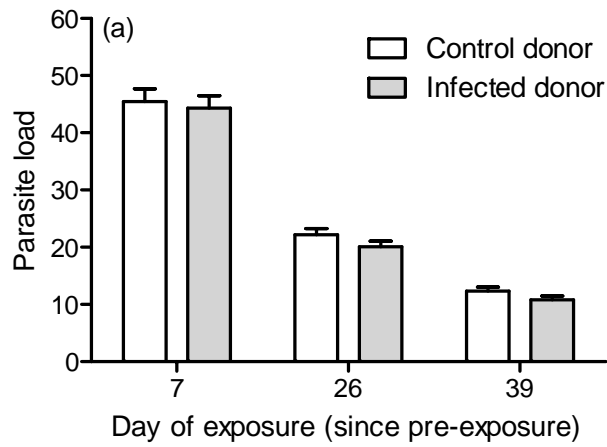
| factors | df denominator | df numerator | F | P |
|---|---|---|---|---|
| treatment | 1 | 453 | 0.03 | 0.854 |
| ratio | 1 | 453 | 0.50 | 0.481 |
| time | 2 | 453 | 265.26 | <0.001 |
| treatment*ratio | 1 | 453 | 0.23 | 0.633 |
| treatment*time | 2 | 453 | 0.49 | 0.614 |
| time*ratio | 2 | 453 | 0.52 | 0.593 |
| treatment*time*ratio | 2 | 453 | 0.23 | 0.797 |
| length | 3 | 453 | 0.21 | 0.649 |

440

441

442    Figure 1

443



444

445    **Fig 1** Parasite load (Least-square means ± SE) of (a) donor individuals and (b) receiver

446    individuals after experimental exposures varying in time since pre-exposure of 'infected donors'.