Experimental infection with the directly transmitted parasite *Gyrodactylus* influences shoaling behaviour in sticklebacks

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Animals usually benefit from joining groups, but joining a group can also come at a cost when members expose themselves to competition and the risk of contracting a contagious disease. Therefore, individuals are expected to adjust grouping behaviour to the ecological circumstances, their own competitiveness and the composition of the group. Here, we used experimental infections and classic binary choice tests to test whether the monogenean flatworm *Gyrodactylus* spp. has the potential to influence shoaling behaviour in the three-spined stickleback, *Gasterosteus aculeatus*, a model organism in behavioural ecology and evolutionary biology. *Gyrodactylus* spp. is a genus of widespread and rather inconspicuous, small (<0.5 mm) ectoparasites on fishes with the ability to cause severe damage to its host. *Gyrodactylus* species infecting sticklebacks have short generation times and those species typically residing on the skin or fins of their hosts are easily spread via body contact. In our experiments uninfected sticklebacks significantly preferred a group of uninfected fish over a group of *Gyrodactylus*-infected fish, while *Gyrodactylus*-infected sticklebacks did not discriminate between the two stimulus shoals with regard to their *Gyrodactylus* infection status. As infected fish were in poorer condition, were less likely to shoal and had a relatively heavy spleen, we suggest a generally reduced health state caused by the infection as a possible indirect mechanism of the altered shoaling preference. Although parasitism has been shown to play an important role in group formation, only a few studies have used experimental infections to directly test its influence on shoaling decisions. Our results show that *Gyrodactylus* spp. can influence shoaling decisions in three-spined sticklebacks and affirm the suitability of the *Gyrodactylus*—stickleback system for studying the role of parasitic infections on host group dynamics.

Forming groups is a widespread phenomenon in animals: associations range from temporary loose aggregations of individuals to eusociality known from hymenopterans, termites and mole-rats (Alexander, 1974). Generally, reduced predation risk (Hamilton, 1971) and more efficient foraging (Clark & Mangel, 1986; Pitcher & Parrish, 1993) are considered the main advantages of being a member of a social group. On the other hand, by joining a group, individuals expose themselves to competition and often increase their risk of contracting a contagious disease. Thus, an individual should adjust its decision to join a certain group not only to the ecological conditions and to the composition of the group with regard to body size, morphology and kinship, but also to its own competitiveness (see e.g. Krause & Ruxton, 2002 for a review). Parasites (referring to macroparasites in this article) play an important role in this context. By definition, parasites cause harm to their host. By impairing certain physical abilities, generally weakening their host, or by changing the appearance of their host, parasites can reduce their host's competitiveness and make it conspicuous. Effects of parasites on their host can ultimately lead to altered group composition if conspecifics are able to identify infected individuals and/or infection affects an individual's tendency to join a group (Krause & Ruxton, 2002; Loehle, 1995).

The detrimental effects that parasites have on their host can vary from hardly noticeable use of resources tolerated by an otherwise healthy host to conspicuous coloration (e.g. visible spots caused by trematodes underneath the transparent skin of fish hosts or in the eye stalks of snails), changes in behaviour (Moore, 2002), host castration or even death. Therefore, the nature of the parasitic infection, in terms of the parasite's virulence, site of infestation, life cycle and mode of transmission (Côté & Poulin, 1995), determines how the social behaviour of a host species can influence the dynamics of a parasitic infection and vice versa. Among parasites with a simple life cycle two different types can be distinguished: mobile

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parasites, such as biting flies on feral horses and *Argulus* spp., a crustacean sucking blood from sticklebacks (Poulin & FitzGerald, 1989; Rubenstein & Hohmann, 1989; Rutberg, 1987), that actively seek new hosts and whose intensity of infection decreases with increasing host group size, and parasites that increase in number when their hosts form larger groups (Côté & Poulin, 1995; Krause & Ruxton, 2002). In terms of their influence on host grouping, parasites in the second category resemble contagious diseases typically caused by microparasites. Their establishment in a group of hosts typically lacks a dilution effect and transmission success often increases in denser host groups as was observed, for example, for intestinal worms in feral horses (Rubenstein & Hohmann, 1989) or for viviparous gyrodactylids on fish (Boeger, Kritsky, Pie, & Engers, 2005; Johnson, Lafferty, van Oosterhout, & Cable, 2011).

A large body of data on social behaviour and its interaction with parasitic infections has been gathered by studying different fish species, predominantly those living in freshwater habitats (see Barber, Hoare, & Krause, 2000 for a review). Here, we look at the possible impact of *Gyrodactylus* spp. on the shoaling decisions of three-spined sticklebacks, *Gasterosteus aculeatus*. *Gyrodactylus* spp. is a widespread and rather inconspicuous ectoparasite on fishes (fresh and salt water, see Bakke, Cable, & Harris, 2007 for a review). The monoxenous (one host life cycle) parasite is directly transmitted via body contact between hosts. Viviparous *Gyrodactylus* species, such as those infecting sticklebacks, give birth to a fully developed embryo that already contains a second embryo. Owing to this special mode of reproduction and the direct transmission via body contact, single worms can initiate an epidemic which is why parasitologists often refer to *Gyrodactylus* as a microparasite. Still, to avoid confusion with conventions established among biologists that allocate parasites to the terms micro- and macroparasite based on their size, in this paper we refer to *Gyrodactylus* spp. as a macroparasite. Some *Gyrodactylus* species have been shown to cause severe damage to their specific host, *Gyrodactylus salaris* on wild and farmed salmon in Norway being the most prominent example due to severe losses in fish stocks since the 1970s (Bakke et al., 2007). Pathogenicity in this genus is strongly dependent on the *Gyrodactylus* species (see e.g. Cable & van Oosterhout, 2007). Most studies on the interaction of *Gyrodactylus* and shoaling behaviour of its fish host have been done on guppies and mainly on the *Poecilia reticulata*—*Gyrodactylus turnbulli* system. In guppies, *G. turnbulli* causes abnormal swimming behaviour and clamped fins, both clearly visible symptoms, before infected fish die (Cable, Scott, Tinsley, & Harris, 2002). Female guppies usually shoal more than males and transmission of *Gyrodactylus* is more easily facilitated among interacting conspecifics (Richards, van Oosterhout, & Cable, 2010; Stephenson, van Oosterhout, Mohammed, & Cable, 2015; but see Richards, van Oosterhout, & Cable, 2012). Experimental infection showed a negative effect on shoal cohesion in studies by Croft et al. (2011), and Hockley, Wilson, Graham, and Cable (2014), but Richards et al. (2012), working on the same species, but a different stock, found infected guppies formed even tighter shoals than uninfected guppies. To our knowledge, whether individual guppies (or any known host for *Gyrodactylus*) would discriminate infected from uninfected conspecifics in shoal choice decisions has never been tested directly. For our experiments, we chose the three-spined stickleback. Sticklebacks are a widely distributed host for *Gyrodactylus* (see e.g. Kalbe, Wegner, & Reusch, 2002; Malmberg, 1970; Özer, Öztürk, & Öztürk, 2004; de Roij & MacColl, 2012; Sulgostowska & Vojtkova, 2005) and their shoaling behaviour has been well studied (see e.g. Frommen, Hiermes, & Bakker, 2009 and citations therein), which makes this species particularly interesting for studies on the impact of parasites on host—host interactions. Sticklebacks form loose shoals during their nonreproductive phase (Wootton, 1976) and their shoaling decisions are known to be influenced by group composition, for example with regard to body size (Hoare, Krause, Peuhkuri, & Godin, 2000), as well as by the nutritional state of the choosing individual (Frommen, Luz, & Bakker, 2007). Parasites have also been recognized as a factor interfering with shoaling behaviour in sticklebacks. In shoal choice tests, uninfected sticklebacks significantly preferred shoals of uninfected conspecifics over shoals containing individuals infected with either the ectoparasitic copepod *Argulus canadensis* (see Dugatkin, FitzGerald, & Lavoie, 1994), *Schistocephalus solidus* (see Barber, Downey, & Braithwaite, 1998) or *Glugea anomala* (see Ward, Duff, Krause, & Barber, 2005). In contrast to *Gyrodactylus* spp., these parasites cause clearly visible signs of infection such as a swollen abdomen (*S. solidus*) or white cysts several millimetres in diameter (*G. anomala*), or are conspicuous themselves because of their body size (*A. canadensis*). A possible impact of *Gyrodactylus* spp. on the behaviour of sticklebacks has not been tested. Compared with guppies or salmonids, consequences of infection are usually not as severe in sticklebacks (see e.g. Konijnendijk, Raeymaekers, Vandeuren, Jacquemin, & Volckaert, 2013; Lester, 1972; de Roij, Harris, & MacColl, 2010) and low infestations are usually assumed to be tolerated by an otherwise healthy host. Dynamics of *Gyrodactylus* infections can be complex due to the parasite's mode of reproduction and because hosts differ in their susceptibility. On a newly infected stickleback responding to the infection the worm population often first increases before the highest level of infection is reached and the population declines again until the infection is eliminated (Bakke et al., 2007; de Roij et al., 2010). Still, *Gyrodactylus* spp. infecting three-spined sticklebacks cause immune reactions in their host (Lester, 1972) and increase mortality (Lester & Adams, 1974). Therefore, uninfected fish would clearly benefit from avoiding infected conspecifics if this reduces infection risk.

In this study, we tested whether three-spined sticklebacks are able to distinguish between *Gyrodactylus*-infected or uninfected conspecific shoals, and if so, whether their shoal choice is influenced by their own *Gyrodactylus* infection status. We used experimentally infected sticklebacks and quantified shoaling preferences in binary shoal choice tests. We hypothesized that, given that sticklebacks are able to distinguish between infected and uninfected conspecifics, uninfected individuals would avoid contact with infected fish. For infected fish the situation is not that clear. On the one hand, individuals already struggling with an infection should avoid increasing their parasite load and the potential costs associated with it. On the other hand, infection may be demanding in terms of energetic expenditure and reduce an individual's competitiveness. In this case it could pay an individual to shoal with weak(er) competitors. Indeed, a preference for poor competitors has been found in minnows, *Phoxinus phoxinus* (Metcalfe & Thomson, 1995). Thus, we expected infected individuals not to show a clear preference for one of the shoals.

METHODS

Origin, Disinfection and Maintenance of Fish

Adult male and female three-spined sticklebacks were caught from a freshwater pond situated in the backyard of the Institute for Evolutionary Biology and Ecology (50°44′ N, 7°4′ E; Bonn, Germany) where all experiments took place. Sticklebacks in that pond show naturally occurring *Gyrodactylus* spp. infections. For the shoal choice experiments approximately 230—300 fish were caught in March and between June and October 2010 using minnow traps and were carried in buckets to the building (distance < 40 m). Sticklebacks not showing any sign of reproductive activity were disinfected by placing them in a 0.015% formalin solution for 40 min. Formalin is commonly used against ectoparasites on fish

and has proven suitable for removing *Gyrodactylus* spp. (see e.g. Boeger et al., 2005; Soleng & Bakke, 1998). We gave this chemical preference over more specific anthelmintic treatments to remove other ectoparasites such as *Trichodina* spp., a ciliate gliding on the stickleback's skin and at high intensities causing skin irritations and mucus hyperproduction through tactile stimuli (Colorni, 2008), as well. Fish appeared to behave normally during and after the formalin bath and did not show any sign of being harmed by the chemical. Twenty-four hours after the formalin treatment, we visually checked disinfection success under $45\times$ magnification (also see Origin of *Gyrodactylus* spp. and Laboratory Infections for details). Fish were randomly assigned to one of the four treatment groups: 'focal fish infected', 'focal fish uninfected', 'stimulus fish infected' and 'stimulus fish uninfected'. During the experimental period fish were kept in groups of up to 35 fish in glass aquaria (see Appendix Table A1 for dimensions). Infected as well as uninfected focal fish were held in two tanks each to avoid testing for tank effects instead of treatment effects. Each aquarium was equipped with a filter and an airstone, at a water temperature of 15 ± 1 °C and a 16:8 h light:dark cycle. Once a week 50% of the water was replaced by fresh tap water. Additionally, dirt was removed from the bottom of each tank and siphoned water was replaced every day. Aquaria were visually as well as olfactorily isolated from each other to prevent contact between focal fish and stimulus fish. Sticklebacks were fed chironomid larvae once a day, not to satiation, thereby preventing overfeeding while at the same time providing a regular food supply.

Origin of Gyrodactylus spp. and Laboratory Infections

Parasites originated from the pond from which experimental fish were taken and from a freshwater pond in Euskirchen near Bonn, Germany (50°38′ N, 6°47′ E). Molecular identification of single specimens of both ponds using the Internal Transcribed Spacer 1 rDNA region (ITS1) indicated that *Gyrodactylus arcuatus* might be the predominant species in the Bonn pond and *Gyrodactylus gasterostei* in the Euskirchen pond (Rahn & Bakker, n.d.). Still, it is not unlikely that both ponds harbour a community of different *Gyrodactylus* species (Raeymaekers, Huyse, Maelfait, Hellemans, & Volckaert, 2008). Therefore, we refer to '*Gyrodactylus* spp.' throughout this article. We assumed single *Gyrodactylus* worms had the same effects on their host, no matter which species they belonged to, especially since in both ponds *Gyrodactylus* is mostly found on the fins and skin of its host and only seldom between the gills (A. K. Rahn, personal observation).

We infected disinfected fish with *Gyrodactylus* spp. by introducing infected sticklebacks ('donor fish') into the 'infected' treatment group aquaria. Owing to the parasite's ability to rapidly cause an epidemic, infection spread fast within the group tanks. Before 'donor fish' were introduced into the treatment group tanks, they were marked by spine clipping. 'Donor fish' were not used in the experiments.

To avoid the spread of *Trichodina* spp., as 'donor fish' we used formalin-disinfected and under controlled conditions *Gyrodactylus* spp.-reinfected sticklebacks. For this purpose a group of sticklebacks caught and disinfected together with the other experimental fish was placed into a separate 'donor tank'. Single highly infected sticklebacks from the ponds were freshly killed by decapitation followed immediately by cutting the brain, and their fins, if the only parasites they bore were *Gyrodactylus* spp., were cut off. Fins and disinfected sticklebacks were brought into close proximity in a water-filled petri dish under a microscope (Leica WILD M313, $45\times$ magnification) which was illuminated by a cold-light source (Schott KL 1500). This way, single worms were given the opportunity to actively move from one host to the other. The procedure was repeated until one to six (mean 3.6) worms had moved onto their new host. Altogether, 19 manually infected individuals were introduced into the 'donor fish' tank to spread the parasite among the 'donor fish'. All infections, as well as all parasite screenings of living experimental fish, were performed in cold tap water in a climatic chamber with an air temperature of 10.5 ± 0.5 °C.

To compare the intensities, i.e. the number of worms per infected fish, of the *Gyrodactylus* infections in our experiments with those naturally occurring in the Bonn pond, we caught and screened 60 additional sticklebacks between 11 and 15 June and 45 additional sticklebacks on 28 and 29 October and examined their body surface under the same conditions as all experimental fish. As for the experiments, only adult fish (standard length ≥ 3 cm) were examined.

Binary Shoal Choice Experiments

Set-up

Experiments were carried out in a glass aquarium (80×35 cm and 40 cm high; Fig. 1) with a water level of 15 cm. Two opposing stimulus shoal compartments, 15×35 cm and 40 cm high, were separated from the middle section by perforated, transparent Plexiglas partitions. Black lines drawn on the bottom of the tank marked choice zones of 10 cm in front of each shoal compartment. A webcam (Video Blaster Webcam 3, Creative-Labs) above the middle section and the program Windows Media Encoder 9.0 were used to record movements of the focal fish. Experiments were performed under constant illumination, at a water temperature of 14 ± 1 °C. In order not to frighten experimental fish by movements outside the test tank the whole set-up was covered by a black curtain. To prevent distraction by air bubbles, we used 1-day-old tap water.

Protocol

Prior to the start of each trial, four stimulus fish infected with at least three living *Gyrodactylus* spp. individuals and four stimulus fish free of any *Gyrodactylus* spp. were size-matched by eye. These stimulus shoals and one either infected or uninfected focal fish were fed chironomid larvae 1 h before they were introduced into the test tank. At the beginning of each trial, shoal fish were placed in their respective compartments and the focal fish was placed at the centre of the middle section in a transparent, perforated cylinder (diameter 11 cm). Video recording was started and initiated a 15 min acclimation period during which grey plastic partitions between the shoal compartments and the middle section prevented visual contact between focal and stimulus fish. At the end of the acclimation period, the grey partitions and cylinder were lifted from outside the black curtain and behaviour was videorecorded for 20 min. After that, all fish were removed from the test tank which was cleaned thoroughly to remove odour of the fish and possibly detached *Gyrodactylus* spp. After each trial, stimulus and focal fish were weighed to the nearest milligram, their standard length, i.e. the distance between the tip of the mouth to the base of the caudal fin, was measured to the nearest millimetre using graph paper, and their body condition factor (CF) was calculated as CF = $100 \times$ mass [g]/length [cm]^3 (Fulton's condition factor as cited in Ricker, 1975). The *Gyrodactylus* spp. on focal and stimulus fish were counted under the microscope and stimulus fish had one dorsal spine cut off, before they were reintroduced into their holding tank. Stimulus fish that had been used for the second time were released into their home pond. Focal fish were killed as described before and screened for ectoparasites as well as endoparasites according to Kalbe et al. (2002). This was done to obtain more exact *Gyrodactylus* spp. counts, since
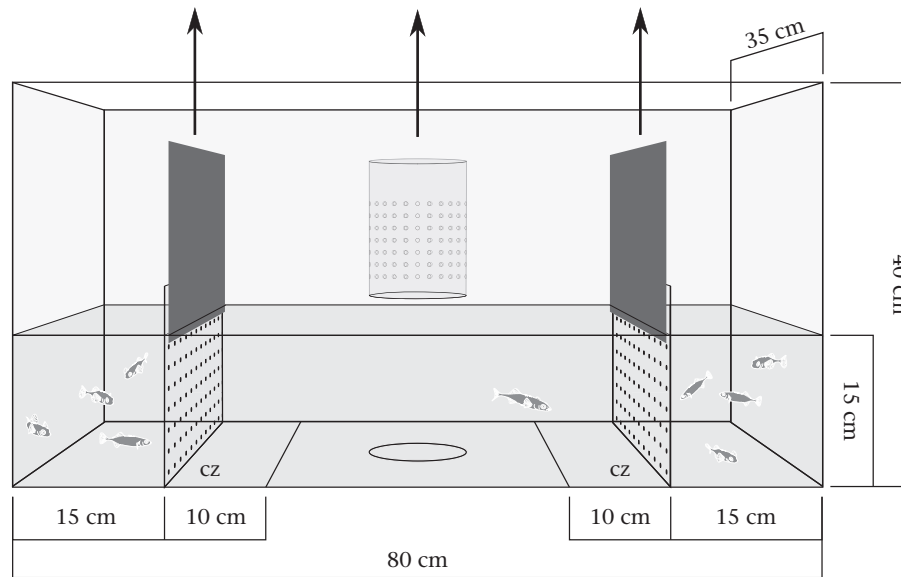
Figure 1. Schematic overview of the experimental set-up during the data recording phase with cylinder and opaque partitions raised. Transparent, perforated (hole diameter 0.5 cm) Plexiglas partitions separate the shoal compartments from the central (focal fish) area. Black felt-tip pen lines drawn onto the bottom of the test tank mark the borders of the choice zones (cz) and the position of the cylinder during the acclimation period. Fish are drawn enlarged for optical reasons.

single individuals of this parasite occasionally invade the body openings of their host and therefore may have remained undetected in the superficial screening, and to exclude other macroparasites (e.g. nematodes) as an undetected confounding variable. During dissection, focal fish were sexed and their livers and spleens were weighed to calculate the hepatosomatic index (I_H) and the splenosomatic index (I_S) according to Wootton, Evans, and Mills (1978) as $I = 100 \times$ mass organ [g]/mass fish [g]. Additionally, we counted the chironomid head capsules in the stomach of the fish.

Between 1 September and 22 October we conducted 21 trials with infected and 21 trials with uninfected focal fish. Whether an infected or uninfected focal fish was to be tested and whether the infected stimulus shoal was placed in the left or right compartment was chosen randomly. By the end of the experimental period nearly all stimulus fish had been used in the trials, leaving only a few fish that could not be assorted to two stimulus shoals of similar mean body size.

Video Analysis

During the 20 min after the cylinder had been removed, the amount of time focal fish spent in the two choice zones and the central compartment was recorded. Preference for one of the shoals was measured as the amount of time focal fish spent in front of the respective shoal relative to the time it spent in both choice zones. Time spent in both choice zones relative to the 20 min test period served as a measure for shoaling tendency. Additionally, the focal fish's activity was measured as the number of switches between the three zones. The person analysing the video files was unaware of the infection status of focal and shoal fish.

Statistical Analysis

Statistical analysis was performed in R 2.12.1 (R Core Team, 2010), except for Mann–Whitney *U* tests which were done in SPSS 15.0 (IBM, Armonk, NY, U.S.A.). Data were tested for normality using the Shapiro–Wilk test. Data significantly (level of significance: $P < 0.05$) deviating from normality were transformed, if possible, or analysed using nonparametric statistics. Given *P* values are two tailed throughout. Paired *t* tests were used for comparisons within trials, i.e. preference by the focal fish for one of the shoals and differences between infected and uninfected shoals in median standard length, mass and body condition. Differences between treatments were compared using unpaired statistics (unpaired *t* tests or Mann–Whitney *U* tests). Single (Pearson or Spearman rank) correlations were performed to test for statistically connected traits. To examine the possible impact of intensity of infection, a linear model ('lm', linear regression model) was used with intensity, measured as square root-transformed numbers of *Gyrodactylus* spp. found on the infected focal fish during dissection, as the dependent variable and body condition, splenosomatic index and time focal fish had spent in front of the infected stimulus fish relative to the total amount of time spent in both choice zones as explanatory variables. Explanatory variables were stepwise removed from the model in order of decreasing *P* values and the resulting models were compared using likelihood ratio tests. Infection intensities were compared between experimentally and naturally infected (both June and October) fish with a Kruskal–Wallis test followed by Mann–Whitney *U* tests.

Eleven trials were excluded from analysis: one because two of the stimulus fish appeared to be gravid, which was discovered after the trial, one because the focal fish never visited the right choice zone during the 20 min period, one because nine *Trichodina* spp. were found on the focal fish after the trial, two because, after the trial, focal fish that were supposed to be 'uninfected' were found to carry one and six *Gyrodactylus* spp., respectively, and six because the median of the body size of their stimulus shoals differed by 2 mm or more (3 mm in one case). Stimulus shoal fish sometimes differed in size because they had been size-matched by eye to keep handling before the trial to a minimum. Analysis was done on 17 trials with infected and 14 trials with uninfected focal fish. Sample sizes are only given when deviating from these values, which was the case for the hepatosomatic and splenosomatic indices, because single organs were disrupted during dissection.

Ethical Note

Experimental infections were necessary to address the central question of this study, but care was taken to minimize possible negative impacts on the fish. The procedure of manually infecting single sticklebacks ('donor fish') and letting them spread the parasite among the experimental fish was chosen to keep the number of individuals that had to be manually infected as low as possible ($N = 19$ compared to an estimated total of 120–150 experimentally infected fish). Short handling times in cold water and the use of a cold-light source for illumination during parasite screenings helped to keep negative impacts of temperature change on fish as well as on parasites to a minimum. Killing fish by decapitation followed by brain destruction is a generally applied and quick (<5 s) method. Shoal and donor fish were released into the institute's pond. Experiments complied with the current laws of Germany and were approved by the regional office for nature, environment, and consumer protection North-Rhine Westfalia (LANUV NRW, reference number 8.87-51.04.20.09.352).

RESULTS

Shoaling Behaviour

Uninfected focal fish spent significantly more time close to the uninfected shoal than to the shoal of infected conspecifics (Fig. 2; paired *t* test: $t_{13} = -2.47$, $P = 0.028$). Infected focal fish did not significantly prefer one of the shoals (Fig. 2; paired *t* test: $t_{16} = 0.45$, $P = 0.662$). Uninfected and infected focal fish chose significantly differently from each other (Fig. 2; unpaired *t* test: $t_{28.5} = -2.08$, $P = 0.047$). Activity did not differ significantly between uninfected and infected focal fish (unpaired *t* test with square root-transformed data: $t_{23.1} = -1.68$, $P = 0.107$), but uninfected focal fish had a significantly higher tendency to shoal (unpaired *t* test:
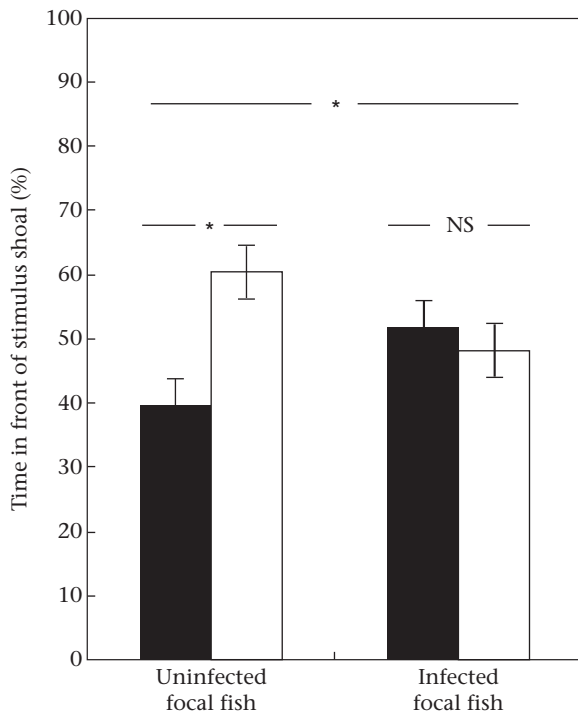
Figure 2. Mean amount of time ± SE that uninfected ($N = 14$) and infected ($N = 17$) focal fish spent in front of the infected (black bars) and uninfected (white bars) stimulus shoals relative to time spent in both choice zones, respectively. *$P < 0.05$.
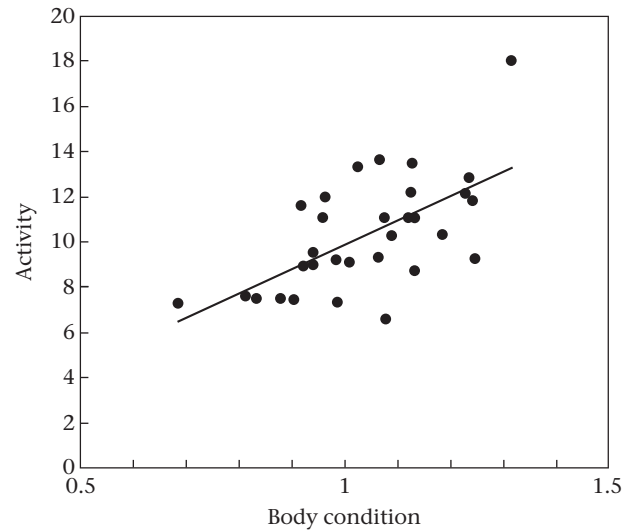
Figure 3. Relationship between activity and body condition of all 31 focal fish. Activity is given as square root-transformed number of zone switches.

$t_{27.2} = -2.63$, $P = 0.014$). Activity was not significantly correlated with shoaling tendency (Pearson correlation with square root-transformed activity data: $r_{29} = 0.09$, $P = 0.627$), but was significantly predicted by the body condition of the focal fish (Fig. 3; Pearson correlation with square root-transformed activity data: $r_{29} = 0.63$, $P = 0.0002$).

Physical Condition

Seven of the 31 focal fish were males (three infected and four uninfected). While uninfected and infected focal fish did not differ significantly in body length (unpaired *t* test: $t_{24.2} = -0.96$, $P = 0.349$), uninfected focal fish were significantly heavier (unpaired *t* test: $t_{28} = -2.53$, $P = 0.017$), had a significantly higher body condition (unpaired *t* test: $t_{28.5} = -3.3$, $P = 0.003$), a higher hepatosomatic index (unpaired *t* test: $t_{25} = -3.47$, $N_{uninfected} = 13$, $N_{infected} = 15$, $P = 0.002$) and a lower splenosomatic index (Mann–Whitney *U* test: $U = 48$, $N_{uninfected} = 11$, $N_{infected} = 16$, $P = 0.048$). Body condition was significantly positively correlated with relative liver mass (Pearson correlation: $r_{26} = 0.52$, $N_{uninfected} = 13$, $N_{infected} = 15$, $P = 0.005$), but was not significantly correlated with relative spleen mass (Spearman rank correlation: $r_S = -0.11$, $N_{uninfected} = 11$, $N_{infected} = 16$, $P = 0.601$). Significantly more chironomid head capsules were found in the stomachs of uninfected focal fish (Mann–Whitney *U* test: $U = 67$, $N_{uninfected} = 13$, $N_{infected} = 15$, $P = 0.039$). Like focal fish, infected and uninfected shoals did not differ significantly in mean body size (paired *t* test: $t_{30} = -1.36$, $P = 0.184$), but fish in uninfected shoals were significantly heavier in both their absolute and relative body mass (paired *t* tests: both $P < 0.005$). Parasite load of infected focal fish was not significantly correlated with body condition or

Table 1
Results of the linear model with number of *Gyrodactylus* spp. on infected focal fish as the dependent variable

Explanatory variable	χ^2	P
Body condition	0.003	0.960
Splenosomatic index	0.440	0.519
Relative time near infected stimulus fish	8.553	**0.011**

See text for further details. $N = 16$. Significant P value is shown in bold.

splenosomatic index (Table 1), but was significantly explained by the relative amount of time infected focal fish spent near the infected stimulus shoals (Table 1).

Comparison Between Experimental and Natural Infections

No macroparasites apart from *Gyrodactylus* spp. were found on or inside the focal fish. Most worms were found on the sticklebacks' fins or on their skin. Only in three fish were *Gyrodactylus* spp. found between the gills (one worm per fish). Before trials, infected focal fish carried between three and 53 *Gyrodactylus* spp. with a median intensity of 12 (first, third quartile 6, 15) worms per fish. This is comparable to natural intensities in the summer if only naturally infected fish with at least three worms (the rule for defining an experimental fish as 'infected') are considered ($N_{focal\ fish} = 17$, $N_{June} = 31$, $N_{October} = 13$; Kruskal−Wallis test: $\chi^2_2 = 3.8$, $P = 0.152$; Mann−Whitney U tests: June versus. focal fish: $U = 228.5$. $P = 0.449$; October versus focal fish: $U = 61$, $P = 0.036$; June versus October: $U = 152.5$, $P = 0.203$; Appendix Fig. A1). Nearly half of the naturally infected fish (prevalence June: 87%; prevalence October: 60%) harboured only one or two worms (40% in June, 52% in October). Thirty-nine per cent (June) and 23% (October), respectively, of the fish naturally infected with at least three worms and nearly 59% of infected focal fish were infected with 10 or more worms (see Appendix Fig. A2 for *Gyrodactylus* spp. frequency distributions). The highest worm load found on stimulus fish was 67 worms.

DISCUSSION

In our shoal choice tests with experimentally infected three-spined sticklebacks, uninfected fish spent significantly more time near a group of uninfected conspecifics than near a group of infected conspecifics. Additionally, uninfected focal fish had a higher tendency to shoal. The results show that three-spined sticklebacks are indeed able to discriminate between conspecifics either infected or uninfected with *Gyrodactylus*, and that they adapt their shoaling decisions accordingly. Moreover, shoaling preferences were in line with our expectations. *Gyrodactylus* spp. has been found to increase host mortality in sticklebacks (Lester & Adams, 1974) and to cause damage to its host's skin, thereby probably increasing the risk of secondary infections (Bakke et al. 2007; but also see Lester, 1972). Therefore, uninfected fish directly benefit from avoiding contact with infected fish as this reduces their own infection risk. Additionally, uninfected fish would also circumvent an increase in predation risk due to oddity effects by avoiding proximity to infected conspecifics, possibly weakened and behaving differently because of the infection. As predicted, no clear preference for either of the stimulus shoals was found in infected focal fish. Like uninfected individuals, fish already infected with *Gyrodactylus* spp. would also benefit from avoiding infected fish since more worms will most likely cause greater damage and weakened fish might attract predators. On the other hand, reduced competitiveness and avoidance of being the odd one in a group of uninfected fish might work against a preference for the uninfected stimulus shoal, eventually resulting in a situation where the infection status of the stimulus fish is not the decisive factor determining shoal choice. Also, the behaviour of the focal fish might not have been independent of the behaviour of the stimulus fish. Exclusion of infected individuals has been observed in many animal taxa including primates (Krause & Ruxton, 2002; Loehle, 1995). For our stickleback−*Gyrodactylus* system it is imaginable that in a situation with direct interactions between an infected individual and a group of conspecifics, shoal members would avoid single, undesirable individuals. In guppies, for instance, shoal

cohesion was reduced if one member was infected (Hockley et al., 2014) and the introduction of single, *Gyrodactylus*-infected fish led to the initiation of more fission events in a larger group of fish than the introduction of an uninfected guppy (Croft et al., 2011). Although physical interactions between focal and stimulus fish were intentionally limited in this study and behaviour of stimulus fish was not tested, stimulus fish possibly reacted differently to infected and uninfected focal fish.

As we used both male and female fish and all experimental fish originated from the same, small pond, one could argue that sex of the focal fish and familiarity might have affected the results. We do not think that this is likely to be the case here. To limit possible behavioural differences between males and females, only fish that did not show obvious signs of reproductive activity were used. Also, the seven males were almost evenly (three infected, four uninfected) distributed among the focal fish of both treatments. Similarly, we assume that familiarity among focal and stimulus fish did not differ between the two treatments, because the pond is rather small and, prior to disinfection, all experimental fish were probably more or less familiar with each other.

From the parasite's point of view, the reduced shoaling tendency of infected hosts reduces the chances of transmission, because *Gyrodactylus* spp. can be easily transmitted via direct body contact between hosts, and population growth has been shown to increase when potential hosts were kept in groups rather than isolated (Boeger et al., 2005). The reduced shoaling tendency of infected sticklebacks is therefore in favour of the host, not the parasite. A reduced shoaling tendency of infected compared with uninfected fish is in agreement with studies on guppies infected with *Gyrodactylus* spp. (Croft et al., 2011) and with studies on mosquito fish, *Gambusia affinis*, and banded killifish, *Fundulus diaphanus*, infected with trematodes causing the 'black spot disease' (Krause & Godin, 1994; Tobler & Schlupp, 2008), but differ from observations made by Ward et al. (2005) on *Glugea*-infected sticklebacks. Similarly to our results, Ward et al. found uninfected individuals preferred uninfected conspecifics while infected individuals did not seem to distinguish between infected and uninfected individuals. Compared with uninfected fish, the *Glugea*-infected fish, however, showed a higher tendency to shoal, which was discussed as an attempt to mitigate a higher predation risk, due to the conspicuous white cysts caused by the parasite, by joining a group of conspecifics. According to Milinski (1985), infection with *Glugea* does not seem to reduce competitiveness in sticklebacks. The differing results emphasize the importance of taking the specific nature of a respective parasitic infection into account when hypothesizing about parasitic influence on shoaling behaviour (see e.g. Barber et al. 2000 and Côté & Poulin, 1995). Since we assume *Gyrodactylus* spp. reduces competitiveness of its host by increasing its energy expenditure and reducing its general condition, the reduced shoaling tendency of infected sticklebacks is consistent with our expectation and can be explained as avoidance of competition. Since infected fish could still gain a net benefit from shoaling when predation risk is high and advantages of joining a group outweigh costs due to competition, it might be interesting to test whether the shoaling behaviour of infected and uninfected fish is influenced by the presence of predator cues.

The mechanism underlying the observed shoaling preferences was not examined in the present study. Sticklebacks may perceive the worms themselves and try to avoid them or the effect of an infection with *Gyrodactylus* spp. on shoaling decisions may be purely based on indirect cues. The perforated, transparent partitions between the stimulus and the focal fish compartment allowed visual as well as olfactory contact between focal and stimulus fish and gave the focal fish the opportunity to assess the health status of the stimulus shoals. By determining the overall health status of the

experimental fish we aimed at testing whether *Gyrodactylus* spp. had measurable effects on the sticklebacks' health, which would be indicative of an indirect mechanism underlying the observed shoaling decisions. Body condition and relative liver mass, which can be seen as measures of short-term energy reserves (Chellappa, Huntingford, Strang, & Thomson, 1995), were significantly correlated and lower in infected fish. Infected fish also had a lower absolute and relative body mass and a lower hepatosomatic index. This indicates that infection with the ectoparasite brought about metabolic costs for the sticklebacks. Experimental evidence for an effect of *Gyrodactylus* spp. on the body mass of sticklebacks is scarce, but Eizaguirre, Lenz, Kalbe, and Milinski (2012) found a link between *Gyrodactylus* load and loss of body mass in laboratory-bred sticklebacks that had been kept in mesocosms placed in the natural habitat for a period of 10 months. In our study, infected focal fish were in poorer body condition and had fewer chironomid head capsules in their stomach pointing to a reduced general condition along with reduced food intake, which is often found to accompany parasitic infections (see e.g. Arneberg, Folstad, & Karter, 1996; Crompton, 1984; Kyriazakis, Oldham, Coop, & Jackson, 1994; van Oosterhout, Harris, & Cable, 2003). Additionally, infected sticklebacks had higher splenosomatic indices. The relative spleen mass is often used as a measure of the activity of the immune system: previous studies have found enlarged spleens to be associated with parasitic infections in different fishes (Lefebvre, Mounaix, Poizat, & Crivelli, 2004; Seppänen, Kuukka, Voutilainen, Huuskonen, & Peuhkuri, 2009). Since *Gyrodactylus* spp. is known to cause an immune response by the host (Bakke et al., 2007; Lester, 1972), the higher splenosomatic indices in this study suggest an activation of the immune system caused by the infection. As some animals are able to recognize infected conspecifics by specific odours associated with infection (e.g. Hughes, Helsen, Tersago, & Leirs, 2014; Kavaliers & Colwell, 1995), further studies could test whether *Gyrodactylus*-altered shoaling behaviour is triggered by olfactory or visual cues.

Parasite load was uncorrelated with body condition or splenosomatic index, showing that in this study the mere fact of being infected, rather than the intensity of infection, was responsible for the differences in physical condition between infected and uninfected fish. The more worms that infected focal fish harboured the more time they spent near the infected stimulus shoals. This could indicate that only high infestations lead to altered shoaling behaviour while a potential influence of low worm numbers is outweighed by advantages of shoaling with healthy conspecifics. Thus, intentionally excluding low worm burdens from the natural full spectrum of infection intensities may have revealed a stronger effect of *Gyrodactylus* spp. than would be expected in natural situations. Given the dynamic infection cycle of *Gyrodactylus* spp. (Bakke et al., 2007), it is unlikely that sticklebacks encounter groups of conspecifics consisting purely of either infected or uninfected fish. Pérez-Jvostov, Hendry, Fussmann, and Scott (2012) found an interaction between predation regime and *Gyrodactylus* prevalence within natural habitats of Trinidadian guppies, which disappeared in flow channel experiments without predator cues. Although predation is a factor known to promote shoaling behaviour in guppies, and increased shoaling favours transmission of *Gyrodactylus* (Croft et al., 2011; Johnson et al., 2011; Richards et al., 2010, 2012), a direct link between an impact of *Gyrodactylus* on shoaling behaviour and how it is affected by predator cues has not yet been tested directly (but see Stephenson et al., 2015 for a correlational study). Therefore, it would be interesting to examine the influence of *Gyrodactylus* spp. on shoaling behaviour in situations in which individuals encounter much more heterogeneous groups of conspecifics in diverse ecological scenarios in order to reveal the relative importance of *Gyrodactylus* spp. for the occurrence of infection-associated behavioural change.

Conclusion

We found that the ectoparasite *Gyrodactylus* spp. had considerable effects on sticklebacks' shoaling decisions and overall health and immune status. These are causal effects as fish had been experimentally infected and nearly all infected fish were used in the experiments. Future studies that take different ecological and social conditions into account and examine possible mechanisms underlying the shoaling decisions found in the present study could elucidate the relative importance of *Gyrodactylus* spp. for shoaling behaviour of three-spined sticklebacks. Our results stress the suitability of the *Gyrodactylus*–stickleback system for studying evolutionary consequences of host–parasite interactions.

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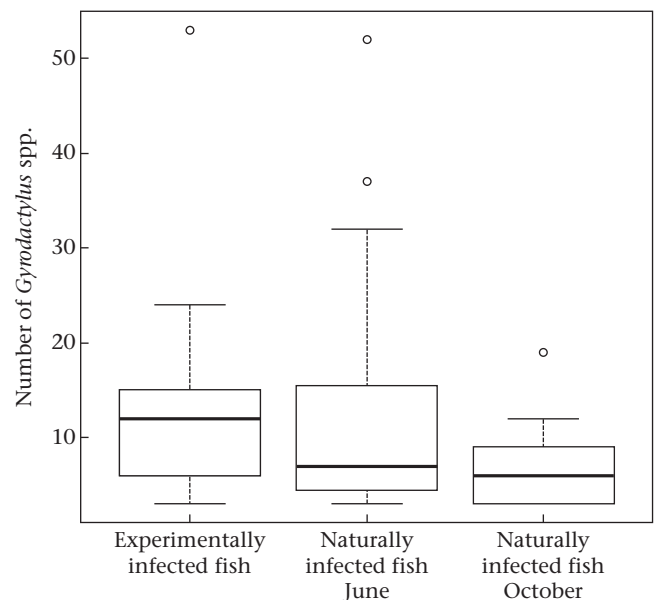
Appendix

Figure A1. Intensities of *Gyrodactylus* infections on experimentally infected focal fish (*N* = 17) and naturally infected sticklebacks caught in June (*N* = 31) and October (*N* = 13) 2010 shown as median, quartiles, 1.5× interquartile range and outliers. Only fish harbouring at least three worms were considered. See text for details and statistics.
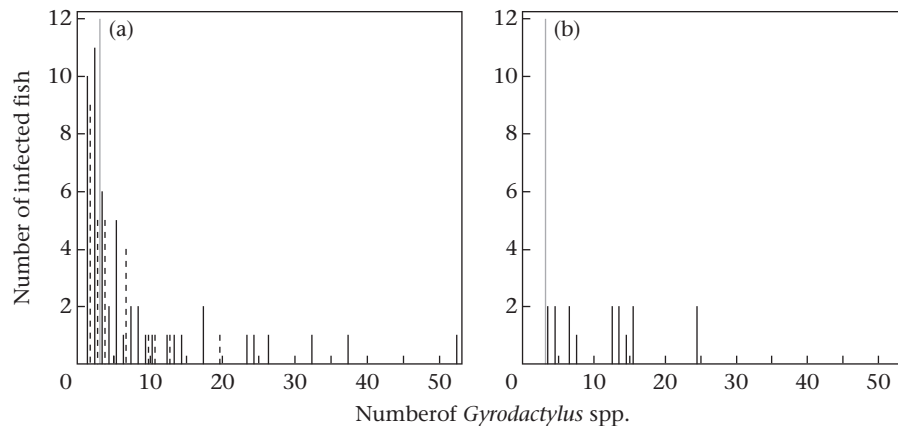
Figure A2. Distribution of *Gyrodactylus* infection intensities among (a) naturally (solid bars June, interrupted bars October) and (b) experimentally infected sticklebacks. Grey line depicts 'three-worms threshold' (see text for details). Note that only data of infected focal, not infected stimulus fish are shown, since stimulus fish were used more than once.

Table A1
Dimensions of the holding tanks

Tank	Dimensions (cm×cm×cm)	Water level (cm)
Focal fish untreated	60×45×30	25
Shoal fish untreated	60×45×30	25
Disinfected focal fish	65×50×30	25
Disinfected shoal fish	65×50×30	25
Uninfected donor fish	70×40×35	30
Infected donor fish	70×40×35	30
Uninfected focal fish (1)	70×35×35	30
Uninfected focal fish (2)	70×35×35	30
Infected focal fish (1)	70×35×35	30
Infected focal fish (2)	70×35×35	30
Uninfected shoal fish (until 14 Sept 2010)	80×45×30	25
Uninfected shoal fish (from 14 Sept 2010)	100×35×30	25
Infected shoal fish	80×45×35	25