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

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Abstract

Group living animals can gain protection against parasitic infections through social contacts with previously infected conspecifics (social immunisation). Recent research suggests that such protective effects can be induced through visual or chemical cues released by infected individuals, resulting in anticipatory immune upregulation among group members. Here, we study cue-induced social resistance in rainbow trout *Oncorhynchus mykiss* exposed to a trematode parasite, the eye-fluke *Diplostomum pseudospathaceum*. We established groups of naïve individuals (receivers) that were paired with previously infected individuals (donors) at different ratios of donors to receivers and at different time points since donor exposure to capture varying concentrations of the anticipated cues. While the pre-infection elevated resistance among the donors, there was no evidence of social transfer of resistance, regardless of the ratio of donors and receivers in a group or the time since the pre-infection. The results suggest that resistance through social signalling may be system-specific and requires further study into the generality of the phenomenon as well as the nature of the cues involved.

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

Introduction

Parasites are expected to play an important role in the evolution of sociality as group living is typically accompanied by both health-related costs and benefits (reviewed in Côté & Poulin 1995; Kappeler et al. 2015). For example, social interactions between group members increase the risk of contracting contagious parasites (Alexander 1974; Côté & Poulin 1995; Rifkin et al. 2012; Patterson & Ruckstuhl 2013; Kappeler et al. 2015). On the other hand, the risk of infection with non-contagious parasites acquired from the environment can be lower in groups due to a decreased *per capita* attack rate with increasing group size (dilution effect, Poulin & FitzGerald 1989; Mooring & Hart 1992), or due to improved parasite avoidance, possibly through increased vigilance and information sharing (Stumbo et al. 2012; Mikheev et al. 2013).

Recent research has revealed that group-living can also confer protection against contagious parasites through social immunisation, where naïve group members show improved resistance to parasites after social contacts with previously exposed group mates (reviewed in Masri & Cremer 2014). This could result from immune priming following a low-dose parasite transfer from infected individuals to naïve group members (Konrad et al. 2012) or from a direct transfer of antimicrobial compounds between individuals (Hamilton et al. 2010). Moreover, two recent studies suggest that social immunisation can also be induced through cues perceived by naïve individuals in infected group mates that cause anticipatory immune upregulation. For example, naïve rainbow trout (*Oncorhynchus mykiss*) shoaling with conspecifics that have recovered from a recent nonlethal bacterial infection show improved survival after a challenge with more lethal doses of the same pathogen (Mothersill et al. 2015). The nature of the cue aiding in transfer of resistance is unknown, but it appears to be released as response to

pathogenic stress (Mothersill et al. 2015). Further, humans visually perceiving symptoms of infectious disease upregulate their immune system compared to individuals that perceive non-disease-related threats (Schaller et al. 2010). In fact, many animals show clear signs of infection, such as altered behaviour or changes in appearance or olfactory identity, that are perceived by conspecifics (reviewed in Hart 1990; Kavaliers et al. 2004; Curtis 2014) and could be used to assess infection risk. Given the general nature of these cues, we propose that social immunisation may not only protect gregarious animals against contact-transmitted diseases, but also against infectious parasitic stages prevailing in the environment. For example, although group members carrying non-contagious parasites do not pose a direct infection risk, they may signal the risk of acquiring such parasites from the environment, making anticipatory defence reactions beneficial.

As immune functions incur a number of costs (Sheldon & Verhulst 1996; Lochmiller & Deerenberg 2000; Graham et al. 2005), the cues used for preventive upregulation must be reliable. The risk of exposure to free-living parasitic stages can be variable and unpredictable, particularly for mobile hosts that move in and out of infection areas. Here, the proportion of infected individuals within a group and/or the intensity of infection in individual group members may indicate parasite prevalence in the environment and consequently, general infection risk. This could be mediated by the strength of cue emission in the group with a certain threshold at which immune upregulation becomes cost effective for naïve conspecifics. Further, the reliability of cues indicating infection with parasitic stages acquired in the environment may decrease with the age of infection, as older infections do not necessarily coincide with the presence of parasites in the environment. However, if cue emission is induced by pathogenic stress (see Mothersill et al. 2015), its occurrence or strength may vary from the initial exposure to the appearance of symptoms and possibly clearance, depending on the specifics of each host-parasite interaction.

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

Here, we study cue-induced immunisation against the trematode *Diplostomum pseudospathaceum*, in relation to infection prevalence within host groups and the time from the initial parasite exposure of the host group members. The parasite's life-cycle involves asexual reproduction in the first intermediate snail host, a second intermediate fish host that serves as transmission vehicle (no reproduction) and finally sexual reproduction in the final bird host (Chappell et al. 1994). Free-living stages (cercariae) emerge in large numbers from the snail hosts and upon encounter penetrate the skin or gills of the fish. Then, they move towards its eye lenses, causing damage to body tissues and blood vessels during migration (Erasmus 1959; Ratanara-Brockelman 1974). Fish hosts have been shown to suffer from pathogenic stress caused directly by the acute invasion of the parasite, as heart rates can increase for several days following exposure (Laitinen et al. 1996), and activity decreases (Gopko et al. 2015). In the host's eye lens, the parasites develop to metacercariae (the bird-infecting stage) within 4-8 weeks that induce eye cataracts (Chappell et al. 1994; Karvonen 2012). These cataracts impair host vision (Shariff et al. 1980) and consequently affect fish physiology and behaviour (Seppälä et al. 2005b;a; Karvonen & Seppälä 2008; Seppänen et al. 2008; Voutilainen et al. 2008), but in a different manner than acute invasion. Although, this eye-fluke is one of the most common fish parasites in both natural populations (Chappell et al. 1994; Valtonen & Gibson 1997) and aquaculture conditions (Chappell et al. 1994; Karvonen et al. 2006), the prevalence of infection varies greatly among host species and populations (Chappell et al. 1994; Valtonen & Gibson 1997; Rellstab et al. 2011; Karvonen 2012), leading to variation in the ratio of infected to uninfected individuals. As infection prevalence among snail hosts is also variable among

populations, but generally low (Louhi et al. 2013) and cercarial shedding is seasonal (Karvonen 2012), fish hosts experience variable encounter rates in space and time.

Many fish species, including rainbow trout, aggregate in groups that include extensive social interactions (shoals; Pitcher & Parrish 1993; Delcourt & Poncin 2012), mediated by visual, chemical, mechanical, electrical and acoustic communication (Rosenthal & Lobel 2006). Whether infections with *D. pseudospathaceum* can be communicated among group members is unknown, but there is evidence suggesting that fish are able to recognize infections with other non-contagious parasites (Barber et al. 1998; Tobler & Schlupp 2008) and that they can transfer stress via chemical cues (Toa et al. 2004; Vavrek & Brown 2009; Barcellos et al. 2011; Giacomini et al. 2015). We established experimental shoals of rainbow trout that were composed of naïve individuals (receivers) and already infected individuals (donors) in different ratios (30:10 and 10:30) and exposed them to *D. pseudospathaceum* five days after shoal establishment. We repeated this setup at different time points after the original infection of the donors. Based on the anticipated stress-related emission of cues indicating infection risk, we predict that (i) receivers show socially induced resistance to *D. pseudospathaceum* and such effects are stronger when cues most likely coincide with the presence of the parasite in the environment, i.e. (ii) in donor-biased compared to receiver-biased groups and (iii) when infections are recent.

Methods

Experimental animals

Juvenile rainbow trout (size selected, average body mass 2.9 g) were obtained from a fish farm in Finland on 23 June 2015. The farm is supplied with groundwater, which ensured that all individuals were free of *D. pseudospathaceum* infection. During the experiment, the fish were kept in aerated ground water (17°C) and fed daily with commercial fish pellets.

Cercariae of *D. pseudospathaceum* were obtained from naturally infected snail hosts *Lymnaea stagnalis*, collected from Lake Vuojärvi (Central Finland, 62° N, 25° E) during 22 – 28 June 2015. The snails were maintained individually at 4 °C and were fed with lettuce *ad libitum*. Before each experimental infection (see below), 12-14 snails were transferred to room temperature and allowed to shed cercariae for a maximum of 3 hours. The cercarial suspensions of all snails were combined and parasite density was estimated by counting the number of parasites in ten 1 ml samples.

All procedures performed in this study were in accordance with the ethical standards of the Finnish Regional State Administrative Agency (License code: ESAVI/4415/04.10.07/2014).

Experimental setup

Rainbow trout were haphazardly divided into six randomly assigned tanks (500 l), two of which housed 290 individuals each (receivers) while the remaining four housed 230 individuals each (donors). On 27 June, receivers were marked by clipping the adipose fin under anaesthesia (MS-222) so that they could be separated from donors in the experimental groupings. The donors were also anaesthetised, but returned to their tanks without fin clipping.

On 28 June, the water volume in all six tanks was lowered to 100 l. To produce the donors, fish in two randomly selected tanks were each exposed to an estimated number of 2300 parasite cercariae (10 cercariae per fish; ‘infected donors’) while the two other donor groups

were sham exposed with ground water without parasites ('control donors'). Receiver groups were not exposed. After 30 minutes, the water volume in all tanks was brought back to 500 l.

Fish groups consisting of donors and receivers were established at three different time points after the exposure of donor individuals: 2 days post-exposure (p.e.) capturing the initial stress effects of the infection, 21 days p.e., when parasites were still developing, and 34 days p.e., when parasites were fully developed and inducing cataracts. Each time, 80 'infected donors', 80 'control donors' and 160 'receivers' were haphazardly selected from the holding tanks and distributed among eight other tanks, each containing 40 individuals in 180 l of water. The groups were formed so that four tanks had combinations of 'infected donors' and 'receivers' in proportions 30:10 (2 tanks) and 10:30 (2 tanks), while the other four tanks had combinations of 'control donors' and 'receivers' in equal proportions and replication.

After five days of social contact within a tank, all group members were exposed individually to *D. pseudospathaceum* in small containers with 500 ml of water and 100 cercariae. After 30 minutes of exposure, all fish were returned to their groups for 24 hours to allow parasite establishment in the eye lenses. Subsequently, all fish groups were euthanized with an overdose of MS-222 anaesthetic, measured for length and dissected for parasite numbers. Dissection was conducted blind to the treatment applied to each group. Metacercariae established during the pre-exposure and the re-exposure of 'infected donors' could be differentiated by their clear size difference (Sweeting 1974). Fish length increased with time (GLM, $X^2 = 772.8$, $P < 0.001$), but did not differ between infected donors, control donors and receivers grouped with infected and control donors ($X^2 = 4.8$, $P = 0.184$, interaction not significant).

Statistics

Parasite load (both eyes combined) was analysed using generalized linear mixed models (GLMM) with Laplace approximation and negative binomial probability distribution. Parasite load (excluding parasites from the pre-exposure of ‘infected donors’) was entered as dependent variable and treatment (‘infected donors’, ‘control donors’, ‘receivers’ and ‘control receivers’), ratio of donors to receivers (30:10 and 10:30), time since pre-exposure (7, 26 and 39 days) and all interactions were entered as fixed factors, and fish length as covariate. Each fish group was labelled with an individual ID, which was included as random factor ($N = 24$) to account for potential group effects. The analysis revealed a negative effect of fin-clipping on parasite resistance, as fin-clipped receiver fish had significantly higher parasite loads than both control donors and infected donors ($P < 0.019$ for all pairwise *Bonferroni* corrected comparisons of donor and receiver fish). This was unexpected as adipose fin clipping in salmonid fish is considered to be non-invasive with negligible effects (Use of Fishes in Research Committee 2014). To exclude this effect, two separate models, one including the two donor groups (‘infected donors’ and ‘control donors’) and the other the two receiver groups (grouped with ‘infected donors’ and with ‘control donors’) were used. Further, due to a miscalculation, one group (round 2, 30 control donors : 10 receivers) consisted only of 29 individuals. However, as the ratio of donors to receivers was comparable to the original setup (21 control donors : 8 receivers), the group was included into the statistical analyses. Thus, the final sample sizes were 240 for ‘infected donors’, 231 for ‘control donors’, 240 for ‘receivers’ and 238 for ‘control receivers’. All analyses were conducted using SAS (v. 9.4).

Results

All ‘infected donors’ harboured fully developed metacercariae following the pre-exposure with an average of ($\bar{x} \pm \text{SE}$) 8.1 ± 0.2 parasites per fish (range 2-17). These infections activated host resistance and resulted in a reduced parasite infection success among the ‘infected donors’ compared to ‘control donors’ (Table 1, Figure 1a). The reduction in parasite load among the ‘infected donors’ also increased with time from 2.7% one week post exposure, to 10.4 % four weeks post exposure and 13.6 % six weeks post exposure (Figure 1 a), but this change was not statistically significant (Table 1). Parasite load also decreased with time since pre-exposure in both ‘infected donors’ and ‘control donors’ (Table 1, Figure 1a), which was most likely caused by a decreasing parasite infectivity with time. The ratio of donors to receivers in a group did not affect parasite load among donor individuals (Table 1). Finally, parasite load was negatively related to fish length (Table 1), a pattern that is commonly observed in this system.

In contrast, parasite load did not differ between receivers grouped either with ‘infected donors’ or ‘control donors’ (Table 2, Figure 1b) suggesting absence of transfer of infection resistance. Parasite load decreased again with time (Table 2, Figure 1b), but there was no interaction with treatment, indicating that the time since pre-exposure in donors had no effect on the result. There was also no effect of the ratio of donors to receivers in a group (Table 2).

Discussion

Recent research suggests that infection resistance of fish to bacteria can be induced in naïve individuals without an actual contact with the pathogen through cues emitted by previously infected conspecifics (Mothersill et al. 2015). Here, we did not find such an effect in rainbow

trout exposed to the trematode *D. pseudospathaceum*. Resistance was comparable for individuals that had been grouped either with infected conspecifics or with uninfected control individuals. Resistance was also not affected by the ratio of infected individuals in a group or the time from their initial exposure. Overall, this suggests that the occurrence of cue-induced resistance may be system-specific and requires more study as to the exact mechanisms.

In the rainbow trout - *Vibrio* system, the cue perceived by receiver individuals caused an increase in cellular calcium (Mothersill et al. 2015). A similar response was observed by receiver fish paired with conspecifics that had been exposed to physical stressors, such as radiation (Lyng et al. 2000; Mothersill et al. 2006). Other studies have also shown that chemical cues emitted by infected individuals and individuals experiencing other forms of stress can induce similar responses in conspecifics. For example, female mice exposed to urine of both, males infected with a sporozoan or nematode parasite and physically stressed males, show decreased sensitivity to pain mediated through increased opioid levels (Kavaliers & Colwell 1993; Kavaliers et al. 2006). Although, the main explanation for this response is facilitation of behavioural infection avoidance (Kavaliers et al. 2004), opioids also play a role in immune signalling and may thus be involved in anticipatory immune upregulation (Penn & Potts 1998 and references therein). Generally, fish can perceive stress induced by various sources in conspecifics and consequently produce a physiological stress response (Toa et al. 2004; Vavrek & Brown 2009; Barcellos et al. 2011; Giacomini et al. 2015). Thus, cues released in consequence of stress could be good candidates for immunisation through social signalling. However, our findings suggest that this needs to be verified in different systems.

The perception of a cue inducing protection against infection likely depends on the strength of its emission within a social group. This may vary not only with the ratio of donors to

receivers, but also with exposure doses experienced by the donors and the resulting infection intensities. For example, in some fish species, physiological responses associated with exposure to *D. pseudospathaceum* have been observed only at high exposure doses (Laitinen et al. 1996). Although exposure doses and the resulting parasite loads in the present study were in the range of those expected (dose) or observed (load) under natural conditions (e.g. Valtonen & Gibson 1997), they were on the lower end of the range, as donor individuals harboured on average four parasites per eye after the pre-exposure. Consequently, pathogenic stress levels of donors may have been too low to induce socially triggered resistance in receivers. However, a resistance response was elicited earlier in rainbow trout grouped with conspecifics that likely experienced only moderate pathogenic stress, as these had been exposed to a nonlethal dose of bacteria and had already recovered from the infection (Mothersill et al. 2015).

Using cues emitted by infected conspecifics to upregulate personal immune responses can offset the increased risk of contracting contagious parasites in groups and may thus be seen as an adaption to compensate health related costs of sociality (Masri & Cremer 2014). If so, selection pressures on social immunisation against non-contagious parasites may be low. First, infections with non-socially transmitted parasites may not reliably signal infection risk to conspecifics, as they do not necessarily coincide with the presence of infective stages in the environment. For example, in the present system with infection hotspots and highly mobile hosts, infection risk may be too variable and unpredictable to make cue-induced immunisation cost-effective. However, infection risk also varies seasonally, as cercarial production in the snail hosts is temperature regulated (Karvonen 2012). In northern latitudes, for example, infection risk prevails only during 3-4 months each year (Karvonen et al. 2004). Thus, infection in others, particularly if recent, may signal the onset of cercarial shedding and thus, an overall risk of

infection. Second, grouping is expected to provide other benefits against free-living parasitic stages. In our study system, infection intensities with *D. pseudospathaceum* decrease with group size, possibly due to a dilution effect (Karvonen et al. 2005). Other experiments have also demonstrated a decreased exposure risk to trematode parasites in shoaling versus solitary fish (Stumbo et al. 2012). Additionally, groups are also more efficient in behaviourally avoiding *D. pseudospathaceum* compared to solitary individuals (Mikheev et al. 2013), possibly due to an enhanced potential for parasite detection ("many eyes" theory; Treherne & Foster 1980; Lima 1995). However, as sociality enhances the possibility of acquiring information from others, it may provide additional protection against virulent non-contagious parasites present in a group's environment.

In conclusion, our results do not support the prediction that group living induces social resistance to a trematode infection. Social immunisation is an emerging field of research that may have important implications for disease dynamics and, owing to a natural vaccine effect, for the management of natural and captive populations. However, the mechanisms of cue-induced social immunisation are not well understood. More studies are needed to gain insights into the generality of the phenomenon in different host-parasite systems and the nature of the cues involved in protective immune stimulation.

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Table 1 General linear mixed model (GLMM) analysis of parasite load in ‘infected donors’ and
‘control donors’, explained by treatment (pre-exposure and sham exposure), ratio of donors to
receivers in a group (30:10 and 10:30), time since pre-exposure (7, 26 and 39 days) and fish
length. Fish group is included in the model as a random factor.

factors	<i>df</i> denominator	<i>df</i> numerator	<i>F</i>	<i>P</i>
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

Table 2 General linear mixed model (GLMM) analysis of parasite load in receivers, explained by treatment (grouped with ‘infected donors’ and with ‘control donors’), ratio of donors to receivers in a group (30:10 and 10:30), time since pre-exposure of donors (7, 26 and 39 days) and fish length. Fish group is included in the model as a random factor.

factors	<i>df</i>	<i>df</i>	<i>F</i>	<i>P</i>
	denominator	numerator		
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

Figure 1

Fig 1 Parasite load (Least-square means $\pm$ SE) of (a) donor individuals and (b) receiver individuals after experimental exposures varying in time since pre-exposure of ‘infected donors’.