Parasitic infection of the eye lens affects shoaling preferences in three-spined stickleback

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The ability to compete with conspecifics and to adequately respond to visual stimuli of group mates are important prerequisites for profiting from group benefits such as confusion of predators and greater efficiency in acquiring food. By impairing their host's physical abilities or making the host conspicuous, even non-contagious parasites that do not pose a direct risk of infection can interfere with group dynamics. *Diplostomum pseudospathaceum*, a widespread parasite of freshwater fishes, infects the eye lens and can impair the vision of its fish host. To test whether this eyefluke affects competitiveness and/or shoaling behaviour in three-spined stickleback (*Gasterosteus aculeatus*), experimentally infected fish were kept in mixed groups comprising infected and uninfected sticklebacks under limited food availability in semi-natural outdoor tanks. Change in body mass over time was measured and sticklebacks were given the choice to shoal with uninfected conspecifics or a mixed group in binary shoal choice experiments. Surprisingly, uninfected sticklebacks spent significantly more time with mixed shoals than with uninfected shoals while this preference was not found in infected sticklebacks. Infection did not significantly affect body condition or immune parameters indicative of stress level (relative spleen mass, granulocyte-to-lymphocyte ratio). The results suggest that sticklebacks can distinguish mixed from uninfected groups, but that they are also able to tolerate potential detrimental effects of infection. Whether uninfected fish can benefit from shoaling with infected but non-contagious conspecifics remains to be tested. Although the present data do not indicate a significant effect of infection on competitiveness, this should be examined further.

ADDITIONAL KEYWORDS: *Diplostomum* – fish – *Gasterosteus aculeatus* – parasitism – social behaviour – tolerance

INTRODUCTION

Parasitic infections can have a significant influence on grouping behaviour (reviewed by Barber, Hoare & Krause, 2000; Krause & Ruxton, 2002). Being part of a group usually involves several advantages, such as reduced predation risk, more efficient acquisition of food sources and reduced energetic costs (e.g. Krause & Ruxton, 2002). However, parasites can reduce the benefits of gregariousness in several ways that either directly affect grouping tendencies of infected individuals or make them less attractive group mates

for uninfected conspecifics. Hosts of directly transmitted parasites, for example, should be avoided by uninfected individuals to reduce the probability of infection, as has been shown in three-spined sticklebacks, *Gasterosteus aculeatus* (Ward *et al.*, 2005; Rahn, Hammer & Bakker, 2015).

Generally, to what extent a parasite can affect grouping behaviour depends largely on its effect on the appearance and physical capabilities of the host (Krause & Ruxton, 2002) and is therefore system-specific. Conspicuousness caused by an infection, such as altered coloration (Seppälä, Karvonen & Valtonen, 2005a; Ondrackova *et al.*, 2006) or abnormal behaviour (Lafferty & Morris, 1996), potentially increases predator attraction (e.g. Landeau & Terborgh, 1986; Bakker,

Frommen & Thünken, 2017) for 'odd' individuals ('oddity effect', Ohguchi, 1978) and for their shoal mates (Landeau & Terborgh, 1986). Consequently, uninfected group members could counteract an increased risk of predation by preferring to associate with uninfected individuals even in the absence of a direct risk of infection, i.e. where trophically transmitted parasites are involved. Evidence of avoidance of hosts of non-contagious parasites has been found in mosquitofish (Tobler & Schlupp, 2008), killifish (Krause & Godin, 1996) and sticklebacks (Barber, Downey & Braithwaite, 1998). In these systems, infection comes with clearly visible phenotypic changes (black spots on/in the skin or swollen abdomen), but parasites that do not cause oddity in infected individuals are also able to interfere with group dynamics by influencing their hosts' tendency to join a group of conspecifics. Grouping behaviour often goes along with competition, especially where resources are finite (Krause & Ruxton, 2002). Parasites can negatively affect competitiveness of their hosts by causing physical impairments and thereby increase the relative costs of grouping. Some fish parasites have been shown to impair buoyancy (Lobue & Bell, 1993), or affect sensory organs (Chappell, Hardie & Secombes, 1994) or the central nervous system of their hosts (Lafferty & Morris, 1996; Shirakashi & Goater, 2001). Uninfected individuals, on the other hand, might even benefit from grouping with weak competitors (Metcalfe & Thomson, 1995), particularly if these do not raise the conspicuousness of the group.

The present study examines the effects of the digenean trematode *Diplostomum pseudospathaceum* on the shoaling behaviour of three-spined sticklebacks. *Diplostomum pseudospathaceum* is a widespread, trophically transmitted endoparasite of freshwater fish (Chappell *et al.*, 1994). Its life cycle includes snails and fish as intermediate hosts, and piscivorous birds as final hosts. In its fish host, the parasite is found in the eye lenses. Unlike many other macroparasites described in the literature, it does not cause any obvious phenotypic alterations (but see Rintamaki-Kinnunen, Karvonen, Anttila & Valtonen, 2004; Seppälä *et al.*, 2005a) and is protected from the immune system of its fish host for most of the time (Streilein, 1987; Niederkorn, 2011). The parasite is able to induce the formation of cataracts that can ultimately lead to complete blindness (Shariff, Richards & Sommerville, 1980). In cyprinids and salmonids, infections with *Diplostomum* spp. can have severe consequences for food intake (Crowden & Broom, 1980; Voutilainen, Figueiredo & Huuskonen, 2008), predation risk (Seppälä, Karvonen & Valtonen, 2005b; but see Seppälä, Karvonen & Valtonen, 2006), oxygen consumption (Voutilainen *et al.*, 2008), standard metabolic rate (Seppänen *et al.*, 2008) and growth (Kuukka-Anttila *et al.*, 2010). Knowledge of the interactions between *Diplostomum* spp. and three-spined

stickleback has for the most part been limited to studies on taxonomy and distribution (e.g. Kuhn *et al.*, 2015; Locke *et al.*, 2015), and immunology (e.g. Scharsack *et al.*, 2007; Franke *et al.*, 2014; Haase *et al.*, 2014), whereas behavioural aspects have largely been ignored – except for one study (Owen, Barber & Hart, 1993), which found that a low number of *Diplostomum* metacercariae (sum of lens- and retina-infecting eyeflukes per fish: 7–34) was associated with a reduced reactive distance to prey (live *Daphnia* spp.).

The aim of the present study was to determine whether *D. pseudospathaceum* affects shoaling decisions in three-spined sticklebacks, and whether infection with the parasite results in physical disadvantages, when infected sticklebacks compete with uninfected fish for food. A possible role of eyefluke infections in shoaling decisions has not been evaluated using binary shoal choice trials, either in sticklebacks or in other fishes. Most studies examining the impact of parasites on host shoaling decisions have tested for preferences between purely uninfected shoals vs. shoals comprising only infected individuals, which is an unrealistic choice given that parasite prevalences are seldom either 0 or 100%, but rather lie between these values. Additionally, studies that make use of experimental infections have often been carried out under conditions particularly favourable for parasite development. Here, preferences of uninfected and of infected individuals for uninfected or mixed shoals were tested and experimental fish were kept in outdoor tanks under semi-natural (winter temperature) conditions. If infection with *D. pseudospathaceum* causes detectable effects on hosts, uninfected sticklebacks should prefer shoals of uninfected fish over mixed shoals. Given that even low numbers of eyeflukes might affect stickleback behaviour (Owen *et al.*, 1993), it could be assumed that infection impairs visual acuity or goes along with stress responses as an indirect result of infection even in the absence of cataracts. This could result in reduced growth under limited food conditions compared to uninfected conspecifics.

METHODS

ORIGIN AND MAINTENANCE OF STICKLEBACKS BEFORE INFECTIONS

Experimental fish were taken from a pool of approximately 320 three-spined sticklebacks maintained at the Institute for Evolutionary Biology and Ecology (University of Bonn, Germany). Young-of-the-year had been caught in a small freshwater pond in Euskirchen near Bonn (50°38′N, 6°47′E) in November and December 2012 (minnow traps: galvanized steel mesh, Gee's G40 M, G48 M, Tackle Factory, Fillmore, NY, USA) and were kept in an aerated, large outdoor tank (750 litres) with

constant freshwater supply (3 L min⁻¹). Sticklebacks were fed chironomid larvae *ad libitum* three times a week. The pond is isolated from other water bodies in a forest. We do not know whether *Diplostomum* spp. exists in the pond, but based on their shape only new *Diplostomum* spp. infections from the experiments were found during dissections (Kalbe & Kurtz, 2006). All sticklebacks were treated with Gyrodol 2 (praziquantel, JBL, Neuhofen, Germany) to remove the ectoparasite *Gyrodactylus* spp. Success of this disinfection treatment was confirmed by checking a randomly selected subsample of 50 sticklebacks for *Gyrodactylus* infections under a microscope (40× magnification, S 8 APO, Leica, Wetzlar, Germany), which was illuminated by a cold light source (KL 1500, Leica).

DIPLOSTOMUM INFECTIONS AND MAINTENANCE OF STICKLEBACKS IN OUTDOOR TANKS

Infections took place in mid-January 2013. A protocol similar to that of Kalbe & Kurtz (2006) was applied. Fifteen lab-bred *Lymnaea stagnalis* (kindly provided by M. Kalbe) that had been multiclonally infected with *D. pseudospathaceum* were placed in individual 200-mL beakers under a light bulb to induce cercarial shedding. After 2.75 h cercariae were pooled and 150 cercariae per fish were transferred to small (20-mL) plastic beakers filled with tap water. Sticklebacks were placed individually in 1-litre boxes filled with 800 mL of tap water and infected by placing the small plastic beaker with parasites (pure tap water for sham infections) in the 1-litre box. Before sticklebacks were released into holding tanks, they were individually marked by spine clipping and their body masses (to the nearest milligram) and standard lengths (distance between the tip of the mouth and the end of the caudal peduncle; measured to the nearest millimetre using graph paper) were measured. Sticklebacks were transferred to new tanks within 48 h of parasite exposure. Within the first 10 days after the infection, five sticklebacks of the uninfected treatment and four (two infected, two uninfected) of the mixed treatment died. These fish were replaced with sticklebacks that had been (sham) infected as described, but with cercariae pooled from 14 of the 15 snails. Therefore, a total of 38 sticklebacks were exposed to cercariae for the present study.

Following the infections, sticklebacks were kept in groups of six fish [12 groups of six uninfected fish ('uninfected' treatment) and 12 mixed groups of three infected and three uninfected fish ('mixed' treatment)] for 11 weeks before the shoal choice experiments began (at the beginning of April). During the winter season, i.e. at temperatures below 10 °C, development of *Diplostomum* metacercariae is usually halted

(Sweeting, 1974). Above 10 °C, metacercariae require between 3 weeks (Seppälä *et al.*, 2005b) and 2 months to become infective (Sweeting, 1974; Whyte, Secombes & Chappell, 1991), depending on the ambient temperature. To examine the effects of *D. pseudospathaceum* on the shoaling behaviour of its host when it can be assumed to be most relevant, experiments were carried out in winter. Outside the breeding season, most sticklebacks are found in loose schools (Keenleyside, 1955; Wootton, 1984) of a few individuals to up to several hundred fish (Peuhkuri, Ranta & Seppä, 1997; Poulin, 1999; Barber, 2003) while reproductively active individuals do not tend to shoal during the breeding season. Experimental fish (standard length 3.0–3.7 cm) were chosen from the stock tank so as to homogenize body sizes within groups and between treatments. Groups were kept in visually isolated 22-litre plastic tanks (39 × 28 cm, water level 20 cm) which were hung in four circular outdoor tanks (diameter 200 cm, 2500 litres). Six holes (diameter 6 cm, covered by green mesh) in the side walls of the plastic tanks enabled constant water exchange. Additionally, each outdoor tank was equipped with a pump (PonDuett 3000, 25 W, 1500 Lh⁻¹, Pontec, Germany) and submersible heaters (Jaeger 3618 and 3614, Eheim, Germany) to keep the water surface ice-free. Sticklebacks were fed two or three drops (*c.* 50–75 larvae) of chironomid larvae from a disposable pipette per tank three times a week. Remaining food was removed after 5–10 min. By the time shoal choice experiments began, natural light conditions had changed from a 9:15-h light/dark cycle to 11:13 h. At that point, sticklebacks did not show any signs of reproductive activity.

SHOAL CHOICE EXPERIMENTS

Set-up

A glass aquarium (70 × 35 cm and 35 cm high, water level 15 cm, see Fig. 1 for a schematic aerial overview of the set-up) covered with grey plastic sheets served as the test tank. On opposite sides of the tank, transparent, perforated partitions separated two shoal compartments (15 × 35 cm) from the central compartment (40 × 35 cm). Next to the transparent partitions, opaque partitions, which could be lifted from outside the set-up, provided a visual barrier between shoal and focal fish during the acclimation period. Black felt-tip pen lines drawn on the bottom of the aquarium marked the borders of 10-cm-wide choice zones adjacent to the shoal compartments. The tank was illuminated by two fluorescent tubes (36 W, True-Light, Germany) which were mounted 70 cm above the bottom of the tank together with a webcam (Pro 9000, Logitech, Fremont, CA, USA) which was connected to a laptop. The whole set-up was surrounded by a black curtain.

A

B
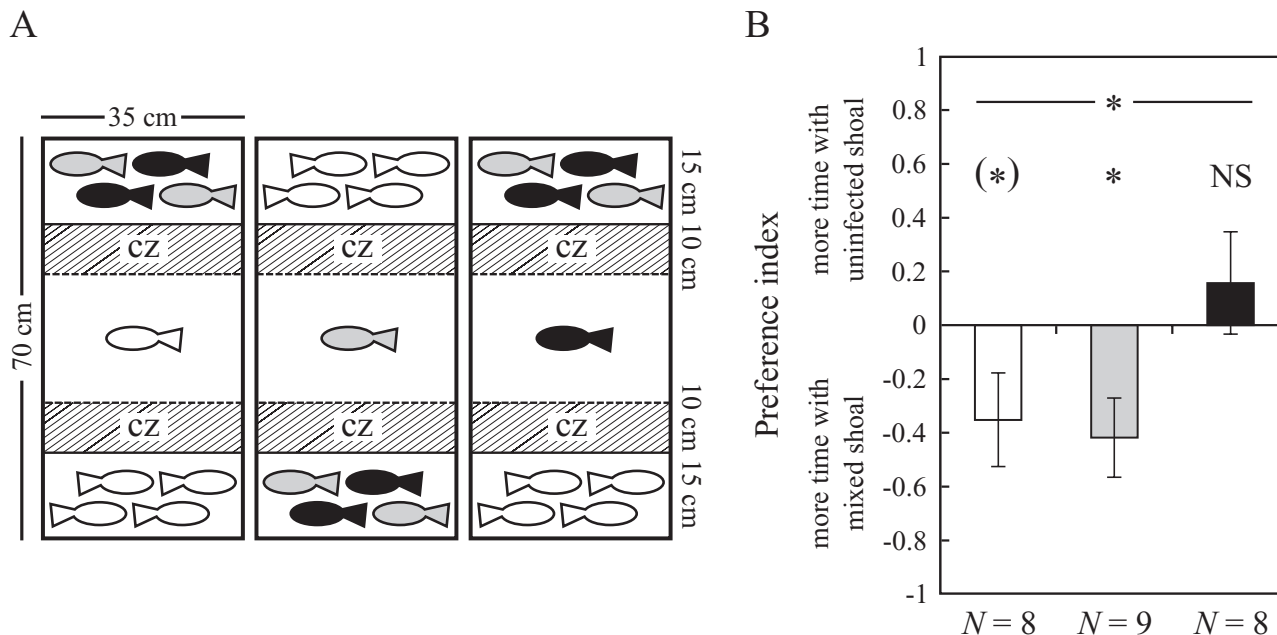
Figure 1. Set-up and main result of the shoal choice experiments. Each pair of shoals was presented to three focal fish [one of each treatment: uninfected fish from uninfected groups (white fish symbol in A and bar in B), uninfected (grey) and infected (black) fish from mixed groups]. (A) Schematic bird's eye view on the shoal-choice tank (height 35 cm, water level 15 cm); cz refers to the 10-cm choice zone in front of each stimulus shoal compartment. The order of focal fish treatments was randomized. (B) Time focal fish spent in front of uninfected and mixed shoal given as 'preference index' (mean ± SE). (*)0.1 > $P \geq 0.05$, *$P < 0.05$, $^{NS}P \geq 0.1$ (significance based on one-sample t tests; see text for details).

Experimental procedure

Three experiments (ten trials per experiment) were performed within a period of 6 days. Experiments differed only in the infection and maintenance treatment (uninfected and kept in uninfected groups, and either uninfected or infected and kept in mixed groups) of the focal fish (total $N = 30$). In each trial, a focal fish was presented two stimulus shoals of four fish each (four uninfected sticklebacks from one of the 'uninfected' group tanks and two infected and two uninfected sticklebacks from one of the 'mixed' group tanks). A total of ten shoal pairs were used and each pair of shoals was presented consecutively to three different focal fish (one of each treatment) in alternating order. In this way, infected and uninfected focal fish were given the choice between a shoal of uninfected individuals and a shoal also comprising infected sticklebacks. At the same time, this was a choice between a shoal of the same and a shoal of a different maintenance treatment ('uninfected' or 'mixed'). Sticklebacks were used only once as focal fish, but some focal fish were used as shoal fish later the same day. Focal and shoal fish always originated from different group tanks to avoid any bias due to prior social interactions. On the day prior to a set of three trials, involved groups were fed as usual and later collected from the outside treatment tanks and placed in aerated plastic aquaria (39 × 22 cm, water level 20 cm) in an air-conditioned aquarium room (15 °C room temperature, 11:13-h light/dark cycle). At the beginning of each trial, the test aquarium was filled with 1-day-old tap water. The opaque partitions were lowered and shoals and focal fish were introduced into their respective compartments. After an acclimation period of 15 min, video recording (Windows Media Encoder 9.0) was started and the visual barriers between focal and shoal fish were raised. To ensure the focal fish had seen both stimulus shoals, behavioural recording started once the focal fish had visited both choice zones and left the second one. At the end of this first trial for a respective shoal pair, focal and shoal fish were removed from the tank, which was cleaned and refilled. Shoal fish were kept in 1-litre boxes and reintroduced into the test tank, this time on the opposite side to avoid side effects. The focal fish of the first trial was weighed and measured as described before and placed back into its group tank. The second and third trial for each shoal pair was performed in the same manner. At the end of the third trial, focal and shoal fish were weighed, measured and then reintroduced into their group tanks.

Video analysis

Behaviour of focal fish was analysed for the first 5 min after the fish had visited both stimulus shoals

for the first time. Time spent in the two choice zones was measured and used to calculate a 'preference index' [(time in front of the uninfected shoal – time in front of the mixed shoal)/(total time in both choice zones)]. Additionally, 'shoaling tendency' (time spent in both choice zones) and 'activity' (number of times the focal fish crossed the lines between choice zones and the central compartment) were recorded. The person analysing the videos was blind with respect to treatment of the fish.

GROWTH AND IMMUNOLOGY

In total, 118 sticklebacks (62 of the 'uninfected' and 56 of the 'mixed' treatment) had survived until the beginning of April, despite hard winter conditions. Of the 56 (28 uninfected, 28 infected) sticklebacks of the 'mixed' treatments, the specific growth rate (SGR) was calculated as SGR = $100 \times (\ln \text{body mass}_{April} - \ln \text{body mass}_{at\ infection})/\text{days}$. To identify potential effects of the infection treatment (either direct or indirect through stress responses) on the immune status of the sticklebacks, 24 'mixed' fish (12 infected, 12 uninfected) were killed by decapitation and destruction of the brain, and then dissected. Spleen mass was weighed to the nearest milligram. The splenosomatic index [spleen mass (g) × 100/body mass (g)] was used as one measure of immune status because the spleen is an important lymphatic organ and swelling of the spleen generally indicates activation of the immune system (Zapata *et al.*, 2006). Despite the very short period of time *D. pseudospathaceum* is exposed to the immune system of its fish host, there is reason to assume that host immune responses towards *Diplostomum* infections are not completely unspecific (Haase *et al.*, 2014; Rauch, Kalbe & Reusch, 2006; Scharsack *et al.*, 2007). Therefore, the granulocyte to lymphocyte ratio (G/L ratio) – a measure of the activation of the innate system in relation to the adaptive immune system – of the head kidney leucocytes was determined by flow cytometry. After the experimental period, all sticklebacks that had survived until mid-April were also dissected to confirm infection with *D. pseudospathaceum*.

Determination of the G/L ratio took place on two days during and directly after the experimental period and was carried out as described by Scharsack *et al.* (2007). In brief, suspensions of head kidney leucocytes were obtained by forcing head kidneys of freshly killed sticklebacks through a nylon mesh (BD Falcon cell strainer, 40-µm mesh size). Cell suspensions were washed twice (4 °C, 5 min, 600 ***g***) with, and resuspended in, 90% RPMI 1640 medium before numbers of intact lymphocytes and granulocytes were determined by flow cytometry (FACSCanto II with software FACSDiva version 6.1.2, both BD Biosciences, Franklin Lakes, NJ, USA).

Only immune and growth data of the 'mixed' treatment (infected and uninfected fish) are reported here because sticklebacks of the 'uninfected' treatment were used in another study (Vitt *et al.*, 2017).

STATISTICAL ANALYSIS

Statistical analysis was conducted in R 3.3.1 (R Core Team, 2013). Kolmogorov–Smirnov tests were used to test for deviation from a normal distribution ($P < 0.05$). All dependent variables (except parasite counts) either met the assumptions of a normal distribution or could be transformed (splenosomatic index). Analyses are based on a total of 25 focal fish (eight uninfected from uninfected groups, nine uninfected from mixed groups, eight infected from mixed groups), because five out of 30 focal fish had entered only one of the two choice zones within 25 min after the visual barriers had been raised. Mean body size, mass and condition of the stimulus shoals were compared using paired *t* tests.

To test whether focal fish of the three different treatments preferred one of the two shoal types, 'preference indices' were tested against 0 using one-sample *t* tests. Linear mixed-effects (LME) models (nlme package; Pinheiro *et al.*, 2017) with 'preference index' as the dependent variable and focal fish treatment as a fixed factor were run to test whether the three different types of focal fish differed in their shoal preference and whether shoal preference was explained by activity, 'shoaling tendency', body size (standard length) or body condition (all as fixed factors). 'Trial' (whether it was the first, second or third trial for a given shoal pair) was included as a covariate and shoal pair as a random factor.

Standard length, body mass and body condition [[mass (g) × 100]/standard length (cm³), Fulton's condition factor as cited by Ricker (1975)] of the focal fish were compared using one-way ANOVAs. An LME with 'shoaling tendency' as the dependent variable, shoal pair as a random factor and 'trial' as a covariate was used to test for differences in shoaling tendency among the three focal fish treatments (fixed factor). Additional Spearman rank correlations with total parasite counts of infected focal fish were used to test for associations between intensity of infection and 'preference index', 'shoaling tendency' and 'activity'.

LME models with SGR as the dependent variable, treatment as a fixed factor and group tank as a random factor were used to test for differences between infected and uninfected fish regarding growth of all sticklebacks of the 'mixed' groups that had survived until April. Additional LMEs tested whether the 24 fish examined for immune status differed in G/L ratio or (log)splenosomatic index. For this, G/L ratio and splenosomatic index were used as dependent variables, infection treatment as a fixed factor and group tank as a random factor.

For all models, significance was determined by step-wise model reduction and likelihood-ratio tests. Fixed factors with $P < 0.05$ were kept in the models. P values are two-tailed throughout. Spearman rank correlations were used to test for associations between parasite numbers (total number per stickleback and number of metacercariae in the least infected eye) and body size, mass and body condition as suggested by Buchmann & Uldal (1994) and Karvonen & Seppälä (2008). An overview of all models used in the analysis is given in Table 1.

ETHICAL STATEMENT

Infection and behavioural experiments were performed in accordance with German legislation and approved by the regional office for nature, environment and consumer protection North-Rhine Westphalia (LANUV NRW, reference number 8.87-51.04.20.09.352).

RESULTS

No cercariae were found in sticklebacks of the 'uninfected' (pure and mixed) treatment groups (one fish was not dissected). All but one stickleback of the 'infected' treatment were infected with at least one cercaria per fish [median infection intensity 13 parasites per individual (first, third quartile: 8, 21, $N = 34$), Supporting Information Fig. S1]. No macroparasites other than *D. pseudospathaceum* were found during dissections of the inner organs. No specific parasite screening of the guts was performed. The stimulus shoal pairs for each experiment were taken from group tanks of the same initial fish size. Consequently, stimulus shoals did not differ significantly in their mean standard length, body mass or body condition (measured after the third trial, paired t tests: $N_{uninfected} = 10$, $N_{mixed} = 10$, all $P > 0.7$).

Focal fish of the three different treatments differed significantly in their shoaling preferences (LME: Δd.f. = 2, $\chi^2 = 9.07$, $P = 0.011$, Table 1, Fig. 1). Uninfected focal fish from mixed groups spent significantly more time with mixed shoals (one-sample t test: $N = 9$, $t = -2.83$, $P = 0.022$, Fig. 1). Uninfected fish from uninfected groups showed a similar trend that failed to reach statistical significance (one-sample t test: $N = 8$, $t = -2.02$, $P = 0.083$, Fig. 1). Infected focal fish did not significantly prefer one of the two shoal types (one-sample t test: $N = 8$, $t = 0.82$, $P = 0.439$, Fig. 1). Focal fish of the three treatments did not differ in standard length, body mass, body condition (one-way ANOVAs: all $F < 1.8$, all $P > 0.2$) or shoaling tendency (LME: Δd.f. = 2, $\chi^2 = 2.45$, $P = 0.294$, Table 1), nor did any one of these measures explain preference for one of the shoal types (LMEs: all $\chi^2 < 3$, all $P > 0.1$, Table 1). Spearman rank correlations showed that parasite load (total number of eyeflukes per stickleback) was not significantly correlated with 'preference index' ($r_S = 0.31$, $P = 0.462$), 'activity' ($r_S = -0.23$, $P = 0.588$) or 'shoaling tendency' of infected focal fish ($N = 8$) although the last showed a negative trend ($r_S = -0.69$, $P = 0.069$).

Infected and uninfected sticklebacks of the mixed treatment groups did not differ significantly in growth (SGR), G/L ratio or (log)splenosomatic index (LMEs: all $\chi^2 < 1$, all $P > 0.4$, Table 1). No significant correlations were found between parasite intensity (total parasites per stickleback and number of eyeflukes in the least infected eye, $N_{mixed\ infected} = 28$) and body size, mass and body condition at the end of the experimental period (Spearman rank correlations: all $r_S < 0.12$, all $P > 0.5$).

Table 1. Results of the linear mixed-effects (LME) models used to analyse the effects of infection on stickleback behaviour, growth and body characteristics

Dependent variable	N_{uninf}	$N_{mix\ uninf}$	$N_{mix\ inf}$	Covariate	Random factor	Fixed factor	Δd.f.	χ^2	P
'Preference index'	8	9	8	Trial	Shoal pair	Activity	1	1.77	0.184
						Shoaling tendency	1	2.56	0.110
						Standard length	1	1.38	0.240
						Body condition	1	0.05	0.822
						Focal fish treatment	2	9.07	**0.011**
'Shoaling tendency' (s)	8	9	8	Trial	Shoal pair	Focal fish treatment	2	2.45	0.294
SGR	–	28	28		Tank	Infection treatment	1	0.62	0.431
G/L ratio	–	12	12		Tank	Infection treatment	1	0.02	0.901
Log$_{10}$ splenosomatic index	–	12	12		Tank	Infection treatment	1	0.26	0.610

Sample sizes for sticklebacks of the 'uninfected' (N_{uninf}), 'mixed uninfected' ($N_{mix\ uninf}$) and 'mixed infected' ($N_{mix\ inf}$) treatment groups are given. SGR, specific growth rate; G/L ratio, granulocyte to lymphocyte ratio; Δd.f., change in degrees of freedom. See main text for definitions of fixed and random factors. Significant ($P < 0.05$) P values are in bold type.

DISCUSSION

Good eyesight is essential for a visual predator and socially interacting animal. Optimal function of the visual system requires transparency of the eye lens and a parasite with the ability to compromise this transparency could severely impair competitive abilities, food intake and social interactions. In the present study, experimental infections with the lens-infecting trematode *D. pseudospathaceum* affected shoaling decisions: shoals that were heterogeneous with respect to the infection status of their members were significantly preferred over uninfected shoals by uninfected sticklebacks, while infected fish did not show a significant preference. However, infections did not result in significantly reduced physical body condition or deviating immune parameters.

The fact that uninfected sticklebacks spent significantly different amounts of time close to uninfected and mixed shoals suggests that uninfected focal fish were able to distinguish both types of shoals. Unusual behaviour of infected shoal fish could be one explanation, but it is also possible that uninfected shoal members showed a particular behaviour towards infected stimulus fish. The preference for mixed over uninfected shoals seems surprising at first glance. Although the parasite is not transmittable between fish, it might still affect the behaviour of its host and make the group more vulnerable to predation. Observations on experimentally eyefluke-infected rainbow trout (*Oncorhynchus mykiss*) revealed that infected animals formed smaller shoals and did not increase shoal cohesiveness after a simulated (avian) predator attack as compared with control fish (Seppälä, Karvonen & Valtonen, 2008; median proportion of the lens covered by parasite-induced cataract 50–75%).

Given that infected fish are not more conspicuous than uninfected fish and do not increase the predation risk (Seppälä *et al.*, 2006), uninfected fish could even benefit from shoaling with potentially weak competitors (Metcalfe & Thomson, 1995) with no risk of contracting an infection. It is not clear whether sticklebacks are able to recognize *Diplostomum* infections inside the eyes of their conspecifics. There is growing evidence that fish (juvenile rainbow trout) are able to perceive free-swimming *Diplostomum* cercariae and can learn to avoid areas where these are present (Klemme & Karvonen, 2016). They were also better at performing this task in a group than alone (Mikheev *et al.*, 2013), which suggests that social information plays a role in avoidance of new *Diplostomum* infections. Performance within a shoal partially depends on vision (Partridge & Pitcher, 1980). The absence of significant effects on shoal preference in infected focal fish indicates that infection might have affected the hosts' ability to identify infected conspecifics.

Overall, the results did not point to reduced competitiveness due to visual impairment caused by the parasite. This is surprising, given that food availability was limited to three feedings per week in the present study and that lens-infecting *Diplostomum* affected prey detection in sticklebacks (*G. aculeatus*, Owen *et al.*, 1993), dace (*Leuciscus leuciscus*, Crowden & Broom 1980) and Arctic charr (*Salvelinus alpinus*, Voutilainen *et al.*, 2008) in feeding experiments. The absence of cataracts in the eye lenses of most experimental fish (only the most heavily infected sticklebacks showed the beginning of opacity) at the end of the experimental period seems the most plausible explanation for this. In infected rainbow trout, the number of eyeflukes in the least infected eye, but not the total number of metacercariae per fish, was negatively correlated with body weight (Buchmann & Uldal, 1994). This correlation could not be confirmed for sticklebacks in the present study nor in an experimental study of whitefish (*Coregonus lavaretus*, Karvonen & Seppälä, 2008). The results of the present study are in accordance with a range of experimental studies that suggest that only heavy, cataract-causing eyefluke infections affect host nutrition and body condition (Karvonen & Seppälä, 2008; Kuukka-Anttila *et al.*, 2010). Experiments were carried out in winter at low ambient temperatures (water temperature inside the group tanks 0–5 °C). At these temperatures, metacercariae still move, but development is generally retarded and larvae become more active and therefore more likely to cause cataracts once temperatures rise above 10 °C (Sweeting, 1974). In experiments with juvenile Arctic charr, exposure to low temperature (9.5 °C), but not optimal temperature (14.5 °C), resulted in lower specific growth rates of eyefluke-infected fish (Voutilainen, Taskinen & Huuskonen, 2010). This could point towards a trend that close to their temperature limits fish have reduced ability to compensate for damage caused by eyeflukes (Voutilainen *et al.*, 2010). Unfortunately, this has seldom been tested. The results of the present study do not support a deteriorating effect of low temperatures on potential impairments caused by the parasite.

One further explanation might be that the food (dead, red chironomid larvae) was too easy to detect and handle and that marginal visual impairments therefore did not result in a competitive disadvantage. As it has repeatedly been shown that parasites can influence food intake (Crompton, 1984; Milinski, 1984; Tierney, 1994; Arneberg, Folstad & Karter, 1996), an interesting question for further studies (both on the intra- and on the interpopulational level) is whether fish change their food preferences when eyeflukes

significantly impair vision. In dace and Arctic charr the increase in the number of failed attacks on live prey was compensated for by a longer period of time spent feeding (Crowden & Broom, 1980; Voutilainen *et al.*, 2008). In the present study, time for feeding and therefore the ability to compensate for failed attempts or food items lost to an uninfected conspecific was limited to 5–10 min. Given the small group sizes and the lack of an effect on body condition, the results could also indicate that the feeding regime was still not sufficiently limited to induce competition.

Diplostomum metacercariae migrate to the eyes and invade the lenses within hours of infection (Chappell *et al.*, 1994). Once inside the eye lens, parasites are protected from the host's immune system due to the immune privilege of this portion of the eye (Streilein, 1987; Niederkorn, 2011). Thus, the parasite is exposed to the immune system of the host only for a short period of time and immune defence is based on (specific) innate immune responses (Haase *et al.*, 2014; Scharsack & Kalbe, 2014). Within the first few days after infection, activation of the innate immune system ceases (Scharsack & Kalbe, 2014). Therefore, potential effects on variables relevant to the immune system were not expected to be the result of a direct influence of infection. G/L ratio is associated with 'stress hormones', such as cortisol (Davis, Maney & Maerz, 2008). An increased relative level of head kidney granulocytes and an enlargement of the spleen due to increased leucocyte synthesis in infected sticklebacks would suggest additional stress as an indirect result of the infection. Experimental *Diplostomum* infections resulted in higher oxygen consumption (Voutilainen *et al.*, 2008) and larger spleens and livers (Seppänen *et al.*, 2009) in Arctic charr. In line with the other traits examined in the present study, the absence of significant effects on G/L ratio and splenosomatic index more than 2 months after exposure to the parasite does not suggest additional energetic costs produced by the infection.

Once metacercariae have reached the infective stage, they can increase their fitness by influencing the risk-averse behaviour of their host and making it more prone to predation by piscivorous birds. Eyefluke-infected dace swam closer to the surface (Crowden & Broom, 1980) and infected rainbow trout were more easily caught by human 'predators' with a dip-net (Seppälä, Karvonen & Valtonen, 2004; Seppälä *et al.*, 2005b), but were not more often caught by real birds (Seppälä *et al.*, 2006). In the present study, the eyeflukes had not reached the infective stage. Additionally, the transmission of *Diplostomum* spp. to its snail or fish host is temperature dependent and usually does not take place below 10 °C (Chappell *et al.*, 1994). The low temperatures in the present study would have prevented the parasite from infecting birds or snails and led to an interruption of the parasite's life cycle. Therefore, a higher risk of predation by piscivorous birds due to impaired vision would not have increased parasite fitness. Thus, under conditions unsuitable for transmission, an absence of significant effects on the physical capabilities of the host lies in the interest of both host and parasite. Furthermore, under the prevailing circumstances, the results do not contradict either the host manipulation hypothesis or the predation suppression hypothesis (e.g. Gopko, Mikheev & Taskinen, 2015). In fact, making its fish host a more attractive group mate would be in accordance with the predation suppression hypothesis if it led to a dilution effect (Pitcher & Parrish, 1993). Future studies investigating the influence of metacercariae at temperatures that are more suitable for parasite growth and transmission should help to assess potential limits of host tolerance.

There are not sufficient parasite screening data for the Euskirchen pond. Yet, due to its isolated location in the middle of a forest, it is not particularly likely that *D. pseudospathaceum* is present in the pond. Therefore, the observed shoal preferences are probably due to general responses to infected conspecifics and not the result of selection. Similar studies using host populations with different prevalences of *Diplostomum* spp. could shed light on the question of whether effects on stickleback group formation are (at least partly) adaptive.

CONCLUSION

In the present study, uninfected three-spined sticklebacks significantly preferred stimulus shoals partially infected with the eyefluke *D. pseudospathaceum* over uninfected shoals while this preference was not found in infected focal fish. Despite this effect on the shoaling behaviour of the experimental fish, laboratory infections did not significantly affect growth or immune parameters. The results agree with the suggestion that unless the parasite causes severe opacities to the eye lens, fish are able to compensate for potential physical disadvantages. The focus in the literature on host–parasite interactions with severe consequences for the host should not hide the fact that the costs of parasitic infections can vary substantially – not only among different host–parasite systems, but also between developmental stages within a parasite species.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Supplementary Figure S1. Distribution of infection intensities (number of eyeflukes per infected individual) of 34 of the 36 fish of the mixed treatment groups. One fish was not dissected and one was free of parasites.