Shoaling as an antiparasite defence in minnows (*Pimephales promelas*) exposed to trematode cercariae

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Summary

1. Individuals that live in groups benefit from increased foraging success and decreased predation. Protection from some types of parasites may provide an additional benefit of group-living. For fish, the extent to which shoaling can reduce an individual’s risk of exposure to the infective stages of parasites is unknown.

2. We tested for antiparasite benefits of shoaling in fathead minnows exposed to larvae (cercariae) of two of their most common species of trematode, *Ornithodiplostomum ptychochelius* and *Posthodiplostomum minimum*. As developing stages (metacercariae) of these trematodes cause reductions in minnow activity, growth and survival, natural selection should favour the evolution of cercariae-avoidance behaviours.

3. We evaluated shoal dimensions in groups of minnows exposed to *O. ptychochelius* and to other chemical/physical stimuli within aquaria. To compare risk of exposure in shoaling vs. non-shoaling fish, we confined groups of minnows into mesh cages in outdoor mesocosms, exposed them to cercariae, then compared mean worm numbers in grouped vs. solitary fish. Lastly, we tested whether fish located within the centre of an artificial shoal reduced their risk of cercariae exposure compared with those along peripheral edges.

4. Minnows distinguished infective cercariae from other potential aquatic threats and responded with activity that reduced the 2-dimensional area of their shoals 15-fold compared with water-only controls. Fish confined within artificial shoals had 3-fold fewer worms than single fish and minnows located within the centre of artificial shoals had significantly fewer worms than those without peripheral minnows.

5. These results show that shoaling reduces a minnows’ risk of exposure to cercariae, either directly via detection of cercariae in the water column followed by behavioural avoidance or indirectly via behaviour-mediated differences in exposure between shoaling vs. non-shoaling fish.

Key-words: avoidance behaviour, fish behaviour, metacercariae, parasite avoidance, parasite detection, parasitism, social living

Introduction

Group-living is common in fishes, with aggregations ranging from small, diffuse shoals to synchronized schools containing thousands of individuals (Pitcher & Parrish 1993; Ward et al. 2008). Evidence from laboratory and field studies indicate that individuals living in groups reduce their risk of predation, typically through dilution and confusion effects, and also increase their rates of food acquisition. In addition, the sharing of social information among individual fish within a shoal enables the highly efficient and rapid transmission of knowledge regarding predation risk and food availability (Ward et al. 2011). Yet, the dilution effects and the ‘many eyes’ effects that make shoaling an effective antipredator strategy should apply equally to the risk of exposure to the infective larvae of certain parasites, so long as the parasites can be detected within the water column (review by Wisenden, Goater & James 2009). Thus, group-living may reduce an
individual’s exposure to the larvae of aquatic parasites that infect fish via free-swimming infective stages (e.g. the obligate penetration stages of trematodes, myxozoans and some parasitic arthropods). The antiparasite benefits of shoaling in fish have not been experimentally evaluated.

Indirect evidence for antiparasite benefits of group-living comes from correlational studies involving social birds and mammals that indicate a negative relationship between the occurrence of mobile parasites and group size (Mooring & Hart 1992; Côté & Poulin 1995; Altizer et al. 2003) and observational studies showing reduced parasite loads in grouped vs. solitary hosts (Wikelski 1999). However, evidence for a direct linkage between group-living and parasitism is limited. When the presence of biting flies was chemically reduced in a population of beef cattle, individual hosts reduced their tendency to form tightly packed groups (Schmidtmann & Valla 1982). Similarly, the ectoparasite Argulus canadensis exhibited fewer attacks on individuals as the size of stickleback groups increased, and individual fish tended to shoal in the presence of the parasite (Poulin & FitzGerald 1989). These results suggest that group-living can protect hosts from mobile parasites. However, experimental tests involving a broader range of host/parasite interactions are required, especially involving parasites that have small, mobile infective stages (Mooring & Hart 1992; Côté & Poulin 1995).

Larval stages of trematode flukes are common in fish, typically encysting as metacercariae within species-specific sites such the eyes, brain, muscle and epidermis (Bush et al. 2001). Many species of fish contain numerous species of these flatworms, each of which can reach population densities in the 100's or 1000's in their individual hosts. Infection occurs via penetration of short-lived, free-swimming stages (known as cercariae) through the host epidermis. Parasitologists, ecologists and fisheries biologists have documented diverse negative consequences of metacercariae infection on fish, ranging from direct effects on host survival (Kelly et al. 2010; Ferguson et al. 2011) to indirect effects on host behaviour, physiological status and reproduction (review by Poulin 2007). Given these substantial costs, natural selection should favour evasive mechanisms to reduce infection risk (Wisenden, Goater & James 2009). Results from several studies support this idea, indicating that fish reduce their overall activity and seek refuge in the presence of cercariae (Poulin, Rau & Curtis 1991; Karvonen, Seppälä & Valtonen 2004). Cercariae penetration has also been shown to elicit an alarm response from shoalmates via minnow chemical alarm cues released from ruptured epidermal cells, and individuals may obtain learned avoidance behaviour through chemo-association (Poulin, Marcogliese & McLaughlin 1999; Wisenden 2000). These results indicate that fish have the potential to detect cercariae within the water column and engage behavioural defences to potentially avoid them. The extent to which shoaling provides anticercarial benefits to individuals is unknown.

The fathead minnow (Pimephales promelas, Rafinesque) is a group-living cyprinid fish that is regionally common and locally abundant throughout most of North America. They are a common intermediate host of various species of trematode that utilize fish-eating birds as definitive host (Chubb 1979; Sandland, Goater & Danylchuk 2001). Several studies have documented a range of physiological and ecological effects on individual fish (Sandland & Goater 2000; Shirakashi & Goater 2005; James et al. 2008). Two of the most common species in minnows, Ornithodiplostomum ptychocheilus (Faust) and Posthodiplostomum minimum (MacCallum) encyst within the brain and the body cavity, respectively (Schleppé & Goater 2004). Both species use Physa snails as first intermediate host, fathead minnows as second intermediate host and piscivorous birds as definitive host. In Alberta lakes, their metacercariae are found only in fathead minnows, typically infecting all minnows within a lake with 20–500 larvae/host (Sandland, Goater & Danylchuk 2001). Both species are amenable to experimental manipulation.

In this paper, we report the results from experiments designed to test the hypothesis that shoaling reduces a minnow’s risk of exposure to trematode cercariae. We performed three experiments to evaluate the anticercariae benefits of shoaling in minnows. First, we compared the dimensions of minnow shoals in aquaria before and after they were exposed to cercariae. We evaluated whether minnows could distinguish cercariae from various stimuli within the water column, and if so, if they could respond behaviourally by decreasing the two-dimensional area of a shoal. In a second experiment, we used outdoor mesocosms containing infected snails to evaluate the risk of exposure in shoaling vs. solitary minnows. For this experiment, we used mesh cages that confined the minnows into artificial shoals, but allowed free-swimming cercariae to pass through. Lastly, we used similar cages to confine minnows with or without peripheral conspecifics to determine whether the central position in a shoal reduced an individual’s risk of exposure to trematode larvae.

Materials and methods

Experiments were performed at the University of Lethbridge, AB, Canada, during summer 2010. All minnows used in these experiments originated from a small pond located in southern Alberta (Stirling Lions Club Fish Pond, AB; 49°30′, −112°32′). We collected large numbers of young-of-the-year (27 ± 1·3 mm) in June 2010. A subsample of 20 YOY indicated a background infection with O. ptychocheilus metacercariae in that year (mean = 6·9 ± 1·8), but no other parasites. Minnows were maintained within two separate 1200-L outdoor mesocosms (108-cm diameter, 120-cm height; Pearson & Goater 2009) prior to experiments.

We used the methods described in Sandland & Goater (2000) to expose fathead minnows to water containing known numbers of cercariae. To ensure that we had large numbers of cercariae available for the exposures to minnows, we infected snails with miracidia of both O. ptychocheilus and P. minimum. The life histories and life cycles of the two species are very similar, and their
cercariae are morphologically identical (Schleppé & Goater 2004). To start, 1-day-old chickens were fed 20 minnow brains containing *O. ptychocheilus* metacercariae or 20 minnow viscera containing *P. minimum* metacercariae from fish collected from a local lake. The metacercariae developed into adults in 72 h, as indicated by the presence of large numbers of eggs in the faeces of each chicken. Eggs were collected from the faeces between days 3–5 post-exposure. Eggs were incubated following Sandland & Goater (2000) and the resulting miracidia were exposed to the F1 generation of laboratory-reared *Physa* snails. Exposed snails (4–6 mm maximum shell length) were housed in 10-L plastic containers at 20 °C, with a 16:8 L/D photoperiod and fed boiled romaine lettuce daily. For experiments 1 and 3, which required exposing individual minnows to known numbers of cercariae, infected snails were placed into glass vials of dechlorinated water under direct light to encourage cercarial release. The cercariae from all infected snails were pooled into a 100-mL flask and diluted to 100 mL with dH2O following methods described in Sandland & Goater (2001). Estimates of the numbers of cercariae/mL were calculated from 3- to 10-mL aliquot samples. The volume containing the required number of cercariae for each dose was then estimated.

**SHOAL DIMENSIONS FOLLOWING EXPOSURE TO CERCARIAE**

The aim of this experiment was to determine whether the presence of cercariae in the water column caused groups of fathead minnows to alter the dimensions of their shoals. We also aimed to test whether minnows could distinguish infective cercariae from other potential novel stimuli in the water column, including other (non-infective) species of cercariae. We exposed groups of five minnows to one of the following six stimuli, each of which was administered into the tanks within 60 mL aliquots: dechlorinated water (control), 500–2–3 h old *O. ptychocheilus* cercariae, 500–2–3 h old *Plagiorchis elegans* (Rudolphi) cercariae, minnow chemical alarm cue from 2 cm² of homogenized fillet, 0.25 g of cleaned and sieved sand (125–400 μm in diameter) and three drops vanilla extract. Eight trials were performed daily over a period of 6 days within four 37-L tanks. Each tank was divided in half with black Plexiglas, so that two replicate trials could be video-monitored at one time. Order effects were controlled by rotating stimuli among the eight replicate tanks.

We used the two types of cercariae to evaluate whether minnows could distinguish between cercariae that are specific to small-bodied cyprinids (*O. ptychocheilus*) from those that are common and sympatric in the same habitats but do not infect fish (*P. elegans*). Cercariae of *P. elegans* develop into metacercariae within a wide variety of aquatic insect larvae (Genov & Samnalieva 1984). *Plagiorchis elegans* cercariae originated from snails collected from Stirling Lions Club Fish Pond.

The concentrations of chemical and physical stimuli were determined from the results of pilot trials completed in the same tanks. Cercariae dosage was selected to parallel our earlier studies (e.g. Shirakashi & Goater 2005), such that each fish could potentially be exposed to approximately 100 cercariae. This rate of exposure results in metacercariae burdens in fish that approximate those mined from the results of pilot trials completed in the same tanks. Our pilot studies also indicated that at this dosage, individual fish did not demonstrate the characteristic shaking and twitching that can be associated with the penetration of large numbers of cercariae (C. P. Goater, unpublished data).

Minnow alarm cue was prepared following methods in Wisenden (2000) and administered to tanks at a concentration known to elicit antipredator behaviour in fatheads. Skin fillets were collected from two fathead minnows, measured to the nearest tenth of a millimetre and placed into dechlorinated water on ice. The fillets were homogenized to release alarm cue from damaged cells, and then the total solution was diluted to 2 cm² skin per 60 mL. The alarm stimulus was kept on ice and introduced in 60 mL aliquots within 15 min of preparation. Vanilla extract and sand particles were used to control for the potential effects of a novel chemical and physical cue, respectively.

Five size-matched minnows were selected at random for each trial. To increase the number of possible replicates that could be video-monitored, each of the four tanks was partitioned into two by an opaque, water-tight piece of Plexiglas. Fish were acclimated within each half tank for 20 h prior to the start of each trial. An open-ended plastic stimulus injection hose (6.4 mm diameter) was wedged into a sponge filter attached to an air supply with a second plastic hose producing a constant stream of air bubbles to mask pressure change during stimulus injection and to allow for rapid dispersion of each stimulus (Wisenden 2000). Just prior to each trial, 30 mL of water was withdrawn through the stimulus hose and discarded to remove any stagnant water within the hose. Another 30 mL was acquired and retained to completely flush test stimuli from the injection hose during stimulus injection. Each trial was video recorded in front of each tank by a camera that was hidden behind a black cloth blind. Trials comprised 11 min in total: a 5-min pre-stimulus period, a 1-min stimulus injection period and a 5-min post-stimulus period.

Following video-capture, the dimensions of each 5-minnow shoal were calculated using ImageJ software (US National Institutes of Health, Bethesda, Maryland, USA) every 15 s during the pre- and post-stimulus periods. The 2-dimensional area (*x* and *y* coordinates) was determined by calculating the perimeter of the polygon made from lines that joined the heads of each fish. A paired sample *t*-test was used to compare means of pre- and post-stimulus polygon area to evaluate changes in shoal dimensions. Here, the average total area of the pre-stimulus polygon was subtracted from the average post-stimulus polygon to assess change in shoal dimensions following stimulus injection. Change in shoaling area was compared among treatments and post hoc comparisons were used to test for differences between selected pairs of stimuli.

**RISK OF PARASITISM WITHIN ARTIFICIAL SHOALS**

The aim of this experiment was to evaluate whether shoaling minnows reduced their rate of exposure to cercariae compared with solitary minnows. Our approach was to mimic natural conditions of exposure as much as possible, whereby cercariae present in the water column had an approximately equal probability of infecting solitary or shoaling fish. We also aimed to parallel natural conditions of exposure so that infected snails would release typical numbers of cercariae following a natural diurnal cycle. To do this, we housed shoaling and solitary minnows (and snails) in large outdoor mesocosms (1200 L; 108 cm diameter × 120 cm height) that had been established following standard procedures (e.g. Pearson & Goater 2009) to contain phytoplankton and zooplankton communities representative of local ponds. Results from our earlier studies showed that fatheads in these mesocosms grow at rates equal to or greater than minnows in natural ponds and mortality is very low (Pearson & Goater 2009).
We used a network of screened containers placed within each of four outdoor mesocosms to confine fish into shoaling or solitary configurations. There were three types of containers: a 1000-cm³ container containing five randomly selected size-matched minnows (L-5, shoaling) to represent the shoaling configuration, a similar-sized container containing one minnow to represent a solitary configuration (L-1; non-shoaling), and a 200-cm³ container to represent a contrasting solitary configuration (S-1; non-shoaling). The purpose of using small and large containers for the solitary fish was to control for potential differences in available space for locomotion in the small vs. large containers.

Cages were constructed with Trical plastic netting (3-mm mesh) fastened at the sides with monofilament fishing line. To control for possible differences in cercarial activity at different water depths, all containers were suspended just below the water surface of the mesocosms. Fishing lines of equal length that were weighted at their distal ends were attached to the bottom of each container to ensure it did not move during the experiment. The positions of the containers within each mesocosm were randomized, and each container was at least 20 cm distant from adjacent containers. Thirteen containers were placed into each mesocosm: Three L-5, five S-1 and five L-1. Containers were placed into each mesocosm on 10 July 2010. Three days prior, two adult P. minimum-infected Physa gyrina had been added to each mesocosm. These snails had been exposed to miracidia 8 weeks earlier and were confirmed to be releasing cercariae prior to their addition to the mesocosms. Following their addition to the mesocosms, the snails are no longer detectable within the complex mesocosm habitat. There is no way to assess cercarial release once the snails are added to the mesocosms and no way of assessing snail mortality.

Fish remained in the mesocosms for a 3-day period of exposure. Thereafter, surviving minnows were housed in 2-L plastic containers labelled according to their cage and mesocosm of origin. Fish had a 16 : 8 L/D photoperiod and were fed commercial flake food ad libitum for 16 days, after which fish were necropsied and metacercarial intensity was assessed. Data from individual minnows in the L-5 treatment cannot be considered independent; therefore, mean metacercarial intensity was determined from the surviving minnows within each shoaling cage. ANCOVA was used to compare mean metacercarial intensity between the two non-shoaling treatments (S-1, L-1) and the shoaling treatment, with the four mesocosms treated as blocks and minnow length at time of necropsy treated as a covariate.

RISK OF CERCARIAE EXPOSURE RELATIVE TO POSITION WITHIN A SHOAL

The aim of this experiment was to determine whether fathead minnows confined to a central position within an artificial shoal would be exposed to fewer P. minimum cercariae than solitary minnows. For this experiment, artificial shoals were constructed from the same 200-cm³ mesh containers described previously. Each shoal was constructed such that a central container was attached by fishing line at its four sides and bottom to make a single floating unit with six subunits comprising the 200-cm³ containers. Thus, a shoaling configuration consisted of a focal minnow in a central container, with an additional minnow in each peripheral container. The solitary configuration consisted of a focal minnow in a central position and none in the peripheral positions. The purpose of the empty peripheral positions around the central minnow was to control for container effects so that individual cercariae could potentially pass through the same number of container walls to reach the focal minnow within the centre.

Both shoaling configurations were placed at one end of a 40-L plastic container containing 30 L aged water. Seventy-five 2-h old cercariae were added to the opposite side of the container 4 h later. Trials were run for 4 h, after which minnows were housed in 2-L plastic containers with a 16 : 8 L/D photoperiod and fed commercial flake food ad libitum for 16 days and then assessed for metacercariae intensity. Trials were run over 3 days for a total of seven replicates. Individual plastic containers were treated as replicates, and mean differences between minnows with and without peripheral conspecifics were assessed. Difference in minnow length at time of necropsy was treated as a covariate.

RESULTS

SHOAL DIMENSIONS FOLLOWING EXPOSURE TO CERCARIAE

There was no significant change in polygon area between pre- and post-stimulus for control (t_5 = 0.50, P = 0.634) or vanilla extract (t_5 = 1.15, P = 0.288) treatments. However, there was a significant change in pre- and post-stimulus area for O. pygchocheilus (t_5 = 4.63, P = 0.002), P. elegans (t_5 = 3.21, P = 0.015), alarm substance (t_5 = 5.12, P = 0.001) and sand (t_5 = 2.53, P = 0.039) following stimulus injection.

The average change in shoal polygon area was strongly affected by treatment (one-way ANOVA, F_{1,42} = 15.735, P < 0.001, Fig. 1). Tukey’s HSD post hoc showed a significantly greater decrease in shoal area of fish exposed to O. pygchocheilus cercariae than for fish in the water controls (P = 0.018), and the vanilla extract and sand treatments (P < 0.001). However, the change in size of O. pygchocheilus-exposed shoals was not significantly different from shoals exposed to minnow alarm substance (P > 0.999). Results of post hoc comparisons showed no significant differences in mean change in shoal size between the control, vanilla extract and sand stimuli (P > 0.50). There was a significant increase in shoal area for shoals exposed to P. elegans cercariae compared with other stimuli (P < 0.05) except for sand (P = 0.224).

RISK OF PARASITISM WITHIN ARTIFICIAL SHOALS

Of the 100 fathead minnows used in this experiment, 86 survived the exposure and the metacercariae development periods, with 5% escaping confinement prior to collection and 9% dying after collection. These minnows were not included in the analyses. There was extensive variation in P. minimum intensity between and within the mesocosms, with minnows harbouring between 8 and 311 metacercariae (Table 1). Metacercarial intensity ranged from 37 to 311 for solitary minnows (large and small cages) and from 8 to 178 for minnows in the artificial shoals. A frequency distribution of worm numbers/host emphasizes this variation (Fig. 2).
When mesocosms were treated as blocks and minnow body length as a covariate, metacercariae intensity was not significantly different between the two non-shoal treatments, that is, the small vs. large containers that contained a single minnow (two-way ANCOVA, $F_{1,21} = 0.04$, $P = 0.948$). There was also no significant effect of the covariate ($F_{1,21} = 0.07$, $P = 0.792$), blocks ($F_{3,21} = 0.04$, $P = 0.173$) or the interaction between treatment and blocks ($F_{3,21} = 0.47$, $P = 0.706$). Because mean metacercariae intensity was the same between the different-sized containers that contained a single fish, we pooled these data to focus our analysis on shoaling vs. non-shoaling fish. With the mesocosms treated as blocks and minnow body length as a covariate, metacercariae intensity was significantly different between shoaling and non-shoaling treatments (two-way ANCOVA, $F_{1,33} = 7.23$, $P = 0.011$), with fish harbouring $70.0 \pm 6.9$ (mean $\pm$ SD) metacercariae in shoaling configurations and $132.4 \pm 13.8$ in non-shoaling configurations. Body length was not a significant predictor of metacercarial intensity ($F_{1,33} = 0.01$, $P = 0.970$). Metacercarial intensities were highly variable among the four ponds, but differences between the mesocosms were not significant ($F_{3,33} = 0.72$, $P = 0.548$) and there was no interaction between treatments and blocks ($F_{3,33} = 1.90$, $P = 0.148$).

**RISK OF CERCARIAE EXPOSURE RELATIVE TO POSITION WITHIN A SHOAL**

Metacercarial intensity was significantly higher in minnows with no peripheral conspecifics compared to minnows with conspecifics in peripheral locations (repeated measures ANOVA, $F_{1,5} = 11.25$, $P = 0.020$, Fig. 3). Difference in body length was not a significant predictor of metacercarial intensity ($F_{1,5} = 1.829$, $P = 0.234$). Minnows without peripheral conspecifics harboured $8.9 \pm 1.5$ metacercariae, whereas minnows with peripheral conspecifics harboured $2.9 \pm 0.3$ metacercariae. Although not included in statistical analysis owing to possible cage effects, mean metacercarial intensity for peripheral fish was $11.5 \pm 1.8$.

**Table 1.** Summary infection characteristics for the effects of shoaling on mean *Posthodiplostomum minimum* metacercariae intensities between ponds and between treatments

<table>
<thead>
<tr>
<th>Pond</th>
<th>Treatment</th>
<th>$n$</th>
<th>Mean ± SE</th>
<th>Range</th>
<th>Median</th>
<th>Cv (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>L-5</td>
<td>3</td>
<td>71 ± 4</td>
<td>63–76</td>
<td>70</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>S-1</td>
<td>3</td>
<td>197 ± 65</td>
<td>86–311</td>
<td>195</td>
<td>57</td>
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<tr>
<td></td>
<td>L-1</td>
<td>4</td>
<td>172 ± 41</td>
<td>72–272</td>
<td>173</td>
<td>48</td>
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<tr>
<td>Pond summary</td>
<td>10</td>
<td>149 ± 29</td>
<td>63–311</td>
<td>125</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>L-5</td>
<td>3</td>
<td>48 ± 8</td>
<td>36–64</td>
<td>43</td>
<td>31</td>
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<tr>
<td></td>
<td>S-1</td>
<td>5</td>
<td>112 ± 24</td>
<td>66–192</td>
<td>90</td>
<td>49</td>
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<tr>
<td></td>
<td>L-1</td>
<td>5</td>
<td>160 ± 35</td>
<td>83–263</td>
<td>133</td>
<td>49</td>
</tr>
<tr>
<td>Pond summary</td>
<td>13</td>
<td>105 ± 21</td>
<td>36–263</td>
<td>83</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>L-5</td>
<td>3</td>
<td>75 ± 12</td>
<td>60–99</td>
<td>67</td>
<td>29</td>
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<td>137 ± 28</td>
<td>81–167</td>
<td>162</td>
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<tr>
<td></td>
<td>L-1</td>
<td>4</td>
<td>110 ± 54</td>
<td>37–267</td>
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<tr>
<td>Pond summary</td>
<td>10</td>
<td>105 ± 23</td>
<td>37–267</td>
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<tr>
<td>4</td>
<td>L-5</td>
<td>3</td>
<td>88 ± 20</td>
<td>49–116</td>
<td>98</td>
<td>40</td>
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<tr>
<td></td>
<td>S-1</td>
<td>4</td>
<td>79 ± 17</td>
<td>52–130</td>
<td>68</td>
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<tr>
<td></td>
<td>L-1</td>
<td>3</td>
<td>100 ± 41</td>
<td>56–181</td>
<td>62</td>
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<td>Pond summary</td>
<td>10</td>
<td>86 ± 25</td>
<td>47–181</td>
<td>63</td>
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L-5 = fathead minnows in artificial shoals; S-1 = solitary minnows in small cages; L-1 = solitary minnows in large cages.

to fish under natural conditions of exposure. The challenge is to extend this approach with a general behaviour that can potentially reduce their specific cercariae within the water column and respond results of this experiment indicate that fish can detect host-'any' cercariae within the water column. Overall, the detection via olfactory cues. James among species. Direct visual observation is possible, as is cercariae, and by extension, how they might distinguish via information transfer and cause the observed contraction of the shoal during exposure. Further, Poulin, Marcogliese & McLaughlin (1999) showed that cercarial penetration may elicit an alarm response in conspecifics by rupturing club cells in the fish epidermis and releasing chemical alarm cues (i.e. Schreckstoff). A cercariae-specific alarm response may also be transferred among shoalmates. Our results are consistent with these observations because the intensity of alarm response was similar for minnows exposed to O. ptychocheilus and minnows exposed to chemical alarm cues.

The contrasting shoaling response of minnows exposed to the two different species of cercariae was striking. In the case of O. ptychocheilus cercariae, minnows formed tighter shoals relative to water controls, whereas those exposed to P. elegans cercariae distributed themselves throughout the aquaria. As our experimental populations of minnows originated from ponds that contained O. ptychocheilus cercariae, it is possible that our results indicating increased shoaling arose as a learned response following their subsequent exposure to O. ptychocheilus, but not to P. minimum. Tests of this hypothesis would require follow-up experiments involving behavioural assays of experienced and non-experienced minnows exposed to cercariae (e.g. James et al. 2008). An alternative possibility is that the process of penetration of even small numbers of O. ptychocheilus may provide the only necessary cue to shoal, whereas in the absence of penetration, cercariae may be perceived as potential food. Size-selected foraging on cercariae has recently been demonstrated in a number of fish/trematode interactions (e.g. Kaplan et al. 2009). Until more is known about learned responses to cercariae under natural conditions, we favour an interpretation based upon cercariae as a potential energy source.

Results from our third experiment support the hypothesis that fish occupying an interior position within a shoal reduce their exposure to trematode cercariae. This is the first experimental evidence to show that position within a shoal can reduce rates of cercariae exposure. Helle & Aspi (1983) showed that reindeer, Rangifer tarandus, located closer to the interior of a herd and those closer to herd mates were attacked less by biting flies than those on the exterior of a herd. Similarly, Grosholz (1994) showed that the marine clam, Transmella tanilla, attached to peripheral edges of a cluster, experienced higher rates of exposure to cercariae of Paravatrematoborealis compared to those attached to central locations. In this case, hosts on the perimeter of host aggregations were considered as `sinks' that diluted the availability of cercariae to the interior hosts. In the case of minnow shoals, a similar mechanism may explain the significant reduction in exposure of central minnows to O. ptychocheilus cercariae, a kind of dilution effect specific to exposure to cercariae.

Many factors contribute to the natural spatial positioning of shoaling fish. Most studies have focused on predation risk and energy requirements as key factors for

Discussion

Results from our first experiment indicate that fathead minnows distinguish infective cercariae from other stimuli and exhibit a behavioural response that is comparable in magnitude to an alarm response indicative of predation. The ability to discern threatening stimuli from non-threatening stimuli is important in determining behavioural avoidance strategies to a wide range of aquatic threats, including the infective stages of parasites (Wisenden, Goater & James 2009). In general, when the numbers of fish in an artificial shoal were kept constant, the dimensions of the shoal decreased significantly during exposure to O. ptychocheilus cercariae. This decrease was greater than it was for novel chemical or physical stimuli, indicating a specific behavioural response independent of a sudden introduction of a visual or olfactory stimulus. The opposite behavioural response was observed when minnows were exposed to non-minnow cercariae, further indicating that the adjustment in shoal dimensions in response to O. ptychocheilus is not a general response to 'any' cercariae within the water column. Overall, the results of this experiment indicate that fish can detect host-specific cercariae within the water column and respond with a general behaviour that can potentially reduce their risk of exposure. The challenge is to extend this approach to fish under natural conditions of exposure.

Little is known regarding the cues that fish use to detect cercariae, and by extension, how they might distinguish among species. Direct visual observation is possible, as is detection via olfactory cues. James et al. (2008) showed that fathead minnows reduced their overall activity in the presence of cercariae cues, but only if they had a prior experience of exposure. As for many other species of trematode cercariae, penetration of O. ptychocheilus through the host's epidermis causes a characteristic shacking and twitching. Potentially, such behaviours may be perceived as a potential 'cercariae' threat by conspecifics via information transfer and cause the observed contraction of the shoal during exposure. Further, Poulin, Marcogliese & McLaughlin (1999) showed that cercarial penetration may elicit an alarm response in conspecifics by rupturing club cells in the fish epidermis and releasing chemical alarm cues (i.e. Schreckstoff). A cercariae-specific alarm response may also be transferred among shoalmates. Our results are consistent with these observations because the intensity of alarm response was similar for minnows exposed to O. ptychocheilus and minnows exposed to chemical alarm cues.

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Fig. 3. Mean (±SE) Posthodiplostomum minimum metacercariae intensity in fathead minnows confined in six-chambered housing units. Minnows were confined into the central chamber with no peripheral fish (non-shoaling) or with peripheral fish (shoaling), or in the peripheral chambers (shoaling).
determining positioning choice of individuals within a shoal. Peripheral and front positions provide greater foraging opportunities and are often utilized by individuals with greater energetic needs, such as larger or malnourished fish (Bumann & Krause 1993; Krause 1993). Predation risk, however, is greater for fish occupying these locations (Bumann, Krause & Rubenstein 1997) and a trade-off arises that balances predation threat and energy needs (Krause 1993). These results suggest that the spatial structure of a shoal is dynamic, although some studies have shown a ‘sustained position preference’ over prolonged periods of time, with innate behavioural traits (e.g. shy vs. bold fish) influencing spatial positioning, and a tendency for larger individuals to occupy positions at the front of a shoal (Bumann & Krause 1993; Bumann, Krause & Rubenstein 1997; Ward et al. 2004; Leblond & Rees 2006). Although our results demonstrate a linkage between spatial position within a shoal and risk of exposure to cercariae, follow-up studies are needed to clarify the temporal component of shoal positioning under natural conditions relative to the temporal pattern of release of cercariae from snails.

The results from field surveys have shown that shoaling fish infected with metacercariae tend to occupy peripheral positions within their shoals (Krause & Godin 1994; Barber & Huntingford 1995). This pattern is typically attributed to alterations in host behaviour and to conspecific segregation that can facilitate transmission to definitive avian hosts. Further, Barber & Huntingford (1995) and Ward, Hoare & Couzin (2002) suggest that metabolic costs associated with infection may also explain the tendency for more heavily infected fish to occupy peripheral positions in a shoal. An alternative possibility suggested by our results is that the pattern of higher burdens in peripheral or solitary fish may be a consequence of their higher rates of exposure to cercariae. It is not possible to distinguish these alternatives until we know more about the stability of individual shoal positions within natural populations. Indeed, it is possible that each of these processes may operate within natural shoals to determine the tendency for peripheral fish to have higher numbers of parasites.

Results from the mesocosm experiment show that individuals located within shoals have, on average, fewer metacercariae than solitary fish. These results are consistent with results from a field study involving shoaling killifish, Fundulus diaphanus, where individuals collected from random positions within shoals had fewer metacercariae than adjacent fish collected outside shoals (Ward, Hoare & Couzin 2002). As for the results from field-based studies such as this, our experimental results are difficult to explain. First, we cannot rule out the possibility of experimental artefacts that differentially affect solitary vs. grouped minnows. This would occur for instance, if individual minnows confined to solitary cages were more physiologically stressed than grouped minnows. We cannot address this possibility until more is known about the linkage between stress and the recruitment of metacercariae in fish. Further, the encounter/dilution scenarios that are typically invoked to explain reduced predation on shoaling fish, and reduced attack rates on grouped mammals by parasitic flies, are unlikely to apply to cercariae. Whereas predators actively seek individual prey located within a group, cercariae tend to rely on passive encounter with their hosts, although behavioural responses to light, water turbulence and shadows can facilitate host/cercariae contact (Haas 2003). Thus, it is unlikely that individual fish in a shoal reduce their rates of exposure by reducing their probability of encounter by actively ‘hunting’ cercariae.

Subtle differences in the behaviours of individual fish could also explain the observed differences in metacercariae intensity between the shoaling and solitary fish. Ward et al. (2011) have shown that mosquitofish within experimental shoals had a reduced rate of turning and that they reduced the tortuosity of their linear paths compared with adjacent solitary individuals, whether or not they were exposed to a simulated predator. For parasites that require passive contact with hosts located within the water column, behaviours associated with increased host activity often lead to increased probability of encounter (Poulin, Rau & Curtis 1991; Karvonen, Seppälä & Valtonen 2004). Thus, subtle differences in host behaviour may provide the most simple explanation for the observed differences in metacercariae intensity between shoaling and non-shoaling fish. If this is the case, the ‘safety in numbers’ that occurs for shoaling fish relative to risk of infection may be a consequence of generalized swimming behaviour of individuals in a shoal. The alternative explanation, that individuals actively assort themselves into tight shoals following detection of cercariae, requires that the behavioural observations arising from the laboratory detection experiment provide a mechanism to explain results of the mesocosm experiment. This interpretation requires caution, especially because different species of cercariae were used in the two experiments.

It is interesting to consider the observed shoaling response to cercariae from the parasite’s perspective. Results from the mesocosm experiment show that the total number of metacercariae recovered within a tank was higher for shoaling fish than solitary fish. Thus by extension, the 100’s or 1000’s of cercariae that are released each day from a single snail are more likely to infect fish that are in a shoal than those outside a shoal, all else (such as host density and size) being equal. Trematode cercariae utilize various strategies to detect and penetrate appropriate hosts, depending on the species (review by Haas 2003). While some cercariae are limited to passive swimming strategies adapted for entering their host’s active space, others respond to features such as water turbulence and shadows (Haas 2003). Thus, it is possible that detection and/or penetration of appropriate hosts may increase for fish in shoals compared with single fish. Under this scenario, the benefit to individual cercariae arises from a
higher probability of transmission into grouped fish on the one hand, but reduction in the number of cercariae that infect an individual fish on the other. Poulin & FitzGerald (1989) showed that increased group size diluted the numbers of attacks of a crustacean ectoparasite on individual hosts, but did not have a negative effect on transmission success. If such a benefit holds for cercariae/fish systems, we might expect specific infection strategies that favour cercariae transmission into shuffling fish.

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