Ecological and host behavioural aspects of parasite dispersal in a simple and a complex host–parasite system

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Die gefährlichste Weltanschauung ist die Weltanschauung derer,
die die Welt nie angeschaut haben.

The most dangerous worldview is the worldview of those who have not viewed the world.

Alexander von Humboldt

Contents

General introduction

General introduction

In 78 countries in the tropical and subtropical areas of Africa, South America, and Asia, many people suffer from schistosomiasis – a disease that goes along with symptoms such as abdominal pain, diarrhoea, and a reduction of cognitive abilities in children (Rollinson and Simpson, 1987; WHO, 2017). Larval stages (cercariae) of the genus *Schistosoma* infect people that get in contact with contaminated water. Inside the human body, adult blood flukes mate and produce eggs that leave the body with the faeces. In the water, the digenean trematodes hatch as ciliated larvae and infect freshwater snails in which they reproduce and develop to infective cercariae (Rollinson and Simpson, 1987). In the Senegal River Basin, the building of dams to generate electricity and to control the influx of sea water reduced salinity and increased alkalinity of the water. Further, migration of river prawns – an important predator of snails – was prevented. This created ideal conditions for the snail host and paved the way for an outbreak of the disease (e.g. Talla et al., 1990; Southgate, 1997). Reintroducing river prawns led to a decrease in parasite prevalence in the human population (Sokolow et al., 2015). Today, control of the snail populations constitutes one pillar in fighting the disease alongside measures such as (preventive) treatment of the population with praziquantel and improving access to clean water (WHO, 2017; Tanser et al., 2018). Population genetic analyses revealed that human host mobility contributed to frequent introduction of schistosomes which again favoured gene flow among the parasites and built the foundation for a high adaptive potential in the parasite (Van den Broeck et al., 2015; also see next paragraph).

Schistosomiasis is just one of many examples that illustrate how a complex interplay of environment, host characteristics, and parasite life-cycle traits shapes the distribution and epidemiology of parasitic diseases, and leads to the main theme of the first part of this thesis (*Chapters I* and *II*) – the relative importance of host dispersal and abiotic factors for parasite distribution. *Chapters III* and *IV* of my thesis concentrate on the influence of parasites on host grouping behaviour – an aspect of animal behaviour with direct consequences for the transmission of contagious parasitic diseases.

The relevance of dispersal and gene flow for host–parasite relationships

Host–parasite relationships are characterised by two parties of which one (the parasite) exploits the other one (the host). While the parasite benefits from this association, infection usually produces costs for the host. Since parasites often affect the resource allocation and

reproductive capacity of their hosts, increase mortality rates in local host populations, and form an integral part in food webs (e.g. Lafferty et al., 2008; Anderson and Sukhdeo, 2011), they constitute an important ecological factor (reviewed e.g. in Lebarbenchon et al., 2009; Schmid-Hempel, 2011). Where host genotypes differ in resistance, parasites also act as selective agents (Haldane, 1949) and are thereby able to induce adaptation and could even drive speciation in their hosts (Buckling and Rainey, 2003; Eizaguirre et al., 2009; Eizaguirre et al., 2011; Schmid-Hempel, 2011). From the parasite's point of view, host populations provide temporally variable environments (Barrett et al., 2008). Adaptation and co-adaptation between hosts and parasites are therefore characterised by host- and parasite-genotype frequencies that oscillate over time ("Red Queen dynamics"; Van Valen, 1973; Thompson, 2005; Schmid-Hempel, 2011). As with local adaptation, antagonistic adaptations of hosts and parasites are determined by a balance between selection and gene flow (Lenormand, 2002). When gene flow (genetic exchange between groups) is restricted in the host, this limits genetic variability and increases susceptibility while genetically more variable populations usually suffer less from parasites (reviewed e.g. in Schmid-Hempel, 2011). On the other hand, if gene flow is high and potentially beneficial genotypes are lost due to gene swamping, this impairs the parasite's potential to adapt to local host populations (Slatkin, 1987; Lenormand, 2002). Generally, intermediate gene flow is expected to maximise adaptation (Gandon and Michalakis, 2002; Lenormand, 2002; Morgan et al., 2005; Barrett and Schluter, 2008; Tigano and Friesen, 2016).

Migration in the sense of movement of individuals between groups that results in genetic exchange – "migration of alleles" – is considered an important mechanism of gene flow (Slatkin, 1985, 1987; Hedrick, 2005). Dispersal is a prerequisite for and correlated with migration. Furthermore, genetic differentiation increases where gene flow among groups is low (Hedrick, 2005). Thus, analyses of population structure – an indirect measure of gene flow – can be used to make assumptions about the geographic dispersal of (subgroups of) a certain species (Slatkin, 1985; Hedrick, 2005) and can give valuable information about the potential of hosts and parasites to coevolve. In *Chaper I* of this thesis, I analysed the population (sub-) structuring of a host and one of its parasites with a complex life cycle. Together with data on local parasite abundances (*Chapter II*), I aimed to find out more about the relative influence of different factors on parasite distribution.

The influence of life cycle and host motility on parasite dispersal

Since parasites, at least temporally, depend on their host, the dispersal of parasites most obviously depends on the host's motility and geographic range, but also on the existence of free-living stages in the life cycle that allow active, host-independent movement (e.g. glochidia, ticks, fleas) and transportation by water current or air (e.g. bacterial pathogens, viruses). Vectors, such as mosquitoes that transmit the malaria pathogen *Plasmodium falciparum*, and paratenic hosts – hosts in which the parasite does not develop further – facilitate dispersal by transporting and transmitting the parasite to the next host (Zander, 1998; Schmid-Hempel, 2011). This highlights the importance of life cycle complexity as a determinant of parasite dispersal. Parasites with a complex, i.e. multi-host, life cycle show higher dispersal rates and often higher genetic diversity compared to parasites that complete their life cycle on/in only a single host species (Barrett et al., 2008; Poulin et al., 2011). Generally, the host with the highest motility in a parasite's life cycle is considered the determinant of dispersal and gene flow in the parasite (Louhi et al., 2010; Van den Broeck et al., 2014; Feis et al., 2015; but see Mazé-Guilmo et al., 2016). Many parasites with a complex life cycle infect birds or terrestrial mammals (including human beings) which carry the pathogens further than fish hosts (Thieltges et al., 2009) and across distances that exceed the range of the intermediate host(s) by far. Consequently, among aquatic parasites, population structures are usually more pronounced in parasites that lack a bird host and complete their entire life cycle in aquatic habitats (Criscione and Blouin, 2004; Blasco-Costa and Poulin, 2013; Feis et al., 2015).

Ecological barriers to parasite distribution

Ecological conditions influence all free-living species, but parasites are affected in several ways: free-living stages and ectoparasites are constantly exposed to the ambient temperature, humidity, and physicochemical properties of their environment. Hence, ectoparasites can be expected to suffer more from changing or adverse local conditions than endoparasites that find a relatively constant environment in their respective host (Zander, 1998). On the other hand, endoparasites and complex life cycle parasites in general indirectly depend on a favourable environment for their host(s) and vectors. In habitats with dynamic environmental characteristics, temporally adverse conditions for the host – like the drying up of water bodies – can be tolerated by parasites with dormant stages (e.g. spores, dormant eggs) such as the bacterium *Pasteuria ramosa* which infects the water flea *Daphnia* (Decaestecker et al., 2004). *Chapter II* of this thesis specifically

examines associations between abiotic habitat characteristics, such as pH and habitat size, and local distribution patterns of ecto- and endoparasites.

As with free-living species, habitat size is usually positively correlated with parasite diversity and prevalence since larger habitats provide space for larger and more diverse groups of potential hosts which again indirectly favours larger parasite populations (Côté and Poulin, 1995; Ebert et al., 2001). Also, habitats and host populations of limited size bear the risk of local extinction of the parasite. The spatial distribution and range sizes of parasites are affected by the migratory behaviour and the geographic range of their host(s) (Poulin et al., 2011; Bozick and Real, 2015; Lange et al., 2015). Invasive host species play a special role in this regard since they are able to alter local parasite faunas by providing new sources of susceptible intermediate hosts (e.g. Sures and Streit, 2001; Goedknegt et al., 2016). Due to spatially changing environmental factors, host migration also alters the temporal parasite community composition of the host. Famous examples are salmon or eels that change their parasitic fauna on their way from freshwater to the sea and back, and migratory birds (reviewed e.g. in Zander, 1998). One important barrier to the dispersal of parasites between freshwater and salt water habitats is the change in salinity (Zander, 1998; Zander and Reimer, 2002; Thieltges et al., 2010).

Flow conditions affect the dispersal and distribution of parasites. Wind e.g. interferes with the free movement of biting flies (e.g. Rubenstein & Hohmann 1989). In aquatic habitats, low velocity habitats often harbour higher parasite prevalence and more diverse parasite communities (e.g. Lenihan et al., 1999; Barker and Cone, 2000; Kalbe et al., 2002; Hallett and Bartholomew, 2008). This might be due to several reasons. Lakes e.g. provide better conditions for lymnaeid snails which are intermediate hosts of trematodes such as *Diplostomum*, which is more prevalent in sticklebacks in lakes than in rivers (Kalbe et al., 2002; Eizaguirre et al., 2012). Also, host-directed movement of free-living stages such as cercariae is impaired at high velocities (Radke et al., 1961; Hallett and Bartholomew, 2008). Further, host individuals of reduced physical condition, which are already infected or more susceptible to disease, might choose less energy demanding low velocity areas (e.g. Hockley et al., 2014a).

Within freshwater habitats, water quality measures such as a lack of dissolved calcium and low pH, i.e. values substantially below 7, are often associated with reduced parasite abundance (e.g. Marcogliese and Cone, 1996; Barker and Cone, 2000; Goater et al., 2005). Also, eutrophication and contamination with metals severely affect populations

of aquatic parasites (see e.g. Blanar et al., 2009 for a meta-analysis). Eutrophication is the result of natural processes, but due to anthropogenic introduction of nutrients into water bodies, it is also an example of the influence of human behaviour on host–parasite interactions. Deforestation e.g. has been shown to lead to increased biting rates by mosquitoes which again might increase local malaria prevalence (Vittor et al., 2006; Gardner et al., 2013). Another important factor in shaping parasite distribution lies in the anthropogenic control of hosts, e.g. the control of molluscan intermediate hosts to prevent the transmission of trematodes (Chappell et al., 1994; Sokolow et al., 2015), which may culminate in the purposeful eradication of selected species using gene-editing technologies (Hammond et al., 2015; Galizi et al., 2016).

With high levels of migration in bird-infecting parasites with a complex life cycle, local infection success is expected to depend on suitable environmental conditions for potential intermediate hosts as well as on genetic factors of local host populations. I examined these aspects in *Chapters I* and *II* of my thesis.

Grouping behaviour under parasitism

Throughout the animal kingdom, many species form groups of varying degrees of temporal and social stability (see e.g. Alexander, 1974; Pitcher and Parrish, 1993; Krause and Ruxton, 2002 for reviews on the topic). Members of a group benefit from a "many eyes effect" that enables more efficient detection of scattered food patches and sooner recognition of predators (Ward and Zahavi, 1973; Treherne and Foster, 1981; Pitcher et al., 1982; Krause and Ruxton, 2002; Davies et al., 2012). Further, groups provide opportunities to share energetic costs regarding aero- and hydrodynamic locomotion, and thermo-regulation (Davies et al., 2012). In theory, one of the most important advantages of joining a group lies in a reduction of the individual predation risk since this is shared in an assemblage of conspecifics ("dilution effect"; Williams, 1966a; Hamilton, 1971; Foster and Treherne, 1981; Morgan and Colgan, 1987; Krause and Godin, 1995). Often, the perception of a threat even triggers the formation of a group (Krause, 1993a; Krause and Tegeder, 1994) and there is experimental evidence that predation pressure acts as a selective force underlying shoaling as an adaptive behaviour (Magurran et al., 1992). In general, larger groups provide more effective protection unless the size of a group itself raises conspicuousness compared to smaller groups or single individuals (Williams, 1966a; Krause and Godin, 1995). Groups of morphologically and behaviourally similar individuals make it difficult for visual predators to detect single prey animals ("confusion

effect"; e.g. Krakauer, 1995; Ioannou et al., 2008). Consequently, animals are expected to choose phenotypically matching individuals as group mates in order to avoid standing out from the group visually ("oddity effect"; Ohguchi, 1978; Landeau and Terborgh, 1986). This explains preferences for shoaling with siblings, which are often phenotypically similar, and/or with familiar individuals that have established dominance hierarchies and more effective anti-predator defences than associations of unfamiliar individuals (e.g. Griffiths and Magurran, 1997; Arnold, 2000; Barber and Ruxton, 2000; Ward and Hart, 2005; Frommen et al., 2007a; Strodl and Schausberger, 2012), but analyses of natural groups show contradictory results (Griffiths and Magurran, 1997; Peuhkuri and Seppä, 1998; Barber and Ruxton, 2000).

Being part of a group can also come at a cost if ressources are limited, which increases competition, or if sick individuals introduce a risk of infection (Krause and Ruxton, 2002). Parasites play a special role in this regard. To what extent a parasite influences the grouping behaviour of its host – and whether the parasite can profit from host aggregations – depends on the parasite's life cycle and route of transmission, and on the kind of harm it causes to its host (reviewed in Barber et al., 2000; Krause and Ruxton, 2002; Mikheev, 2011). Analogous to the "dilution effect" in predator–prey relationships, prevalence of macroparasites that "attack" individuals in a predator-like manner is often reduced in larger host groups (e.g. biting flies on feral horses and crustacean parasites on sticklebacks; Duncan and Vigne, 1979; Rutberg, 1987; Poulin and FitzGerald, 1989; Rubenstein and Hohmann, 1989; also see Mooring and Hart, 1992 for a review) and animals exposed to these parasites are often found to aggregate in larger groups (Mooring and Hart, 1992). Directly transmitted parasites with a simple life cycle and those complex life cycle parasites whose intermediate hosts live together in close spatial proximity usually benefit from large and dense host groups (Rubenstein and Hohmann, 1989; Côté and Poulin, 1995; Poulin, 1999; Krause and Ruxton, 2002; Bagge et al., 2004; Boeger et al., 2005; Johnson et al., 2011). To escape an infection, uninfected animals should avoid (groups of) conspecifics infected with a contagious pathogen. This has been observed, e.g., in guppies (Croft et al., 2011). Animals that harbour a parasitic infection are often of a weaker physical condition. They have lower energy reserves and their competitiveness is reduced. Infected individuals are expected to compensate the consequences of an infection as much as possible to avoid being rejected by conspecifics which would ultimately lead to isolation from the group. Weak animals also often show a reduced tendency to join groups

(Loehle, 1995). Parasites that cannot be spread within a group – mostly trophically transmitted endoparasites – do not pose a direct risk (of infection) to other group members. Yet, they can affect the visual appearance and physical condition of their hosts and thereby indirectly interfere with group dynamics. There is experimental evidence that, under certain circumstances, weak or physically impaired competitors may even be preferred shoal mates (Metcalfe and Thomson, 1995; Frommen et al., 2012), but parasites that cause conspicuous coloration, clearly visible cysts, weak physical condition, emaciation, or abnormal behaviour presumably indirectly attract predators (Lafferty and Morris, 1996; Seppälä et al., 2005a; Ondrackova et al., 2006; Bakker et al., 2017). Therefore, individuals infected with these non-contagious parasites should be avoided by conspecifics as has been observed in several fish species (killifish *Fundulus diaphanus*, Krause & Godin 1996; three-spined sticklebacks *Gasterosteus aculeatus*, Barber et al. 1998; mosquitofish *Gambusia affinis*, Tobler & Schlupp 2008) .

Environmental factors such as predation risk, water current, water temperature and quality, and diet affect shoaling behaviour (e.g. Weetman et al., 1999; Ward et al., 2004; Sneddon et al., 2006; Fischer and Frommen, 2013; Hockley et al., 2014b; Hiermes et al., 2015a). Experimental evidence of intraspecific differences in shoaling behaviour in sticklebacks (Wark et al., 2011) and guppies (Magurran et al., 1992) suggests that ecological factors play a role in the adaptation of social behaviour. The fact that environmental factors can affect the social behaviour of the host, the distribution of the parasite as well as interactions between host and parasite, underlines the importance of controlled laboratory experiments, as performed in *Chapters III* and *IV* of this thesis, for understanding the system-specific impact of a parasite on the behaviour of its host.

The study system

The three-spined stickleback

The three-spined stickleback, *Gasterosteus aculeatus* L. (Fig. 1), is a small (usually less than 10 cm in body length), euryhaline teleost that is found in salt water, brackish water, or freshwater, but also forms anadromous populations that live in the sea and enter coastal brackish water lagoons or freshwater habitats in spring for breeding (Münzing, 1959; Wootton, 1976; Paepke, 1996). Its geographical distribution is limited by temperature and physical barriers such as water falls and (artificial) weirs (Paepke, 1996). Sticklebacks (referring to three-spined sticklebacks in this thesis) are nearly ubiquitous in the Northern

hemisphere with most southern occurrences in California, Spain, and Italy (Wootton, 1984; Bell and Foster, 1994). Frequent colonisation of freshwater habitats and the ability to adapt to local environmental conditions have made *G. aculeatus* a prominent example of adaptive radiation particularly with regard to the number and shape of its eponymous spines and lateral bony plates (e.g. Bell and Foster, 1994; Cresko et al., 2004; Colosimo et al., 2005). The macroparasitic fauna of the three-spined stickleback has been well documented at various places around the world (e.g. Chappell, 1969; Kalbe et al., 2002; Zander, 2007; Natsopoulou et al., 2012) and a lot of research on the ecology and evolution of host–parasite relationships has focussed on this teleost (reviewed e.g. in Barber, 2007; Barber, 2013). Hypothesis-driven correlational analyses and experimental infections have shown that the compositions of parasitic faunas and parasite abundances in sticklebacks are significantly influenced by ecological factors such as water temperature, habitat-type (river, lake), and niche (benthic, limnetic) (e.g. Kalbe et al., 2002; Kalbe and Kurtz, 2006; MacColl, 2009; Karvonen et al., 2013; Karvonen et al., 2015; also see Scharsack et al., 2016 for a recent review). Furthermore, innate immune responses towards certain parasite species can be specific (Rauch et al., 2006; Haase et al., 2014) and stickleback populations exhibit spatial differences in resistance (e.g. Kalbe and Kurtz, 2006; de Roij et al., 2010; Raeymaekers et al., 2011; Kalbe et al., 2016). In conclusion, these findings have fuelled current research on the role of ecologically driven divergence of parasite communities for host (and parasite) speciation (Brunner and Eizaguirre, 2016).

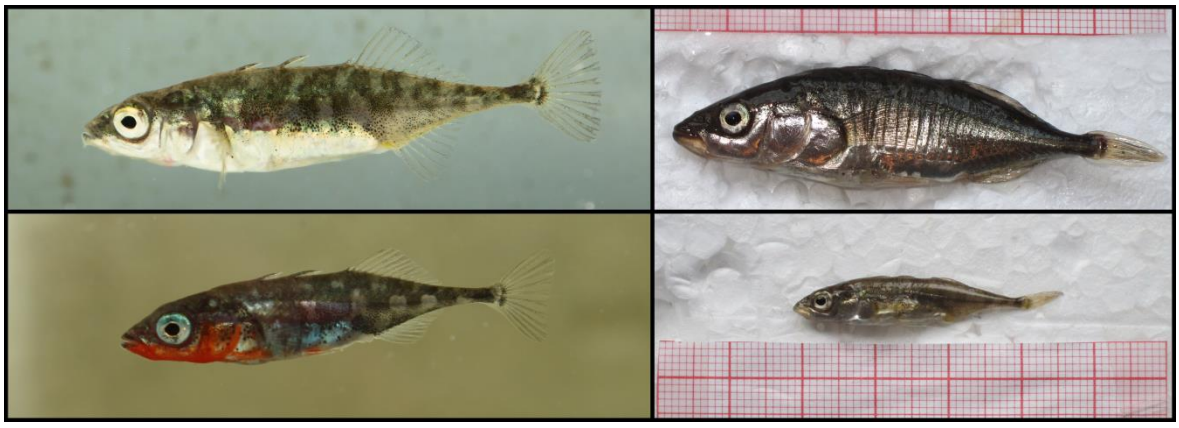

Fig. 1. Female (upper left image) and red-throated male three-spined stickleback (lower left image) from a freshwater lake on North Uist; full-scale images of an anadromous and a freshwater specimen (right panel, upper and lower image, respectively).

Apart from its physiological plasticity and potential to rapidly adapt to different environments, the (reproductive) behaviour of the three-spined stickleback has fascinated generations of ethologists (e.g. Tinbergen, 1952; von Hippel, 2010). During the breeding

season, which is determined by temperature and length of day and typically lasts from late March through early August (Wootton, 1984), stickleback males develop an orange-red nuptial coloration and establish their own territory, which they defend aggressively (reviewed e.g. in Wootton, 1984; Bakker, 1994; Rowland, 1994). The male uses plant material and filamentous algae to build a nest in shallow water. The secretion from the kidney (Jakobsson et al., 1999) that serves as glue reduces bacterial and fungal infections of the eggs (Little et al., 2008). Performing a typical zig-zag dance, the male guides a gravid female to its nest where the female spawns. Once the male has collected clutches of several females (up to ten; Kynard, 1978), it starts fanning oxygen-rich water through the nest and protects the eggs from potential predators (Wootton, 1984).

Non-reproductively active sticklebacks form loose shoals (Keenleyside, 1955; Wootton, 1984) that comprise between less than ten animals and several hundred fish (Peuhkuri, 1997; Poulin, 1999; Barber, 2003). As with many fishes, sticklebacks generally prefer larger shoals (Tegeder and Krause, 1995; Barber et al., 1998; Krause et al., 1998; Fischer and Frommen, 2013; Thünken et al., 2014) and conspecifics of similar body size as shoal mates (Ranta et al., 1992; Peuhkuri et al., 1997; Hoare et al., 2000), but they also decide which shoal they join based on factors like kinship and familiarity (Barber and Ruxton, 2000; Frommen and Bakker, 2004; Ward et al., 2004; Ward and Hart, 2005; Frommen et al., 2007a; Frommen et al., 2007c). Physical condition like gravidity or the nutritional state also influences shoaling decisions (Frommen et al., 2007b; Frommen et al., 2012). Hunger, e.g., mitigates the (positive) influence of group size and familiarity on shoal choice (Krause, 1993b; Frommen et al., 2007b) which is interpreted as an avoidance of competition for limited food ressources. A few studies have examined the influence of parasites on the shoaling behaviour of sticklebacks, but the focus has mainly been on pathogens that severely affect either the visual appearance or the physical capabilities of the host (or both). *Schistocephalus solidus* is a cestode that can grow to the mass of its host inside the body cavity (Arme and Owen, 1967). The swollen abdomen reduces the manoeuvrability and buoyancy of its host (Arme and Owen, 1967; Lobue and Bell, 1993) – an effect comparable to that in gravid females carrying ripe eggs. While gravid females are preferred shoal mates of non-gravid females (but not by other gravid females who compete with them for males; Frommen et al., 2012), uninfected sticklebacks prefer uninfected over *Schistocephalus*-infected sticklebacks (Barber et al., 1998). Avoidance of infected conspecifics has also been observed in sticklebacks infected with the microsporidian

Glugea anomala that causes clearly visible white cysts underneath the skin (Ward et al., 2005), and in sticklebacks infected with the ectoparasite *Argulus canadensis*, a crustacean with a body size of up to 3 mm (Dugatkin et al., 1994). The results are discussed as a form of phenotype matching, i.e. an avoidance of the "oddity effect" (*G. anomala*) and an avoidance of contracting an infection with a highly mobile ectoparasite (*A. canadensis*). Apart from the aforementioned studies, knowledge on the impact of parasites that have less obvious effects on three-spined sticklebacks is lacking. To redress the balance towards studies that also take into account parasites that do not cause severe alterations of the visual appearance and health of their host, I tested the impact of the digenean trematode *Diplostomum pseudospathaceum* and of the monogenean *Gyrodactylus* sp. on the shoaling behaviour of sticklebacks (*Chapter III* and *IV*).

Diplostomum spp.

Diplostomum is a genus of digenean trematodes with a complex life cycle (Fig. 2). Adult worms mate in the intestines of birds (definitive host) that consume infected fish. With the birds' faeces, eggs are released into the water where one small, ciliated larva (miracidium, *c*. 70–140 µm in length, see e.g., Sweeting, 1976; Field and Irwin, 1995) hatches from each egg. These miracidia infect snails (mostly lymnaeids), develop to sporocysts and asexually multiply to hundreds or thousands – depending on the parasite and snail species – of furcocercous (fork-tailed) cercariae (Sweeting, 1976; Chappell et al., 1994). The cercariae actively leave the snail, find a fish host (second intermediate host) and – attracted by a suite of chemical compounds of the fish surface (Haas et al., 2002) – attach to and penetrate the skin. When entering the fish, cercariae loose their tail and sequentially follow different chemical cues (Haas et al., 2007) to migrate to the eyes or the brain (e.g. *D. phoxini*) of the fish within a few hours. Here, they develop to infective metacercariae (Fig. 2) that are able to establish in piscivorous birds.

Site selection within the fish eye appears to be species specific with some species, e.g. *D.* (*pseudo-*)*spathaceum*, infecting the eye lens while others, e.g. *D.* (*pseudo-*)*baeri* or *D. gasterostei*, are located in the vitreous chamber, i.e. the humour or the retina (e.g., Williams, 1966b; Field and Irwin, 1995; Locke et al., 2010a). Eyes of vertebrates are immune privileged sites where immune responses are strictly regulated and from which systemic T-cell effector responses are actively suppressed (Streilein, 1987; Streilein and Stein-Streilein, 2000; McKenna and Vicetti Miguel, 2011). While the interior of the lens is

inert, the retina is vulnerable to inflammation once the blood–retina barrier is breached by pathogens (Gregory, 2011). Therefore, it is thought that lens-infecting *Diplostomum* species, which are found in a large number of fish host species (Kennedy, 1974; Valtonen and Gibson, 1997), are potentially less host-specific than parasites that are located in the retina (Locke et al., 2010b). At the site of penetration and during migration, i.e. for less than 24 h (Ratanarat-Brockelman, 1974), cercariae are exposed to the host's immune system and elicit local innate inflammatatory responses (Ratanarat-Brockelman, 1974; Whyte et al., 1987). Larvae that do not reach the eye are attacked and removed by macrophages by means of phagocytosis (Ratanarat-Brockelman, 1974; Whyte et al., 1990). Innate immune responses are mounted much faster than reactions of the adaptive immune system (Ratanarat-Brockelman, 1974; Kalbe and Kurtz, 2006; Scharsack and Kalbe, 2014), but can be specific with regard to parasite genotype as has been shown in three-spined sticklebacks (Rauch et al., 2006; Haase et al., 2014).

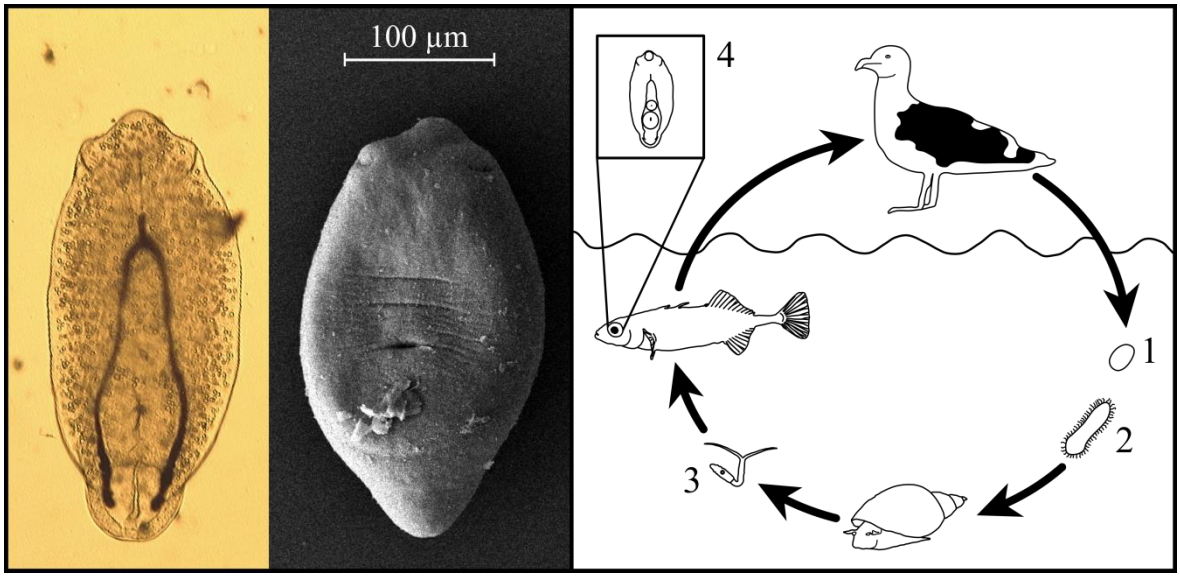

Fig. 2. Light microscope (left) and scanning electron microscope (middle) images of *Diplostomum* spp. metacercariae from the retina of infected sticklebacks; schematic overview of the life cycle and infective stages of *Diplostomum* sp. – 1 egg, 2 miracidium, 3 cercaria, 4 metacercaria.

The pathology of *Diplostomum* infections is most likely an indirect consequence of a degeneration of the lens and local inflammatory responses following rupture of the lens capsule since this causes the formation of cataracts that ultimately lead to complete blindness (Rushton, 1937; Shariff et al., 1980). Thus, impairment is not only determined by the actual number of eyeflukes per infected fish, but also by the amount of time the metacercariae have moved and fed inside the eye (lens), i.e., by their developmental stage. Chronic *Diplostomum* infections affect feeding capability (Crowden and Broom, 1980;

Shariff et al., 1980; Owen et al., 1993; Voutilainen et al., 2008) and often result in reduced body condition (Shariff et al., 1980; Buchmann and Uldal, 1994; Bjerkås et al., 1996; Kuukka-Anttila et al., 2010), emaciation, and increased mortality (Shariff et al., 1980; Brassard et al., 1982) in several species of fish. Further, infected rainbow trout (*Oncorhynchus mykiss*) are darker in body colour than uninfected conspecifics (Shariff et al., 1980; Seppälä et al., 2005a) and their ability to reduce contrast on light-coloured substrates is impaired (Seppälä et al., 2005a). In Atlantic salmon (*Salmo salar*) standard metabolic rate is positively correlated with cataract formation (Seppänen et al., 2008) while chronic infections cause decreased standard metabolic rate, higher masses of spleen and liver (Seppänen et al., 2009), and higher oxygen consumption (Voutilainen et al., 2008) in infected Arctic charr (*Salvelinus alpinus*). Although in naturally infected three-spined sticklebacks even small numbers of eyeflukes (7–34 metacercariae per fish) can affect visual acuity and reduce reactive distance to living prey items (*Daphnia*, Owen et al., 1993), negative effects of *Diplostomum* spp. infections on the health of sticklebacks are less pronounced (Kalbe and Kurtz, 2006) and usually limited to individuals with high infection intensities.

The free-living stages of *Diplostomum* – miracidium and cercaria – are short lived and loose their ability to infect a new host within less than one (cercariae) or two (miracidia) days of emergence from egg or snail (Whyte et al., 1991; Chappell et al., 1994). Therefore, the endoparasite is exposed to the surrounding water for only a short period of time. Infectivity decreases with decreasing water temperature (with a lower limit of 10 °C; Stables and Chappell, 1986; Chappell et al., 1994) and increased water flow (Stables and Chappell, 1986). General habitat characteristics (e.g. open vegetation and attractiveness for piscivorous birds) and water quality influence the distribution of eyeflukes also indirectly by providing suitable conditions for their intermediate hosts. Dissolved calcium can be a reliable predictor of the occurrence of diplostomiasis in a habitat since the snail host often depends on a certain minimum calcium concentration in the water (Curtis and Rau, 1980).

Eyefluke infections and cataracts potentially compromise the ability to escape predation by birds and therefore directly affect the transmission of *Diplostomum* spp. to the definitive host. Therefore, studies on the impact of *Diplostomum* spp. on the (social) behaviour of fishes has so far concentrated on this aspect. Dace (*Leuciscus leuciscus*) spend more time close to the water surface when they are more heavily infected which is

expected to increase transmission (Crowden and Broom, 1980). Infected rainbow trout, which are less bold than uninfected animals (Klemme et al., 2016), form smaller shoals and do not show increased shoal cohesion when attacked by an (artificial) predator from above (Seppälä et al., 2008). Further, chronic infection with *Diplostomum* spp. leads to an increased risk of predation by aerial predators (simulated by experimenters with dip nets; Seppälä et al., 2004; Seppälä et al., 2005b), but not by wild gulls (Seppälä et al., 2006). Apart from tests on prey detection (Owen et al., 1993), possible effects of *Diplostomum* spp. on the behaviour of sticklebacks have not been examined before.

Gyrodactylus spp.

Parasitologists traditionally refer to parasites of small body size, such as viruses, bacteria, protozoa, and fungi, as microparasites, and to (multicellular) organisms, such as parasitic plants and animals, as macroparasites (Reece et al., 2016). This distinction is mainly based on the visibility either with the "naked eye" (macroparasites) or with the help of a microscope (microparasites) and is not absolutely strict with respect to taxonomy (Schmid-Hempel, 2011). Typically, microparasites have much shorter generation times than their respective hosts and they are able to reproduce to uncountable numbers causing epidemics in a new host population while macroparasites have longer generation times and usually can be counted individually, e.g., to determine the intensity of an infection (Schmid-Hempel, 2011). Stickleback-infecting gyrodactylids are viviparous monogenean flatworms (Platyhelminthes) with a direct life cycle and generation times of only a few days (Scott, 1982) depending on the ambient temperature (Jansen and Bakke, 1991). They give birth to a fully grown daughter once that daughter bears inside an embryo (Bakke et al., 2007; Fig. 3). Due to this "Russian-doll"-like mode of reproduction which includes sexual as well as asexual production of embryos (Bakke et al., 2007), and the fact that the ectoparasite is easily transmitted via body contact (either directly or indirectly via the substratum, water column, dead or paratenic hosts; Cable et al., 2002; Olstad et al., 2006; Richards et al., 2012), single *Gyrodactylus* worms can start an epidemic (Scott and Anderson, 1984). As mentioned above, this epidemiological potential is characteristic of microparasites. Yet, to stay with the traditional concept anticipated by biologists of most disciplines, *Gyrodactylus* will be considered a macroparasite throughout this thesis.

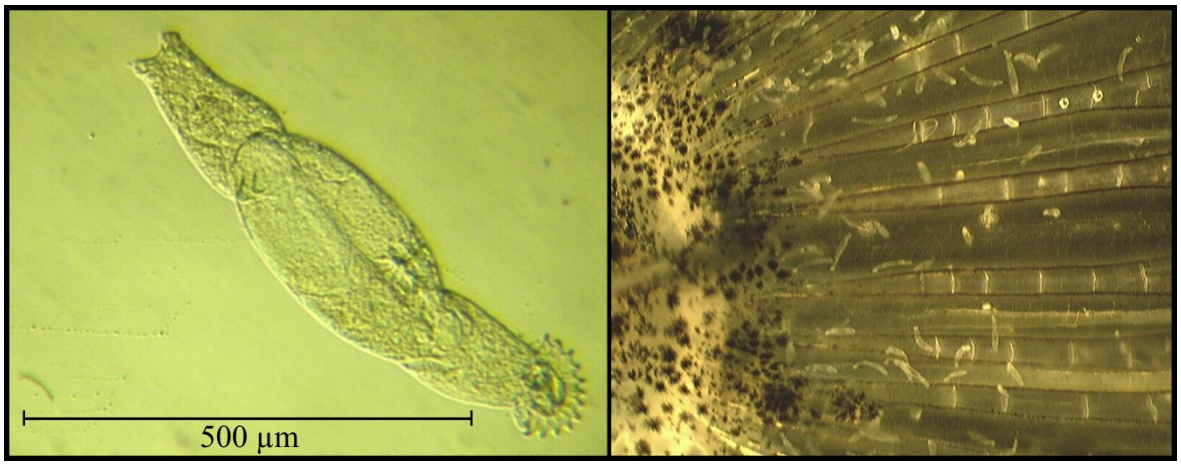

Fig. 3. *Gyrodactylus* sp. with two embryos (left) and on the tail fin of an infected stickleback (right).

Gyrodactylus has gained much interest by parasitologists as well as by fish farmers because of the devastating effects of *G. salaris* on the (salmon) fish industry, particularly in Norway (see e.g., Bakke et al., 2007 and citations therein). The about 0.4–0.7 mm long parasite (Malmberg, 1970; Fig. 3) uses adhesive substances secreted by specific glands (Kritzky, 1978; Whittington et al., 2000) and a special adhesive organ (opisthaptor) with two large hooks (hamuli) and 16 marginal hooks to attach to the fins, gills, or skin of fish (Fig. 3). Attachment causes small damages which pave the way for secondary (fungal or bacterial) infections (Buchmann and Lindenstrøm, 2002; Bakke et al., 2007). The parasite feeds from its host's mucus and epithelial cells. The costs of infection are generally associated with the parasite burden and the general health status of the fish, but the impact of *Gyrodactylus* on the health of its host is species- and even strain-specific (see e.g., Bakke and MacKenzie, 1993; Cable and van Oosterhout, 2007). In Atlantic salmon (*Salmo salar*), e.g., *G. salaris* infections increase mortality to up to 100 % (Johnsen and Jensen, 1992) while the same *Gyrodactylus* species has less or no significant effect on mortalities in brown trout (*Salmo trutta*, Johnsen and Jensen, 1992). Typically, otherwise healthy three-spined sticklebacks can tolerate low infestations without obvious severe costs of infection (Lester, 1972; Lester and Adams, 1974; de Roij et al., 2010). Yet, *Gyrodactylus* infections can elicit an innate immune response (Lester, 1972), lead to increased mucus production and eroded fins (Lester, 1972), and cause higher than usual mortality rates (Lester and Adams, 1974). Further, higher parasite burdens are associated with lower weight gain (Eizaguirre et al., 2012) and body condition (Anaya-Rojas et al., 2016).

Detached *Gyrodactylus* can survive for up to several days (e.g., Olstad et al., 2006), but they are not resistant to drying or freezing and are therefore bound to aquatic habitats.

As an ectoparasite, *Gyrodactylus* is constantly exposed to the ambient water. Thus, its distribution largely depends on suitable environmental conditions but also on the availability and dispersal of susceptible hosts. Many gyrodactylids are considered host specific (Bakke et al., 1992; Whittington et al., 2000) and even site specific with some species most often found on the gills and others on the skin or fins of their host (see e.g., Malmberg, 1970; Raeymaekers et al., 2008). Stickleback populations differ in their resistance to *Gyrodactylus* (de Roij et al., 2010; Raeymaekers et al., 2011; Eizaguirre et al., 2012; Anaya-Rojas et al., 2016; Mahmud et al., 2017). Yet, since *Gyrodactylus* can also make use of less suitable hosts as vectors (Soleng and Bakke, 1998; Cable et al., 2013; Paladini et al., 2014), environmental factors remain a crucial factor for the distribution of this parasite. *Gyrodactylus* occurs in habitats as diverse as fresh-, brackish-, and salt-water habitats, and strains of the parasite are usually well adapted to their habitat of origin. However, changes in water quality regarding salinity (Lester and Adams, 1974; Soleng and Bakke, 1997), metal concentrations (Poleo et al., 2004; Gheorghiu et al., 2007), humic acid (Yamin et al., 2017) and pH (Mahmud et al., 2017) severly affect the distribution of *Gyrodactylus* (Bakke et al., 2007).

Due to its direct mode of transmission and its constant need of new susceptible hosts (Scott and Anderson, 1984; Boeger et al., 2005), it is obvious that *Gyrodactylus* thrives in large (Bagge et al., 2004) and more dense (Johnson et al., 2011) host groups. Effects of *Gyrodactylus* on the behaviour of three-spined sticklebacks have not been examined before, but behavioural implications have been studied in guppies (*Poecilia reticulata*). In this popular aquarium fish, infection with only tens of worms of *Gyrodactylus turnbulli* causes aberrant swimming behaviour, fin clamping, reduced courtship and competitiveness, and high mortality (Cable et al., 2002; Bakke et al., 2007; Kolluru et al., 2009). *Gyrodactylus*-infected guppies reduce shoal cohesion by eliciting more fission events in shoals than uninfected animals do, and they show a reduced tendency to shoal compared to uninfected individuals (Croft et al., 2011; Hockley et al., 2014b), but results seem to differ between wild and ornamental guppies (Richards et al., 2012). It is not clear whether conspecifics are discriminated based on their infection status.

The island of North Uist

The findings that are reported on in the first two chapers of this thesis are based upon three field trips of several weeks to the Scottish island of North Uist. Various characteristics

make North Uist an excellent place to study ecological and evolutionary aspects of sticklebacks and host–parasite interactions, in general, and the questions I strived to answer with this work, in particular. The island provides a range of lakes that differ in water chemistry as well as in their degree of isolation from other habitats, and it is home to a variety of potential (intermediate) hosts.

Fig. 4. Typical oligotrophic, acidic lake (upper image) and alkaline lake in the machair on North Uist.

North Uist is part of the Outer Hebrides, which shield the north-west coast of the Scottish mainland from the North Atlantic. The island is situated approximately 20 kilometres west of the Isle of Skye and measures about 300 square kilometres with maximum distances of about 21 kilometres from north to south and about 29 kilometres from west to east (Thompson, 1999). More than 180 lakes define the landscape of North Uist (Giles, 1983; Fig. 4). Most of which have been isolated since the last deglaciation about 15,000 years ago (Ballantyne, 2010). The soil contains large amounts of peat. Due to humic acid and dissolved tannins, waters in the central, southern and eastern part of the island are acidic, tea-stained, and oligotrophic. On the west and north-west coast of North Uist, additional calcareous shell-sand from the Atlantic forms the basis for the so called “machair“ – fertile grassland with clear, alkaline waters –, which is unique for the Atlantic coast of the Outer Hebrides and Ireland (Whittington and Edwards, 1997). Due to this

gradient of water qualities, the lakes on North Uist differ greatly in biological productivity (Waterston et al., 1979; de Roij and MacColl, 2012).

North Uist possesses a rich avifauna of which piscivorous birds are of interest here not only because of their role as stickleback predators, but also as potential definitive hosts of fish parasites with a complex life cycle. The piscivorous birds on North Uist include red- and black-throated divers (*Gavia stellata* and *G. arcitica*), red-breasted merganser (*Mergus serrator*), Little Grebe (*Tachybaptus ruficollis*), grey heron (*Ardea cinerea*), gulls (black-headed gull, *Larus ridibundus*, common gull, *Larus canus*), and terns (common tern, *Sterna hirundo*, and Arctic tern, *Sterna paradisaea*; Giles, 1981; MacColl et al., 2013) . The fish fauna of the freshwater lakes comprises six euryhaline species: salmon (*Salmo salar*), sedentary populations of brown trout (*S. trutta*) and charr (*Salvelinus alpinus*), eel (*Anguilla anguilla*), and three- and nine-spined (*Pungitius pungitius*) sticklebacks (Campbell and Williamson, 1979; Waterston et al., 1979). Of these species, brown trouts – the main (fish) predator of the sticklebacks, eels, and three-spined stickleback are almost ubiquitous in the freshwater lakes of the island (Campbell and Williamson, 1979).

Three-spined sticklebacks, which have recolonised North Uist from the North Atlantic (Ravinet et al., 2014), are also found as residents in the brackish water lagoons around the island (MacColl et al., 2013) where they temporarily co-occur with anadromous sticklebacks that enter the brackish water lagoons in spring to spawn (MacColl et al., 2013). While most sticklebacks on North Uist are annual, about 10 % experience a second winter (Abdul Rahman and Andrew MacColl unpublished data). North Uist sticklebacks show a range of different morphological types from a slender, spine-less and lateral plate deficient morph to the completely plated morph with a deeper body (Campbell, 1985). The past decades have seen rising interst in the ecological mechanisms behind morphological and behavioural adaptations of the North Uist sticklebacks. Direct and indirect influences of environmental factors such as calcium availability and varying degrees of predation risk have been discussed as putative causes for the phenotypic diversity in body armour and overall body shape (Giles, 1981; Giles, 1983; Spence et al., 2013; MacColl and Aucott, 2014; Smith et al., 2014; Klepaker et al., 2016; Magalhaes et al., 2016). Also, three-spined sticklebacks tend to be generally smaller in lakes where pH is lower and the slightly smaller competitor, the nine-spined stickleback, is less abundant (MacColl et al., 2013). Furthermore, the different degrees of light-transmission in the peat-influenced lakes have

raised some intriguing questions about the relative importance of UV-signalling in different social contexts (Hiermes, 2015; Hiermes et al., 2015b). And, recently, the North Uist sticklebacks have also proven promising subjects to study (ecological) correlates of boldness (Spence et al., 2013; De Winter et al., 2016). Taken together, the geographic mosaic of different habitats, which have been isolated from each other and from the sea for thousands of years, provides a great opportunity to study local host–parasite dynamics and (co-)adaptations within a reasonably small geographical scale.

Thesis outline

This thesis consists of four studies that are written as four independent manuscripts (*Chapter I–IV*). The objectives of these studies are given below. A separate paragraph at the end of this section summarises the contributions of the different co-authors.

The main focus of this thesis was to search for correlational evidence helping to disentangle the influence of life history and ecology on parasite distribution, and to experimentally test whether sticklebacks change their shoaling decisions in the presence of a contagious and/or a non-contagious parasite.

More specifically, I aimed to answer the following questions:

1. Does the population genetic structure of *Diplostomum* lineage 6 support the paradigm that the most motile host in a parasite's life cycle determines its dispersal? – ***Chapter I***
2. Does spatial variation in pH shape stickleback parasite distribution on North Uist or are connectivity between habitats and host dispersal more important? – ***Chapter II***
3. Do the simple life-cycle parasite *Gyrodactylus* spp. and the complex life-cycle parasite *Diplostomum pseudospathaceum* affect shoal choice decisions in three-spined stickleback, and if so, do the effects differ depending on the type of infection? – ***Chapters III*** and ***IV***

In 2012, de Roij and MacColl examined the macroparasitic fauna of the North Uist sticklebacks and found substantial differences among lakes in abundances of single parasite species as well as in parasite community composition which were consistent over two years, but could not be attributed to environmental charactersistics such as pH, calcium availability, or habitat size (de Roij and MacColl, 2012). Unfortunately, the chosen set of lakes weighted acidic lakes much more strongly than alkaline lakes (ten acidic lakes compared to only two alkaline lakes) and did not take into consideration genetic connectivity between host populations although population specific differences in host susceptibility have long been known to shape parasite distribution and although the North Uist sticklebacks are known to differ in their susceptibility, e.g., to *Gyrodactylus* spp. (de Roij et al., 2010). Building on these previous findings, *Chapters I* and *II* are concerned with the ecological and population genetic foundations of the macroparasitic faunae of the North Uist stickleback populations.

Chapter I

I developed the first microsatellite primers for *Diplostomum* lineage 6. In *Chapter I,* I used these markers together with previously published stickleback primers to analyse the population genetic structures of the eyefluke and its second intermediate host. According to the paradigm that the most motile host in a parasite's life cycle determines its population structure, I did not expect to find distinct populations of *D.* lineage 6. In contrast to that, I expected to detect strong population genetic differentiation among the stickleback populations.

Chapter II

Chapter II analysed the spatial distribution of common stickleback macroparasites in 19 freshwater lakes on North Uist in relation to abiotic habitat characteristics, such as pH and lake surface area. Dissimilarity in parasite communities between lakes was tested for correlation with pairwise host population genetic differentiation (measured as pairwise F_{ST} based on microsatellite data) between sampling sites. I hypothesised that the distribution of ectoparasites that are constantly in contact with the surrounding medium and of parasites with calcium-dependent intermediate hosts were not independent of pH. Further, I expected that local host–parasite dynamics would show in a correlation between dissimilarity in parasite communities and host genetic differentiation.

Chapter III

In *Chapter III*, I examined whether the directly transmitted monogenean *Gyrodactylus* affects shoaling behaviour in three-spined sticklebacks using binary shoal choice experiments with experimentally infected sticklebacks. I hypothesised that uninfected individuals would prefer uninfected over infected shoals, which bear a risk of infection. Further, I expected infected sticklebacks not to show a clear preference for either of the two shoal types since healthy sticklebacks might be more competitive than infected ones while the potential harm caused by a few additional parasites might be negligible.

Chapter IV

Diplostomum pseudospathaceum was used in *Chapter IV* to test for potential effects of this not directly transmitted, lens-infecting eyefluke on the shoal choice of three-spined sticklebacks. Experimental studies that test for effects of non-contagious parasites with no or only marginal influence on the appearance of the host on shoaling decisions are rare.

Here, I examined the effect of *D. pseudospathaceum* on sticklebacks that were kept in outdoor tanks under seminatural winter temperature conditions either in purely uninfected or in mixed groups and hypothesised that uninfected sticklebacks should prefer to shoal with uninfected conspecifics.

Author's contributions

Chapter I

Theo C. M. Bakker conceived the study. Anna K. Rahn and Andrew D. C. MacColl contributed to the study design. Anna K. Rahn collected the samples, analysed the data and wrote the manuscript, supported by Theo C. M. Bakker and Andrew D. C. MacColl. Johannes Krassmann contributed to the analysis of the sticklebacks' population structure. Kostas Tsobanidis contributed to establishing and applying the *Diplostomum* markers.

Chapter II

Theo C. M. Bakker conceived the study. Anna K. Rahn contributed to the study design, collected the samples and performed the measurements assisted by Elisabeth Eßer, Stephanie Reher, and Flora Ihlow. Anna K. Rahn analysed the data and wrote the manuscript, supported by Theo C. M. Bakker and Andrew D. C. MacColl.

Chapter III

Anna K. Rahn and Theo C. M. Bakker conceived the study. Daniela A. Hammer contributed to the study design and performed the experiments. Anna K. Rahn and Theo C. M. Bakker analysed the data. Anna K. Rahn wrote the manuscript, supported by Theo C. M. Bakker.

Chapter IV

Anna K. Rahn, Simon Vitt, Ingolf P. Rick, and Theo C. M. Bakker conceived the study. Lisa Drolshagen contributed to the study design and performed the experiments. Anna K. Rahn and Lisa Drolshagen analysed immune parameters, supported by Jörn P. Scharsack. Anna K. Rahn, Ingolf P. Rick, and Simon Vitt analysed the data. Anna K. Rahn wrote the manuscript, supported by Theo C. M. Bakker, Ingolf P. Rick, Simon Vitt, and Jörn P. Scharsack.

The following four chapters of this thesis are written as manuscripts and have been published in scientific journals. This requires that they must be comprehensive in themselves and it makes recurrent descriptions and concordant explanations inevitable at times. Format and layout of the published manuscripts were adapted to the general layout of this thesis, but no changes were made with regard to content. References and corresponding supplementary material of all chapters are listed in separate sections at the end of this thesis.

Chapter I

Strong neutral genetic differentiation in a host, but not in its parasite

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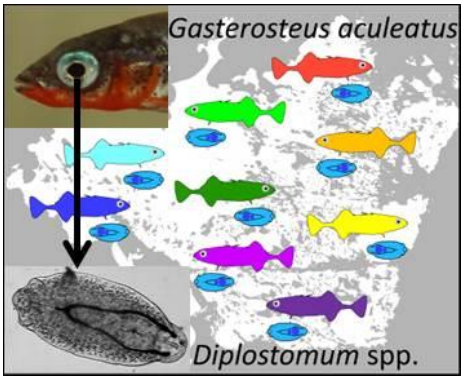
Chapter I

Strong neutral genetic differentiation in a host, but not in its parasite

Anna K. Rahn, Johannes Krassmann, Kostas Tsobanidis, Andrew D.C. MacColl, Theo C.M. Bakker



Graphical abstract

Abstract

The genetic diversity and population structure of a parasite with a complex life cycle generally depends on the dispersal by its most motile host. Given that high gene flow is assumed to hinder local adaptation, this can impose significant constraints on a parasite's potential to adapt to local environmental conditions, intermediate host populations, and ultimately to host-parasite coevolution. Here, we aimed to examine the population genetic basis for local host-parasite interactions between the eye fluke *Diplostomum* lineage 6, a digenean trematode with a multi-host life cycle (including a snail, a fish, and a bird) and its second intermediate host, the three-spined stickleback *Gasterosteus aculeatus* L. We developed the first microsatellite primers for *D.* lineage 6 and used them together with published stickleback markers to analyse host and parasite population structures in 19 freshwater lakes, which differ in their local environmental characteristics regarding water chemistry and *Diplostomum* abundance. Our analyses suggest that one parasite population successfully infects a range of genetically differentiated stickleback populations. The lack of neutral genetic differentiation in *D.* lineage 6, which could be attributed to the motility of the parasite's definitive host as well as its life cycle characteristics, makes local host-parasite co-adaptations seem more likely on a larger geographical scale than among the lakes of our study site. Our study provides a suitable background for future studies in this system and the first microsatellite primers for a widespread fish parasite.

Introduction

In host–parasite interactions both parasites and hosts are expected to adapt not only to changes in their respective environments, but also to changes in each other's defence mechanisms. Since the balance between selection and gene flow is considered the strongest determinant of local adaptation (e.g., Tigano and Friesen, 2016), investigating the rate of genetic exchange among host and parasite populations can help to understand the local adaptive potential in a host-parasite system. Generally, it is assumed that high migration rates and gene flow can hinder adaptation to (temporally stable) habitats where selection by environmental factors is weak (Slatkin, 1987; Lenormand, 2002; Kawecki and Ebert, 2004).While limited gene flow reduces the introduction of maladapted alleles and thus favours local adaptation, genetic drift, which can cause the loss of potentially beneficial alleles, is expected to decrease local adaptation (Blanquart et al., 2012). Host–parasite systems add a further dimension of (reciprocal) adaptations because host populations that adapt their defence mechanisms to the parasites present in their habitat constitute an environment that changes not only in space, but also in time. In temporally variable environments, on the other hand, intermediate levels of gene flow can even maximise adaptation by contributing to genetic variation (Blanquart et al., 2013). Interestingly, a recent meta-analysis found a general trend towards stronger genetic differentiation in hosts than in parasites across a wide range of taxa (Mazé-Guilmo et al., 2016). In light of this, identifying the mechanisms which determine dispersal and genetic differentiation in parasites remains a key question in the study of host–parasite interactions.

The distribution and population structure of a parasite (here we refer to macro-parasites) depends on a range of different factors. Host dispersal is commonly considered the most obvious determinant of parasite dispersal (Blouin et al., 1995). Although gene flow requires physical movement between populations and dispersal is usually expected to correlate positively with gene flow (Räsänen and Hendry, 2008; but see Edelaar and Bolnick, 2012), dispersal per se is not the only factor determining parasite genetic structure (Mazé-Guilmo et al., 2016). Host-specificity and life-history traits like the mode of reproduction, the existence of free-living stages, or life-cycle complexity also affect parasite population structures and genetic diversity (see e.g. Barrett et al., 2008; Blasco-Costa and Poulin, 2013; Mazé-Guilmo et al., 2016 for a review and meta-analyses). Since different factors (partly with opposed effects) act on different stages in the life cycle, parasites with complex (multi-host) life cycles are particularly interesting, in this regard.

By providing additional dispersal opportunities (intermediate/alternate host(s), water current), life-cycle complexity, host specificity, and the presence and number of free-living stages are expected to contribute to weaker parasite genetic differentiation compared to each single host (Mazé-Guilmo et al., 2016). Theoretical models indicate that in parasite species with alternating sexual and asexual reproduction self-fertilisation in the sexual phase results in higher inbreeding coefficients whereas variance in reproductive success among different clones decreases inbreeding coefficients (Prugnolle et al., 2005a). In a recent meta-analysis hermaphroditic parasites were less genetically differentiated than their hosts, which was attributed to a homogenising effect of higher dispersal rates in the (mostly bird-infecting) parasites (Mazé-Guilmo et al., 2016). Quite a few theoretical and empirical studies have focussed on genetic diversity in digenean trematodes, a subclass of parasitic flatworms (Platyhelminthes), which exhibit complex life cycles and comprise many human and livestock infecting species. In general, in digenean trematodes the host with the largest geographic range, i.e. usually the definitive host, is assumed to determine dispersal and genetic structure. This has been shown e.g. in salmon and eel infecting trematodes (Criscione and Blouin, 2004; Blasco-Costa et al., 2012), *Schistosoma mansoni* (Prugnolle et al., 2005b; Van den Broeck et al., 2015), *Diplostomum pseudospathaceum* (Louhi et al., 2010), and in marine trematodes (Feis et al., 2015). Further, parasites completing their entire life cycle in aquatic habitats tend show more pronounced population structuring than parasites which use birds or (terrestrial) mammals as definitive host since these facilitate dispersal across aquatic habitat boundaries (Criscione and Blouin, 2004; Blasco-Costa and Poulin, 2013; Feis et al., 2015).

Here, we investigate the population structure of the digenean trematode *Diplostomum* lineage 6. Adult *Diplostomum* sexually reproduce in the intestines of piscivorous birds either through self-fertilisation or outcrossing (facultative hermaphrodites). With the bird's faeces, their eggs are released into the water where larvae (miracidia) hatch and infect lymnaeid snails. Inside the snail host, miracidia develop to sporocysts which clonally multiply and develop further into cercariae. These leave the snail, penetrate the skin of fish within eight minutes or less (Williams, 1966b) and move within hours to the lens or to the retina. Thus, the parasite is exposed to the immune system of its host only for a short period of time before it reaches the immune-privileged eye. Despite this short time frame, innate resistance of the three-spined stickleback *Gasterosteus aculeatus* L. against *D. pseudospathaceum* is based on genotype-genotype interactions and (indirectly) involves

the adaptive immune system of the host (Rauch et al., 2006; Haase et al., 2014; Haase et al., 2015). Research on host-parasite interactions of *Diplostomum* mainly focuses on lens-infecting species, which form cataracts and can have severe consequences for the competitive ability, growth and mortality of their host, particularly in fish farms (Chappell et al., 1994). *Diplostomum* species infecting the non-lens region have rarely been investigated, although recent molecular studies suggest that *Diplostomum* species diversity within the non-lens region might be higher than previously thought (Locke et al., 2010b; Blasco-Costa et al., 2014; Locke et al., 2015). In the only population genetic study on a *Diplostomum* species of which we are aware, Louhi et al. (2010) analysed the population genetic structure of *D. pseudospathaceum* over a geographic range of > 300 km between sampling sites and failed to detect evidence for population structure despite the presence of population genetic structuring in the snail host *Lymnaea stagnalis* (Puurtinen et al., 2004).

In this study, we aimed to compare the population genetic structure of *Diplostomum* lineage 6 – an eye fluke from the non-lens region in fishes – with that of its second intermediate host, the three-spined stickleback *Gasterosteus aculeatus* L., on the Scottish island of North Uist. The three-spined stickleback has frequently colonised freshwater habitats from the sea and is known to diverge into genetically differentiated populations within relatively short periods of time (e.g., Lescak et al., 2015). Thus, we expected strong population genetic structuring in the fish host, while we expected the parasite's highly motile definitive host to impede the formation of distinct populations in *D.* lineage 6. The three-spined sticklebacks on North Uist have proven interesting models for various research questions in the recent past regarding e.g. morphology (MacColl et al., 2013; Smith et al., 2014), UV-signalling (Hiermes et al., 2015b), patterns of macroparasite distribution (de Roij and MacColl, 2012; Rahn et al., 2016), and spatial differences in susceptibility to a monogenean parasite (de Roij et al., 2010). Therefore, we additionally aimed to establish a useful basis for further studies in this system.

Materials and methods

Study site and sampling

North Uist (Outer Hebrides, Scotland) measures about 300 km^2 and is covered with > 180 lakes (Giles, 1983). Due to the influence of shell sediment and peat, these lakes comprise habitats ranging from alkaline clear water lakes in the west to lakes with acidic tea-stained water in the central and eastern part of the island (Giles, 1983). The lakes were likely

recolonised by sticklebacks from the North Atlantic (Ravinet et al., 2014) during the last deglaciation approximately 15,000 years ago (Giles, 1983; Ballantyne, 2010) and have been isolated from each other ever since. The North Uist sticklebacks are mostly annual with about 10% experiencing a second winter (Abdul Rahman & Andrew MacColl unpublished data). De Roij and MacColl (2012) and Rahn et al. (2016) have examined the distribution of stickleback macroparasites on North Uist and found substantial differences in *Diplostomum* spp. abundances among lakes, which were largely consistent over several years. As these differences could not be explained by general abiotic habitat characteristics such as geographic distance, pH or the amount of dissolved calcium, they were attributed to local host-parasite dynamics. Prevalences (% fish infected) of *Diplostomum* spp. of the non-lens region (present in all lakes sampled in this study, not identified to species level) ranged from 14 to 100% (55, 31.5, 90; median, 1st, 3rd quartiles) (Table 1; see also Rahn et al., 2016).

We caught approximately 21 (median; 20, 25 1st, 3rd quartiles) adult male and female three-spined sticklebacks per sampling location from 19 freshwater lakes and from three coastal lagoons with open access to the sea (see Fig. 1 and Table 1 for sampling locations and sample sizes). Lakes were chosen with the aim of covering a geographically large part of the island as well as a broad spectrum of sampling locations representing the habitat diversity found on North Uist with regard to *Diplostomum* spp. abundance and presumably resistance to parasites (de Roij et al., 2010; de Roij and MacColl, 2012; Rahn et al., 2016), water chemistry, and stickleback morphology. Fish were caught using minnow traps (Jenzi: green nylon mesh (3–4 mm), Gee: galvanized steel mesh, G40 M, G48 M), which were set overnight in shallow water near the shoreline in spring 2010 (April and May) and 2011 (April). This time of the year marks the beginning of the breeding season when marine sticklebacks enter the coastal bays. At the three brackish water sites resident as well as morphologically distinct (significantly larger, fully plated) anadromous sticklebacks were caught. Therefore, we will speak of a total of 25 sampling locations. We additionally collected fish from the freshwater lakes in summer 2012 (August) to obtain sufficient *Diplostomum* spp. sample sizes.

For dissection, fish were killed by decapitation followed immediately by a cut through the brain and placed under a microscope (Novex RZRange, 6.5–45× magnification, illuminated by a cold-light source (Schott KL 1500)). The eyes of the sticklebacks

were carefully checked for metacercariae within the intact lenses as well as outside the lens. Fins and metacercariae were conserved in 98% EtOH and stored at room temperature.

Table 1. Sampling locations (19 freshwater lakes, three coastal lagoons with anadromous and resident fish) with three letter codes (LocID), lake surface area in km^2 (Area), pH, prevalence of infections with *Diplostomum* outside the lens (in %, D_{prev}), and sample sizes of genotyped individuals given as N_s MS number of sticklebacks genotyped at nine microsatellite (MS) loci, N_s mt number of sticklebacks sequenced at cytochrome *b* and control region of the mitochondrial DNA, and N_D MS number of *Diplostomum* spp. genotyped at six microsatellite loci. pH and *Diplostomum* prevalence (based on an average of 20.8 ± 2.3 dissected fish (mean ± standard deviation)) were taken from Rahn et al. (2016).

Location name	Geographic coordinates	LocID	Area	pH	D_{prev}	N_s MS	N_s mt	N_D MS
Aileodair *anadromous*	57°38'7"N, 7°12'54"W	1ana	–	–	0[d]	58	7	–
Aileodair *resident*		1res	–	–	0[c]	28	5	–
Aird Heisgeir *anadromous*	57°34'48"N, 7°24'48"W	2ana	–	–	0[d]	19	6	–
Aird Heisgeir *resident*		2res	–	–	0[d]	20	5	–
nan Clachan *anadromous*	57°38'14"N, 7°24'45"W	3ana	–	–	0[d]	21	5	–
nan Clachan *resident*		3res	–	–	0[d]	19	5	–
Croghearraidh	57°36'54"N, 7°30'40"W	4GRO	0.108	7.94	14[d]	22	5	19
Eubhal	57°37'6"N, 7°29'42"W	5EUB	0.379	7.89	35[d]	20	5	15
nam Magarlan	57°36'10"N, 7°28'54"W	6MAG	0.066	7.19	100[c]	22	5	20
Hosta	57°37'40"N, 7°29'18"W	7HOS	0.247	8.34	14[d]	20	5	22
Sanndaraigh	57°35'12"N, 7°27'48"W	8SAN	0.157	8.10	51[b]	41	5	18
Olabhat	57°39'8"N, 7°26'48"W	9OLA	0.141	7.47	29[d]	20	5	6
na Gearrachun	57°38'34"N, 7°25'18"W	10GEA	0.070	6.89	100[d]	33	5	20
Mhic Gille-bhride	57°36'6"N, 7°24'36"W	11MGB	0.142	6.77	90[c]	21	5	19
a' Charra	57°35'45"N, 7°23'42"W	12ACH	0.093	6.62	95[c]	21	5	17
Mhic a' Roin	57°35'42"N, 7°25'48"W	13MOI	0.064	6.30	15[d]	20	5	6
Dubhasairidh	57°34'54"N, 7°24'12"W	14DUB	0.234	6.67	55[d]	25	5	7
Tormasad	57°33'45"N, 7°19'W	15TOR	0.213	6.87	72[c]	40	5	11
a' Bharpa	57°34'24"N, 7°17'42"W	16BHA	0.482	6.10	30[d]	20	5	5
na Moracha	57°34'30"N, 7°16'18"W	17MOR	0.367	6.53	95[d]	30	5	22
Sgadabhagh[a]	57°35'6"N, 7°14'10"W	18SCD	5.516	6.16	45[d]	20	4	9
nan Ceithir Eilean	57°34'24"N, 7°15'30"W	19EIL	0.033	7.37	90[d]	21	5	20
an Daimh	57°35'35"N, 7°12'35"W	20DAI	0.034	6.87	55[d]	20	4	6
na Maighdein	57°35'42"N, 7°12'6"W	21MAI	0.095	6.30	33[d]	24	5	6
na Buaile	57°38'48"N, 7°11'48"W	22BUA	0.020	6.29	60[c]	20	5	5

[a] Referred to as "South Sgadabhagh" by (Spence et al., 2013).
[b] Average of two sampling years (2010, 2011).
[c] Sampled in 2010.
[d] Sampled in 2011.

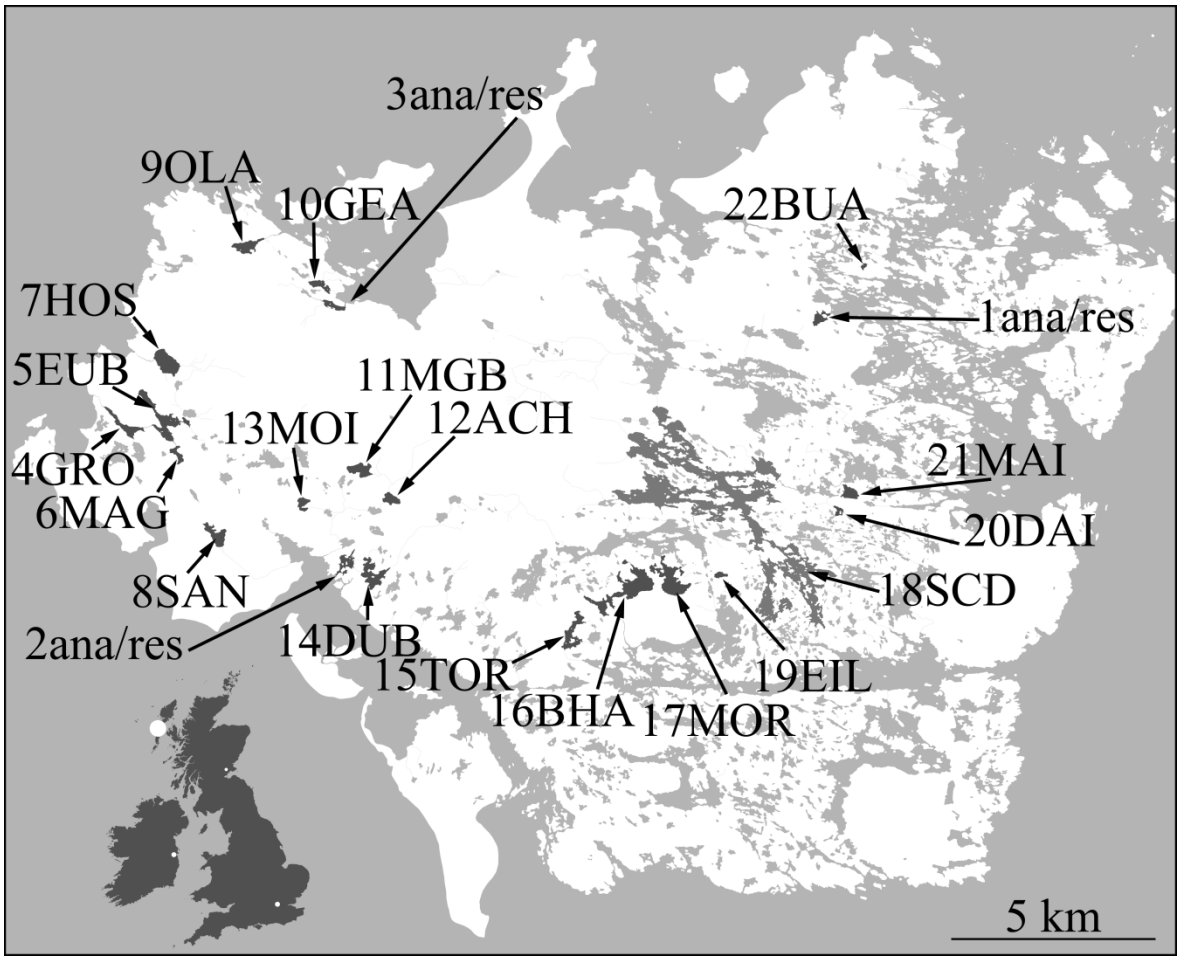


Fig. 1. Distribution of the sampling locations across North Uist. See Table 1 for full lake names.

Microsatellite genotyping of sticklebacks

Amplification

Microsatellite analysis was based on 600 fish caught in spring 2010 and 2011 as well as 25 anadromous sticklebacks from one of the three coastal lagoons (‘Aileodair’) in 2007 some of which had been freshly killed, some had been conserved after they had died in captivity, some had been frozen (−20 °C), and some were stored in 70% denatured EtOH. Genomic DNA was extracted using blood and tissue kits (Macherey and Nagel, Qiagen) following the companies' protocols. DNA concentration was determined using a spectrophotometer (NanoDrop™ 1000, Peqlab) and adjusted to a concentration of 20 ng/µl. DNA samples were stored at −20 °C. Sticklebacks were genotyped at nine microsatellite loci developed at the University of Bern, Switzerland (Gac7010PBBE (Heckel et al., 2002), Gac1097PBBE, Gac1116PBBE, Gac1125PBBE, Gac3133PBBE, Gac4170PBBE, Gac4174PBBE, Gac5196PBBE, Gac7033PBBE (Largiadèr et al., 1999)). DNA was amplified using the tailed primer method (Schuelke, 2000; see Appendix Table A1 for

detailed PCR conditions). PCR products were analysed on a CEQTM 8800 capillary sequencer (Beckman Coulter) with GenomeLabTM GeXP (version 10.2) software. To estimate the reliability of our genotyping method, 10% of all analysed samples (62 randomly chosen fish) were genotyped again. Ambiguities were found for five individuals at one locus each, resulting in an error rate of 0.9%.

Analysis

Allele frequencies were checked for possible scoring errors using the program Micro-Checker (van Oosterhout et al., 2004; 1000 randomisations, Bonferroni correction). The web-based version of Genepop (Genepop on the web 4.2, Raymond and Rousset, 1995; Rousset, 2008) was used to test for linkage disequilibrium as well as for deviation from Hardy-Weinberg equilibrium (10,000 steps dememorization, 1000 batches, 10,000 iterations) and to calculate the inbreeding coefficient F_{IS} according to Weir and Cockerham (1984). Observed and expected heterozygosity (Nei's unbiased gene diversity, Nei, 1987), and pairwise F_{ST} values as a measure for genetic differentiation between sampling locations were calculated in Arlequin 3.5.1.3 (Excoffier and Lischer, 2010; 1000 permutations). Expected heterozygosities of the freshwater populations were regressed against lake surface areas (determined from a 1:25,000 Ordnance Survey map using ImageJ 1.45s; Rasband, 1997-2009) in R3.0.1 (R-Core-Team, 2013). Spearman rank correlations were used as surface area data significantly deviated from normal distribution ($P < 0.05$, Shapiro–Wilk test).

Due to the colonisation history of the island, we followed a Bayesian cluster assignment approach to infer population structure using the programs STRUCTURE 2.3.3 (Pritchard et al., 2000; Falush et al., 2003) and BAPS (Corander and Marttinen, 2006; Corander et al., 2008). Cluster analyses were based solely on allele frequencies. Spatial information was not considered. STRUCTURE analysis was run using an admixture model with correlated allele frequencies with 10^6 Markov Chain Monte Carlo (MCMC) repetitions preceded by a burn-in of 100,000 repetitions. One to 20 clusters were assumed and each number of clusters (K) was tested five times. The most likely K was estimated using the Delta K method (Evanno et al., 2005) as implemented in STRUCTURE Harvester (Earl and von Holdt, 2012). As Delta K indicated a first level of population structure for K = 4 clusters, we additionally performed a hierarchical structure analysis following Coulon et al. (2008; see Appendix Fig. A1). For finding mean cluster

membership coefficients of the five runs for each individual, we used the LargeKGreedy method in CLUMPP (Jakobsson and Rosenberg, 2007; random input order, 1000 repeats). Admixture analysis in BAPS was based on 100,000 simulations. The number of reference individuals per cluster was set to ten. Maximum numbers of clusters from one to 20 were tested ten times.

The microsatellite primers used in this study have proven informative in several other studies, but according to Colosimo et al. (2004) and DeFaveri et al. (2011) loci 4174 and 1125 may be linked to variation in number and pattern of lateral plates (but also see Mäkinen and Merilä, 2008). As North Uist fish differ strongly in these traits (Giles, 1983; Campbell, 1985; Spence et al., 2013; MacColl and Aucott, 2014; Smith et al., 2014), using these loci might have biased our analysis and potentially resulted in overestimating population structure. We therefore additionally ran our STRUCTURE analysis without these loci.

To visualise genetic relationships among fish from the different lakes, a Neighbor-Joining tree was constructed using the software package PHYLIP and the programs therein (Felsenstein, 2013). First, allele frequencies were boostrapped 1000 times using SEQBOOT. The newly generated data sets were then used to calculate pairwise genetic distances (Cavalli-Sforza's and Edwards' chord distance DC, Cavalli-Sforza and Edwards, 1967) in GENDIST. NEIGHBOR and CONSENSE (all PHYLIP) were used to assemble a consensus tree based on majority criteria. The final tree was visualised in FigTree 1.3.1 (Rambaut, 2006).

Mitochondrial DNA sequencing of the sticklebacks

Amplification

Mitochondrial DNA analysis was based on five randomly chosen individuals per sampling location and three morphologically deviating fish found in two of the coastal areas (one partially plated, ‘Aird Heisgeir’, two of intermediate body size compared to anadromous fish and residents, ‘Aileodair’, 128 fish in total, Table 1). We considered these sample sizes sufficient as theory suggests that even small samples can describe distribution patterns of allele frequencies and limit standard deviations of haplotype and nucleotide diversity (Tajima, 1983).

Partial sequences of the cytochrome *b* and control region of the mitochondrial DNA were amplified using the primers published in Mäkinen and Merilä (2008).We did not

make use of the nested primer method suggested by the authors. Separate PCRs were carried out for cytochrome *b* and control region sequences respectively. PCR conditions can be found in Table A1 of the supporting information. Amplification success was confirmed on 1.5% agarose gel before purified (MN NucleoSpin® PCR clean-up kit) PCR products were sent to a commercial sequencing service (LGC Genomics GmbH, Berlin).

Analysis

Electropherograms of the raw sequences were visually checked for ambiguities and manually edited and aligned in BioEdit 7.2.5 (Hall, 1999). Final cytochrome *b* sequences (1014 bp) and sequences of the control region (453 bp) were concatenated to a sequence with a total length of 1467 bp. Diversity indices (haplotype diversity (Hd), nucleotide diversity (π) (Nei, 1987) and average number of nucleotide differences (k) (Tajima, 1983)) were calculated in DnaSP 5.10.01 (Librado and Rozas, 2009). Arlequin was used to calculate average pairwise nucleotide differences between sampling locations and to compare these with pairwise F_{ST} values calculated from microsatellite data using a Mantel test with 1000 permutations. A median-joining network of all haplotypes that occurred at least twice in the data set was constructed using the program Network 4.6.1.3 (http://www.fluxus-engineering.com; Bandelt et al., 1999; Polzin and Vahdati Daneshmand, 2003). Epsilon was set to 10 as suggested by the program's manual (page 17) and all variable sites were weighted equally.

Establishing microsatellite primers for Diplostomum *spp.*

To our knowledge, no microsatellite primers have so far been published for any *Diplostomum* species from the non-lens region of the eyes of freshwater fish. The only available primers for *Diplostomum* spp. are those Reusch et al. (2004) published for the lens infecting *D. pseudospathaceum*. We therefore tested their applicability for our *Diplostomum* species and additionally developed own primers. For this, a pooled DNA sample of metacercariae from stickleback eyes was enriched for simple sequence repeats and sequenced. Sequences suitable for primer design were checked against published fish sequences and tested for amplification on stickleback DNA. Please refer to the supplementary material for a more detailed description of the procedure. Five markers proved to be *Diplostomum* spp. specific, i.e. they yielded a product within the size range expected from sequencing for *Diplostomum* spp., while not amplifying stickleback DNA. Final PCR conditions can be found in Table A2 of the Appendix.

For primer tests and subsequent genotyping, DNA was extracted by incubating individual metacercariae for two hours at 56 °C in a lysis solution consisting of 0.25 µl 1 M Tris (pH 8), 0.05 µl 0.5 M EDTA, 0.625 µl 20% SDS, 24.075 µl H_2O (LiChrosolv®, Merck), and 2.27 µl Proteinase K (20 mg/ml). After incubation samples were vortexed for 20 s, incubated for 15 min at 100 °C, vortexed for 20 s, and shortly centrifuged before 25 µl of 20% Tween 20 were added. Samples were stored at −20 °C.

Microsatellite genotyping of Diplostomum *spp.*

We analysed only one metacercaria per infected stickleback to keep the *Diplostomum* spp. individuals analysed in this study as genetically independent as possible. As *Diplostomum* spp. reproduces clonally inside its snail host and snails are able to release hundreds of cercariae at a time – *Lymnaea stagnalis*, for example, has been shown to shed several thousand *D. spathaceum* cercariae per day (Karvonen et al., 2004) – it is theoretically possible that one individual stickleback contracts several genetically identical parasites. We tested metacercariae from all infected fish caught for this study until either a target sample size of 20 worms per lake had been successfully genotyped at at least five of the six loci or until all available worms had been tested. In total 253 metacercariae from North Uist were successfully genotyped. In addition, to examine geographically extended population structure, we genotyped 26 metacercariae from 26 sticklebacks caught on Iceland (65°37′42″N, 16°55′17″W), which were kindly provided by Frederik Franke.

Analysis

Considering all 253 metacercariae as belonging to one population, we estimated expected and observed heterozygosity, linkage disequilibrium and indications of possible scoring errors for each locus using the same programs and settings as for the stickleback analysis. As this study is the first application of the new markers, we also calculated PIC values (polymorphism information content, Botstein et al., 1980) using the Microsatellite Toolkit (Park, 2001) for Microsoft® Excel. Genetic diversity at the different sampling locations as well as the degree of population genetic structuring was estimated as described for the sticklebacks.

Molecular Diplostomum *species identification and marker specificity*

Morphological *Diplostomum* species identification based on metacercariae is nearly impossible. We therefore confirmed species identity of our samples and three additional metacercariae from the non-lens region of three nine-spined sticklebacks, *Pungitius*

pungitius, from lake 8SAN by sequencing the barcode region of the cytochrome *c* oxidase subunit 1 (*cox*1) of the mitochondrial DNA using the PlatdiploCOX1 primers published by Moszczynska et al. (2009; see Appendix for details).

Results

Population structure of the sticklebacks

Microsatellite analysis

Genotyping success was 99.4% (4 of the 625 fish could not be genotyped at one locus each). For one locus (Gac7010PBBE) scoring errors due to stuttering were suspected. Furthermore, for all loci the presence of null alleles was suspected, due to a general excess of homozygotes. These results did not occur (except for the null alleles at locus Gac1097PBBE) when only anadromous fish were considered in the analysis. No significant evidence for large allele dropout or linkage disequilibrium between the loci was found. Significant deviations from Hardy–Weinberg equilibrium were found at four sampling locations (13MOI, 17MOR, 18SCD, 21MAI; Table 2). Observed heterozygosity was significantly lower than expected heterozygosity at these locations and inbreeding coefficients were positive but small, ranging from 0.059 to 0.175 (Table 2). Expected heterozygosity was significantly positively correlated with lake surface area (Spearman rank correlation: $r_S = 0.84$, $N = 19$, $P < 0.0001$, Fig. 2) indicating limited genetic diversity in small lakes. This correlation stayed significant if 18SCD was excluded (Spearman rank correlation: $r_S = 0.81$, $N = 18$, $P < 0.0001$) and also if the regression was based on the 15 freshwater population clusters suggested by the Bayesian analyses (see below, Spearman rank correlation: $r_S = 0.82$, $N = 15$, $P < 0.001$). In this case, mean expected heterozygosities were regressed against the sum of the surface areas of the contributing lakes.

In general, pairwise F_{ST} values (Supplementary Table A4) as well as Bayesian cluster analyses (Fig. 3) clearly show the presence of structuring into distinct freshwater populations. No significant genetic differentiation was found between western lakes 4GRO, 5EUB and 6MAG (same cluster, all $F_{ST} < 0.01$), and between 11MGB and 12ACH (same cluster, after Bonferroni correction, $F_{ST} = 0.017$). Between 17MOR and 18SCD there was only little ($F_{ST} = 0.022$) but significant genetic differentiation. Fish in 18SCD showed signs of admixture as only eleven of the 20 genotyped individuals could be assigned to a certain cluster (proportion > 0.5, STRUCTURE). Of these, seven were

assigned to the same cluster as 17MOR fish. Pairwise F_{ST} values and Bayesian clustering analysis did not suggest population structuring among the anadromous fish, but significant reproductive isolation from resident fish caught at the same sampling locations was found with the highest value (F_{ST} = 0.051) found between anadromous and resident sticklebacks at the north-western site (3ana/res in Fig. 1).

Population assignments by BAPS (16 clusters) and STRUCTURE (17 clusters) generally showed similar patterns. However, BAPS assigned fish from 13MOI, 19EIL and 20DAI to distinct clusters, while STRUCTURE assigned 19EIL and 20DAI fish to the same cluster, although genetic differentiation between fish of these lakes was high (F_{ST} = 0.328). Also, 17 of the 20 13MOI fish were assigned to the same cluster as 11MGB and 12ACH (two lakes in the same catchment as 13MOI; F_{ST} 13MOI–12ACH = 0.236, F_{ST} 13MOI–11MGB = 0.207) with an average proportion of 0.6. Fish from 16BHA formed their own cluster in STRUCTURE, but not in BAPS. Both programs clearly separated resident fish caught at the north-western site (3ana/res) from all other fish, but resident fish from the southwest (2ana/res) were only assigned to their own cluster by STRUCTURE. Resident fish from the north-eastern site showed high degrees of admixture as 14 (BAPS) and 19 (STRUCTURE) of the 28 analysed fish could not be assigned to a cluster at all (proportions < 0.5).

Table 2. Summary of basic diversity indices calculated from microsatellite data and mtDNA sequences given as N_s MS (number of sticklebacks genotyped at nine microsatellite (MS) loci), A (average number of alleles per locus rounded to the nearest integer), H_e (expected heterozygosity), H_o (observed heterozygosity), deviation from HWE (Hardy-Weinberg equilibrium (χ^2, df degrees of freedom, *P*, *P* values significant after Bonferroni correction printed in bold)), mean F_{IS} (inbreeding coefficient), N_s mt (number of fish for which composite mtDNA sequences were obtained, see text for details), h (number of mtDNA haplotypes), Hd (Haplotype diversity), SD (standard deviation), π (nucleotide diversity), k (average number of nucleotide differences). Statistics are given for all sample origins separately as well as for all anadromous, resident, and freshwater fish treated as one population, respectively.

					HWE								
LocID	N_s MS	A	H_e	H_o	χ^2	df	*P*	F_{IS}	N_s mt	h	Hd ± SD	π ± SD	k
1ana	58	18	0.86	0.84	16.17	18	0.581	0.027	7	6	0.95±0.10	0.0049±0.0007	7.2
1res	28	14	0.88	0.85	29.53	18	0.042	0.026	5	5	1.00±0.13	0.0022±0.0004	3.2
2ana	19	14	0.90	0.86	28.47	18	0.055	0.041	6	4	0.80±0.17	0.0031±0.0008	4.5
2res	20	13	0.89	0.87	13.87	18	0.737	0.021	5	2	0.40±0.24	0.0014±0.0008	2.0
3ana	21	14	0.88	0.86	21.19	18	0.270	0.024	5	5	1.00±0.13	0.0060±0.0011	8.8
3res	19	10	0.84	0.82	19.16	18	0.382	0.024	5	2	0.40±0.24	0.0011±0.0007	1.6
4GRO	22	11	0.80	0.75	28.95	18	0.049	0.065	5	4	0.90±0.16	0.0027±0.0013	4.0
5EUB	20	11	0.81	0.84	12.69	18	0.810	-0.039	5	3	0.80±0.16	0.0015±0.0006	2.2
6MAG	22	10	0.80	0.80	17.94	18	0.460	-0.007	5	4	0.90±0.16	0.0008±0.0002	1.2
7HOS	20	10	0.83	0.79	16.18	18	0.580	0.038	5	4	0.90±0.16	0.0012±0.0003	1.8
8SAN	41	13	0.82	0.82	15.46	18	0.630	0.006	5	3	0.70±0.22	0.0008±0.0003	1.2
9OLA	20	7	0.63	0.57	24.00	18	0.155	0.086	5	2	0.60±0.18	0.0004±0.0001	0.6
10GEA	33	11	0.74	0.73	18.04	18	0.453	0.024	5	4	0.90±0.16	0.0010±0.0002	1.4
11MGB	21	8	0.70	0.70	9.33	18	0.952	-0.004	5	2	0.40±0.24	0.0014±0.0008	2.0
12ACH	21	7	0.64	0.61	21.07	18	0.276	0.062	5	3	0.80±0.16	0.0008±0.0002	1.2
13MOI	20	8	0.69	0.59	70.83	18	**<0.0001**	0.175	5	2	0.40±0.24	0.0014±0.0008	2.0
14DUB	25	10	0.79	0.79	16.84	18	0.534	0.004	5	4	0.90±0.16	0.0019±0.0008	2.8
15TOR	40	11	0.80	0.81	17.86	18	0.465	-0.019	5	4	0.90±0.16	0.0008±0.0002	1.2
16BHA	20	9	0.81	0.76	22.82	18	0.198	0.064	5	2	0.40±0.24	0.0003±0.0002	0.4
17MOR	30	12	0.82	0.75	∞	18	**<0.0001**	0.096	5	2	0.40±0.24	0.0003±0.0002	0.4
18SCD	20	13	0.87	0.78	∞	18	**<0.0001**	0.101	4	3	0.83±0.22	0.0017±0.0007	2.5

Table 2 continued

					HWE								
LocID	N_s MS	A	H_e	H_o	χ^2	df	*P*	F_{IS}	N_s mt	h	Hd ± SD	π ± SD	k
19EIL	21	5	0.56	0.57	21.37	18	0.261	-0.036	5	2	0.60±0.18	0.0012±0.0004	1.8
20DAI	20	4	0.56	0.54	17.41	16	0.359	0.020	4	2	0.50±0.27	0.0007±0.0004	1.0
21MAI	24	9	0.77	0.72	48.57	18	**0.0001**	0.059	5	1	0±0	0±0	0
22BUA	20	3	0.45	0.44	13.47	14	0.490	0.045	5	1	0±0	0±0	0
anadromous	98	22	0.88	0.85	24.17	18	0.150	0.032	18	12	0.92±0.05	0.0046±0.0005	7
resident	67	19	0.90	0.85	44.70	18	**0.001**	0.056	15	8	0.73±0.12	0.0026±0.0004	4
freshwater	460	28	0.89	0.71	∞	18	**<0.0001**	0.204	93	38	0.96±0.01	0.0037±0.0002	5
all	625	32	0.90	0.75	∞	18	**<0.0001**	0.169	126	53	0.97±0.01	0.0039±0.0002	5.7

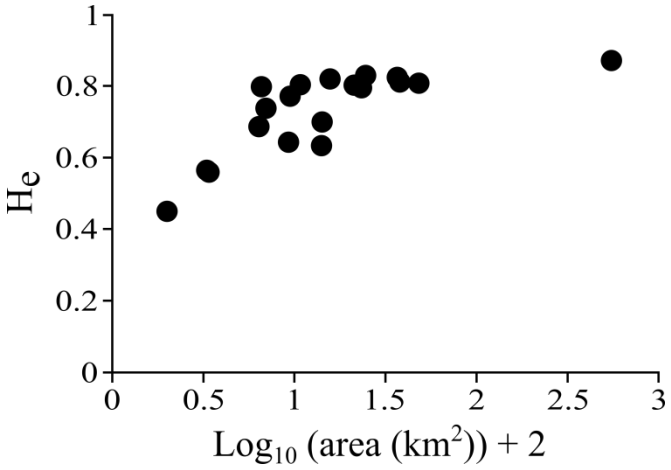


Fig. 2. Relationship between lake surface area in km^2, given as log(area)+2, and expected heterozygosity calculated from stickleback microsatellite data.

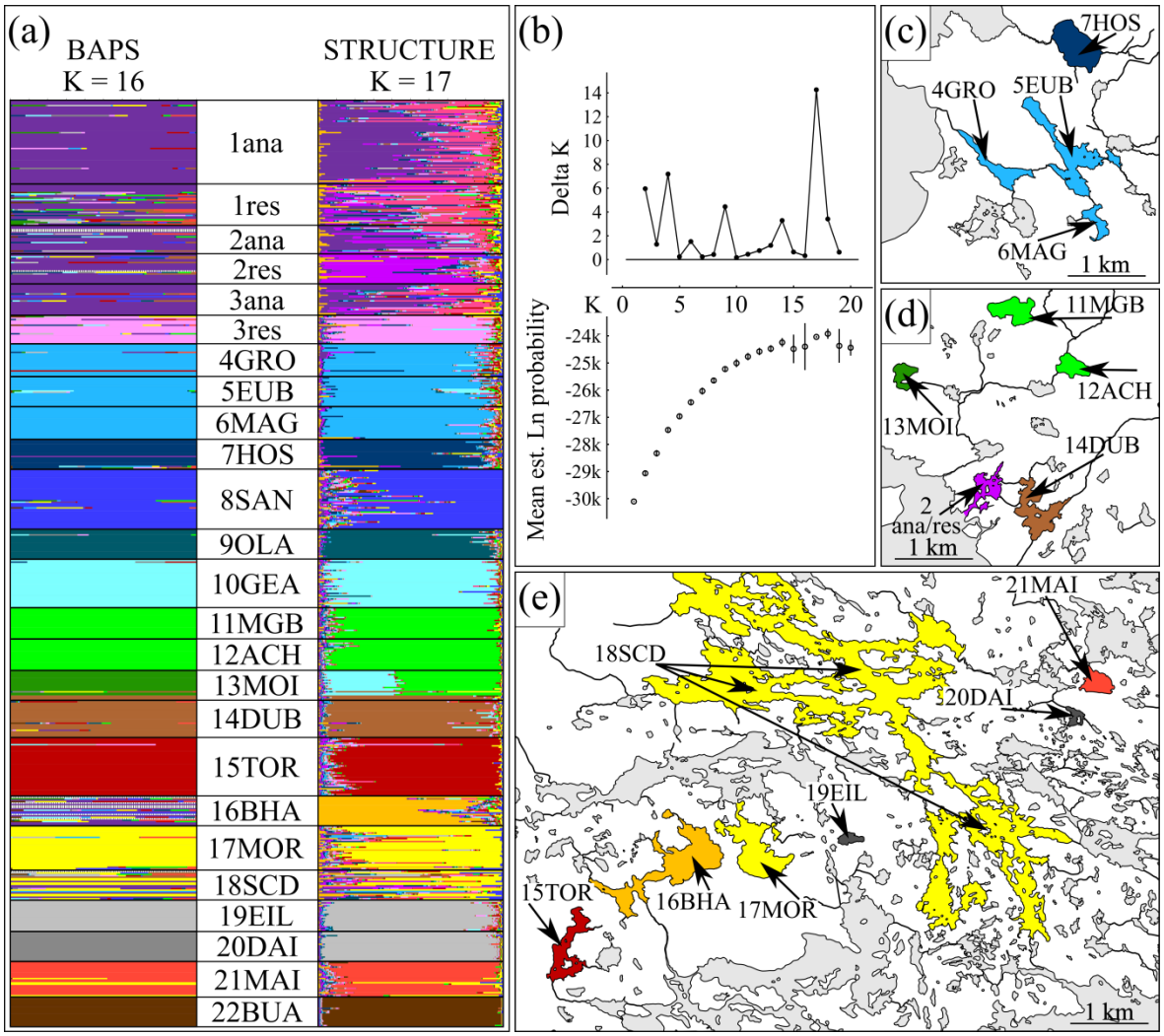


Fig. 3. Results of the Bayesian cluster analysis based on nine microsatellite loci. (a) Cluster membership proportions of the sticklebacks according to BAPS and STRUCTURE, (b) Delta K values and Ln probabilities (mean of five runs with standard deviation), (c)–(e) regional maps depicting sampling locations contributing to population clusters and connecting streams; sampled lakes have been coloured for better visibility.

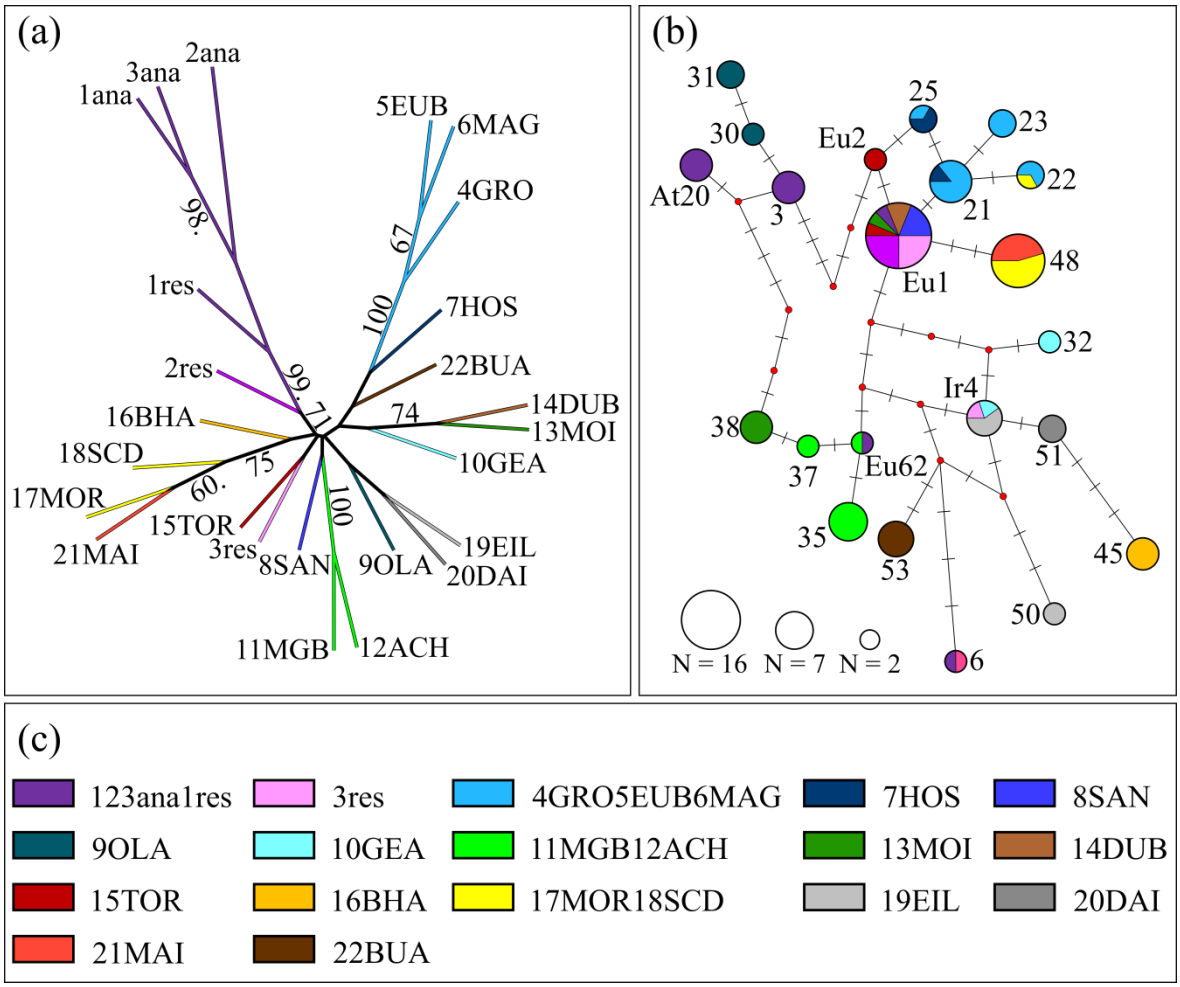


Fig. 4. Visualisation of the relationships among sticklebacks of 25 sampling locations on North Uist. (a) Neighbor-Joining (NJ) tree based on Cavalli-Sforza's and Edwards' chord distance calculated from microsatellite data. Bootstrap (1000×) values ≥50% are given next to branching points. (b)Median-Joining (MJ) network based on composite mitochondrial (cytochrome *b* and control region) haplotypes. Red dots depict median vectors, dashes depict mutation steps. Numbers correspond to haplotype numbers in Table A5, i.e. 3 = NU3 etc. Haplotypes identical to published sequences retained their original names (See text for details.). Circle widths relate to haplotype frequency (three examples are shown). Note that only haplotypes occurring at least twice in the data set were considered. (c) Colour codes used for NJ tree and MJ network. Coding is based on Bayesian clustering results and was applied to all fish caught at the respective sampling sites, regardless of an individual's cluster membership.

Excluding the two loci that might be linked to plate morphology resulted in an estimated number of two clusters according to Delta K (Supplementary Fig. A2), assigning fish of the freshwater lakes 4GRO, 5EUB, 6MAG, 10GEA, 11MGB, 12ACH, 13MOI, 15TOR, and 22BUA to one cluster and all brackish water fish together with fish from the remaining freshwater lakes to another cluster. For K=17, STRUCTURE results showed a similar pattern to that based on all nine loci (Supplementary Fig. A2) with the exception that the 17 13MOI fish mentioned earlier were now assigned to their own cluster with an average proportion of 0.5.

In over 90% of all generated Neighbor-Joining trees anadromous fish as well as resident fish from the coastal lagoon in the Northeast of the island were assigned to the same branch. Also, fish from lakes 4GEO, 5EUB and 6MAG, and fish from 11MGB and 12 ACH originated from a common branch (Fig. 4). Bootstrap support for close relatedness of fish from lakes 13MOI and 14DUB, and from lakes 17MOR, 18SCD and 21MAI was 74% and 75%, respectively (Fig. 4).

Mitochondrial DNA analysis

Analysis of mitochondrial DNA was based on 126 individuals, because cytochrome *b* sequences were incomplete for two fish (one 18SCD, one 20DAI). Overall, 53 different haplotypes with 54 polymorphic sites were found, resulting in a sequence divergence of only 0.39%. Comparison with composite haplotypes previously published by Mäkinen and Merilä (2008) and Ravinet et al. (2014) revealed that ten haplotypes of the North Uist fish correspond to sequences from the European, Irish and Trans-Atlantic lineage (see Supplementary Table A5 for all haplotypes from this study and their GenBank accession numbers). Although mean haplotype diversity was relatively high (0.7±0.3, mean±standard deviation over all samples), this was mostly due to differences in only a few nucleotides (0–9, average diversity per sampling location) resulting in a very low mean nucleotide diversity (π) of 0.0015±0.0014 (Table 2). The correlation between pairwise F_{ST} values calculated from microsatellite data and average differences in mitochondrial DNA nucleotide diversity was positive, but failed to reach statistical significance (r = 0.24, P = 0.081). Although genetic differentiation was not very pronounced at the mitochondrial DNA level, the median-joining network shown in Fig. 4 generally supported the population clusters of the microsatellite analysis.

Population structure of Diplostomum *spp.*

Polymorphism of the new microsatellite loci

All six markers were polymorphic with five to fifteen alleles per locus (see Table A3 of the Appendix for general marker characteristics). Diga4 was difficult to interpret due to heavy stuttering. To avoid overestimating polymorphism, we reduced its genotype profile to six different patterns thereby artificially increasing homozygosity at this locus. There was no significant indication of large allele dropout or linkage disequilibrium between the six loci. Generally, fewer heterozygotes were detected than would have been expected by chance.

Therefore, scoring errors due to stuttering or the presence of null alleles were suspected at all loci (stuttering: all markers except for Diga3).

Molecular *Diplostomum* species identification and marker specificity

*Cox*1 sequences could be obtained for 260 of the 279 individuals that were included in the analyses. All worms, including the three worms from nine-spined sticklebacks, were identified as *Diplostomum* lineage 6 (following naming from Blasco-Costa et al. (2014). This name is most likely a synonym for *D. gasterostei* (Williams, 1966b)), which was first described in three-spined sticklebacks from Scotland. Closest similarity was found to samples from Norway collected by Kuhn et al. (2015). Five of the metacercariae that could not be genotyped at any of the six loci were also sequenced at the barcode region and were identified as *D. baeri* 2 sensu Georgieva et al. (2013).

Population structure *Diplostomum* spp.

Observed heterozygosity was significantly lower than expected at nearly all sampling locations (see Table 3) resulting in relatively high inbreeding coefficients. The only pair-wise coefficients of genetic differentiation (F_{ST}) that remained significant after Bonferroni correction were found between individuals from Iceland and lakes 8SAN, 15TOR, and 19EIL, and indicated moderate genetic differentiation (F_{ST} (ICE–8SAN) = 0.062, F_{ST} (ICE–15TOR) = 0.078, and F_{ST} (ICE–19EIL) = 0.073, respectively; Appendix Table A4). *Diplostomum* spp. samples were best clustered into four groups according to the Evanno-method (note that K(optimal) = 1 is not possible with this method). Generally, the results of the Bayesian cluster analysis did not indicate structuring into distinct populations and differentiation between worms from Iceland and from North Uist was only marginal (Fig. 5).

Table 3. Summary of basic diversity indices calculated from microsatellite data, N_D MS number of *Diplostomum* spp. genotyped, A average number of alleles per locus rounded to the nearest integer, H_e expected heterozygosity, H_o observed heterozygosity, deviation from HWE (Hardy-Weinberg equilibrium (χ^2, df degrees of freedom, *P*, *P* values significant after Bonferroni correction printed in bold)), mean F_{IS} (inbreeding coefficient).

					HWE			
LocID	N_D MS	A	H_e	H_o	χ^2	df	*P*	F_{IS}
4GRO	19	6	0.64	0.49	36.43	12	**<0.001**	0.219
5EUB	15	5	0.68	0.49	30.16	12	0.003	0.233
6MAG	20	5	0.59	0.38	61.04	12	**<0.001**	0.296
7HOS	22	6	0.66	0.40	∞	12	**<0.001**	0.367
8SAN	18	6	0.65	0.49	34.55	12	**<0.001**	0.223
9OLA	6	5	0.67	0.56	10.54	10	0.394	0.174
10GEA	20	6	0.67	0.44	∞	12	**<0.001**	0.251
11MGB	19	5	0.65	0.43	47.75	12	**<0.001**	0.323
12ACH	17	6	0.70	0.57	41.42	12	**<0.001**	0.152
13MOI	6	4	0.71	0.38	26.20	12	0.010	0.481
14DUB	7	4	0.63	0.33	40.48	12	**<0.001**	0.506
15TOR	11	4	0.59	0.38	35.64	12	**<0.001**	0.259
16BHA	5	4	0.57	0.43	12.90	10	0.229	0.262
17MOR	22	6	0.66	0.46	52.89	12	**<0.001**	0.314
18SCD	9	5	0.72	0.55	22.56	12	**0.032**	0.254
19EIL	20	6	0.63	0.40	61.68	12	**<0.001**	0.332
20DAI	6	3	0.60	0.64	15.10	10	0.129	-0.145
21MAI	6	5	0.72	0.47	25.81	12	0.011	0.359
22BUA	5	3	0.74	0.56	19.83	10	0.031	0.271
ICE	26	5	0.61	0.48	45.27	12	**<0.001**	0.178
all	279	10	0.67	0.46	∞	12	**<0.001**	0.285

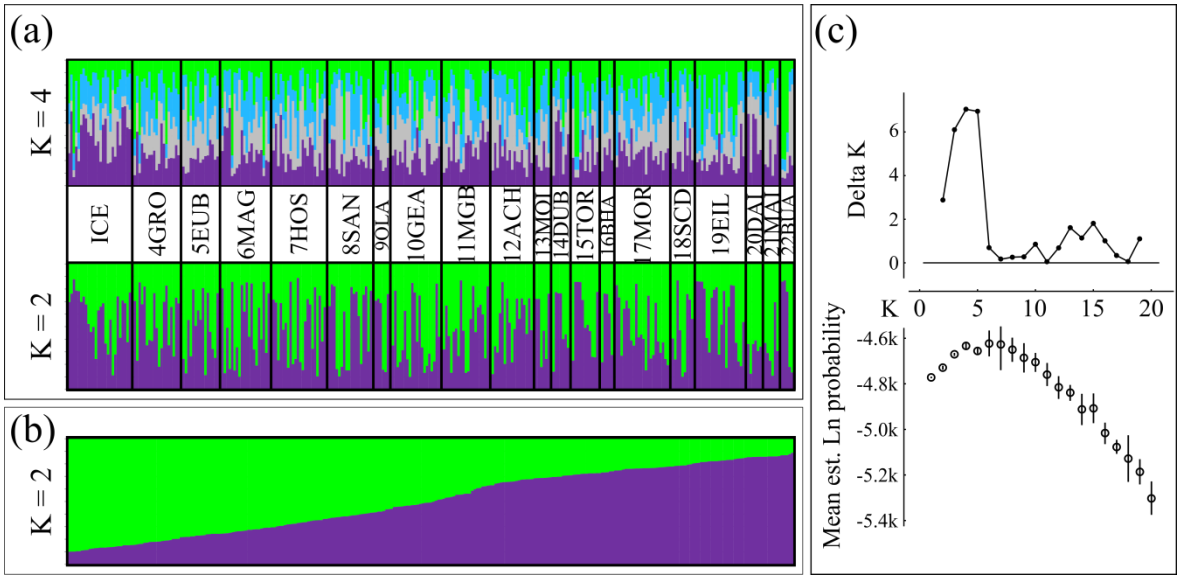


Fig. 5. Results of the Bayesian cluster analysis based on six *Diplostomum* spp. microsatellite loci. (a) Cluster membership proportions for K=4 clusters as suggested by Delta K values and for K=2 clusters sorted by sampling location, (b) results for K=2 sorted by cluster membership proportion, (c) Delta K values and Ln probabilities (mean of five runs with standard deviation).

Discussion

Population structure of the sticklebacks

As expected, our results show strong neutral genetic differentiation in the North Uist sticklebacks. Cluster analyses suggest the presence of different levels of population structure: some lakes seem to occasionally receive gene flow from the sea, while others are completely isolated. This was indicated by the cluster membership proportions for K = 4 clusters (Fig. A1), but also by the high degree of admixture in lake 18SCD, which is indirectly connected to the sea through streams and neighbouring lakes. Small streams connecting 18SCD and 17MOR seem to facilitate genetic exchange between the fish in these lakes, which are genetically isolated from fish in other freshwater lakes. The same applies for lakes 11MGB and 12ACH, and for lakes 4GRO, 5EUB, and 6MAG (Fig. 1). Although spatial information was not considered in the analyses, lakes 7HOS, 8SAN, 9OLA, 10GEA, 14DUB, 15TOR, 21MAI, and 22BUA clearly form distinct populations. If strong population structures are present in a data set, this can affect the clustering algorithms in a way that subtle population structures might not be detected. This seems to be the reason why STRUCTURE assigned fish from lakes 19EIL and 20DAI to the same cluster despite significant evidence for differentiation between the two lakes provided by pairwise F_{ST} values and the BAPS analysis. That fish of 19EIL and 20DAI belong to separate populations is also supported by mitochondrial data (Fig. 4) as well as by differences in morphology (19EIL: ventral spines not present, 20DAI: ventral spines present; Giles, 1983; Spence et al., 2013). The positive correlation between lake surface area and expected heterozygosity, which mainly seemed to be driven by lakes 22BUA, 19EIL, and 20DAI (Fig. 2), point to an influence of genetic drift, brought about by small population sizes, on genetic differentiation. The present results suggest that the anadromous sticklebacks around North Uist belong to a single population. Differentiation from resident sticklebacks was significant but relatively low (highest F_{ST} = 0.051), which is comparable to a study on Irish anadromous and resident sticklebacks (F_{ST} = 0.07; Ravinet et al., 2015). That BAPS and STRUCTURE detected substantial proportions of admixture among the saltwater fish and (at least BAPS) did not assign resident fish to separate clusters as clearly as freshwater fish, might indicate occasional gene flow. The network analyses revealed striking similarity of the relationships between mitochondrial composite haplotypes and population clusters derived from microsatellite genotypes. Given the lower mutation rates of mitochondrial DNA compared to nuclear loci, this underlines the results of the microsatellite analysis and confirms the presence of strong population genetic structuring.

Population structure of Diplostomum *and conditions for local host–parasite (co-)-adaptations*

Bayesian cluster analysis as well as small (mean F_{ST} = 0.04) and mostly not significant pairwise F_{ST} values indicated the absence of population genetic structuring of *D.* lineage 6 on the island of North Uist despite evidence for strong neutral genetic differentiation in its fish host in the same area. Significant F_{ST} values between Iceland and 8SAN, 15TOR, and 19EIL indicate that the newly established markers were able to detect (weak) genetic differentiation between Iceland and North Uist. Our observation is congruent with the study by Louhi et al. (2010) on the lens-infecting *D. pseudospathaceum*. Despite a geographic range of 300 km, the authors did not find evidence for population genetic structuring. The lack of structuring into distinct populations on a relatively small island is not surprising for a bird-infecting parasite (Blasco-Costa and Poulin, 2013) – especially, since some of the fish-eating birds on North Uist (e.g. gulls, terns, divers; Giles, 1981) are migratory and presumably disperse the parasite over large geographic areas. Also, this result supports theoretical predictions that parasites with complex life cycles are generally less structured than their (intermediate) hosts (Mazé-Guilmo et al., 2016). Further, our results would be in line with the hypothesis that less host-specific parasites show weaker genetic differentiation than their single hosts. Although recent surveys have suggested a narrow fish host range of *D.* lineage 6 (Locke et al., 2010a; Blasco-Costa et al., 2014) – to this date, it has only been found in *G. aculeatus* – we can confirm that this *Diplostomum* species infects at least two different stickleback species.

The lack of population genetic structuring in *D.* lineage 6 does not completely rule out parasite local adaptation. An increasing number of studies have shown that gene flow does not necessarily disrupt local adaptation and that it can even promote adaptation (see e.g. Tigano and Friesen, 2016 and citations therein). But in that case, natural selection favouring local genotypes must have been strong as gene flow is generally assumed to hinder local adaptation (Lenormand, 2002; Kawecki and Ebert, 2004; Räsänen and Hendry, 2008). It appears more likely that gene flow across (freshwater) habitat boundaries provides the parasite with the genetic diversity necessary to successfully infect a range of genetically differentiated host populations.

The absence of population genetic structuring does not suggest local adaptation of the parasite to local fish populations as a cause of the different *Diplostomum* spp. abundances found in de Roij and MacColl (2012) and Rahn et al. (2016). Instead, it is

possible that the stickleback populations differ in their *Diplostomum* susceptibility. However, our results indicate that such differences in susceptibility, should they exist, would be the result of adaptation to a diversity of *D.* lineage 6 genotypes rather than to specific genotypes. Spatial heterogeneity in host resistance to a certain parasite genotype would have led to a non-random distribution of parasite genotypes and therefore parasite genetic differentiation within the fish host despite continuous mixing in the bird host (Edelaar and Bolnick, 2012). Additional analyses of genotypes of immune relevant genes, e.g. those of the major histocompatibility complex (MHC; but see Scharsack and Kalbe, 2014), in relation to parasite abundances could shed light on the mechanisms responsible for *Diplostomum* spp. distribution patterns. Alternative explanations include the distribution of the snail host, site preferences of the fish-eating birds (e.g. gulls and terns; Giles, 1981), which serve as definitive host, and/or the direct or indirect influence of abiotic conditions (de Roij and MacColl, 2012; Rahn et al., 2016).

Louhi et al. (2010) found inbreeding coefficients to be low in *D. pseudospathaceum* (between −0,029 and 0,050). This was attributed to high numbers of parasites and high genetic diversity among parasites inside the intestines of the definitive hosts, *Larus argentatus* and *L. canus* (common gull and herring gull, respectively; Karvonen et al., 2006; Louhi et al., 2010). Given the high dispersal rates and frequent encounters of worms from distant lakes owing to the mobility of the definitive host, the significant and positive inbreeding coefficients found in this study (0.289 across all samples) appear counterintuitive. Self-fertilisation within the bird host, probably due to low prevalence and/or diversity in the definitive host, which again might partially be due to clonal reproduction in the snail host, seems the most likely reason (Prugnolle et al., 2005a). Such an influence of prevalence on parasite mating patterns and, as a consequence, parasite genetic differentiation (Barrett et al., 2008) has been found e.g. in the malaria parasite *Plasmodium falciparum* (Anderson et al., 2000). All but one (Diga4) of the newly developed markers were polymorphic and fairly good to analyse. Still, our approach does not allow to decide whether homozygosity was high because of the presence of null alleles (David et al., 2007) or whether the presence of null alleles was suspected because of the high number of homozygotes. The fact that five of the metacercariae which had not yielded a product with any of the markers were identified as *D. baeri* 2 suggests that the markers could be used as a tool for discriminating *D.* lineage 6 and *D. baeri* 2.

Our results are congruent with the hypotheses that predict high gene flow and low genetic differentiation in hermaphroditic parasites with complex life cycles including free-living stages, several host species, and birds as final hosts. The lack of neutral genetic differentiation in the parasite makes local host–parasite co-adaptations between *D.* lineage 6 and its fish host seem more likely on a larger geographical scale than among the lakes of a relatively small island.

The microsatellite primers established for this study are the first for *Diplostomum* lineage 6 and can provide a useful tool for studying host–parasite interactions with this geographically widespread parasite found in three-spined and nine-spined sticklebacks. Additionally, our description of the stickleback population structures could be used for choosing genetically independent lakes for studies investigating the ecological causes underlying the evolution of sticklebacks on this island and elsewhere.

Data accessibility

Stickleback mitochondrial haplotypes are available as separate cytochrome *b* and control region sequences under GenBank accession numbers KT971020–KT971072 and KT971073–KT971125, *Diplostomum* microsatellite sequences under GenBank accession numbers KT971126–KT971130. Stickleback and *Diplostomum* microsatellite genotypes are available from the Mendeley Digital Repository DOI: 10.17632/rr434xd2dm.1 and DOI: 10.17632/5tftys6ww5.1. *Diplostomum* Cox 1 sequences can be found on GenBank (accession numbers KX037874–KX037915 and KX140051–KX140055).

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Chapter II

Distribution of common stickleback parasites on North Uist, Scotland, in relation to ecology and host traits

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Chapter II

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Abstract

Analysing spatial differences among macroparasite communities is an important tool in the study of host–parasite interactions. Identifying patterns can shed light on the underlying causes of heterogeneity of parasite distribution and help to better understand ecological constraints and the relative importance of host and parasite adaptations. In the present study, we aimed to find correlational evidence that the macroparasite distribution patterns on the Scottish island of North Uist, which had been described by de Roij and MacColl (2012), are indicative of local processes rather than an unspecific influence of habitat characteristics. We therefore reinvestigated parasite abundances and tested for associations with habitat characteristics and host traits. Distribution patterns of the most common parasites were largely consistent with the observations of de Roij and MacColl (2012). In accordance with the published results, we found that the most obvious abiotic habitat characteristic varying among the lakes on the island, pH, did not statistically explain parasite abundances (except for eye fluke species inside the lens). Instead, we found that genetic differentiation between host populations, measured as pairwise F_{ST} values based on available microsatellite data, was significantly correlated with dissimilarity in parasite community composition. Our results indicate that individual lake characteristics rather than physicochemical variables shape parasite distribution on this island, making it an ideal place to study host–parasite interactions. Furthermore, additionally to geographic distance measures taken from maps, we suggest taking into account connectivity among freshwater habitats, indirectly measured via fish population structure, to analyse spatial distribution patterns.

Introduction

Identifying constraints imposed by environmental factors on the spatial distribution of free-living organisms remains a key question in understanding (their) evolution. Parasites (here we consider macroparasites) are also limited in their dispersal by (abiotic) environmental factors, but in addition depend on the availability – and therefore on the spatial distribution – of suitable hosts (see, e.g., Bozick and Real, 2015 for a recent review). Furthermore, the interactions between hosts and parasites themselves can be affected by environmental changes like increase of temperature (global warming) or eutrophication (e.g., Brunner and Eizaguirre, 2016). In the study of host–parasite interactions and host–parasite coevolution in particular, it is therefore important to characterise the biotic and abiotic circumstances that determine the dispersal and infection success of a certain parasite. In addition to the abundance of intermediate hosts (e.g., Sures and Streit, 2001; Sokolow et al., 2015), use of different niches within the same habitat (MacColl, 2009; Eizaguirre et al., 2011) or host genetic factors (Lange et al., 2015) can lead to different parasite communities of one host species. On the other hand, parasites can also act as selective agents and promote local adaptation of their hosts (Stokke et al., 2002; Schmid-Hempel, 2011). Local adaptation requires that hosts and parasites co-occur at a place for long enough so that resident hosts (genotypes) can gain an advantage over non-resident hosts (Williams, 1966a; also see Feis et al., 2016, for an example). Numerous studies on three-spined sticklebacks (*Gasterosteus aculeatus* L.), a model organism in evolutionary biology and ecology (Wootton, 1976, 1984; Bell and Foster, 1994; Östlund-Nilsson et al., 2007; von Hippel, 2010) and the host species of the present study, have shown that local adaptation can lead to spatial differences between populations in resistance against parasites (e.g., Kalbe and Kurtz, 2006; de Roij et al., 2010; Raeymaekers et al., 2011; Konijnendijk et al., 2013; Scharsack et al., 2016). Further, spatial differences in parasite distribution can be due to factors such as geographic distance (Poulin, 2003) or differences in physicochemical variables (Goater et al., 2005; Thieltges et al., 2010). These abiotic factors can act on parasites either directly or indirectly, e.g. by providing more or less suitable conditions for their (intermediate) hosts. It can be assumed that habitat characteristics that directly affect parasites (e.g., temperature, salinity, pH, pollution) have a greater impact on pathogens that are constantly in contact with the surrounding medium (ectoparasites or free-living stages of endoparasites) than on endoparasites that are ‘protected’ by their host (Blanar et al., 2009).

Here, we examine the distribution patterns of parasites of three-spined sticklebacks from several lakes on the Scottish island of North Uist. This system is particularly interesting for studying host–parasite interactions, because the numerous isolated lakes on the island comprise a wide range of different habitats. A published survey of the macroparasitic fauna of sticklebacks from North Uist found temporally (over two years) consistent differences in parasite distribution patterns (de Roij and MacColl, 2012). Although five prominent habitat characteristics – lake surface area, pH, the concentration of calcium ions, chlorophyll A concentration, and dissolved organic carbon content – were analysed, none of these factors could explain differences in parasite abundances, leaving individual lake characteristics as the most reasonable explanation.

With the present study, we aimed to reinvestigate the distribution of the most common stickleback parasites on North Uist in relation to abiotic factors and host traits. In detail, we (i) analysed associations of infection with pH in a more balanced choice of lakes (7 alkaline and 12 acidic lakes compared to 2 alkaline and 10 acidic lakes in de Roij and MacColl, 2012) and (ii) compared our data to published infection data to see whether general distribution patterns had been consistent over more than two years, i.e. over several stickleback generations. As fish parasites can be assumed to be directly (ectoparasites) or indirectly (suitability for intermediate host(s)) influenced by the quality of the ambient water, we hypothesised that parasite distribution would not be independent of pH. In addition, we compared differences in parasite community composition with neutral genetic differentiation (measured as pairwise F_{ST} based on available microsatellite data) between host populations and hypothesised that common distribution patterns could be indicative of local host–parasite dynamics.

Materials and methods

Sampling

The island of North Uist is relatively small (about 300 km^2) and covered with more than 180 lakes (Giles, 1983), most of which have been colonised by sticklebacks from the sea since the last deglaciation about 15,000 years ago. The lakes in the western part of the island are characterised by shell sediment, with alkaline, clear water, while the lakes in the central and eastern part are influenced by peat and thus tea-stained and more acidic (Giles, 1983). A population genetic analysis of the sticklebacks of North Uist revealed restricted gene flow and strong genetic differentiation among the fish populations (Rahn et al., unpublished data).

To cover a broad spectrum of different habitats, approximately 20 (20.8 ± 2.3, mean ± standard deviation (sd)) three-spined sticklebacks (*Gasterosteus aculeatus* L.) per sampling location were collected from 19 different freshwater lakes and from 3 different brackish water lagoons (see Fig. 1 and Table 1 for sampling locations and number of dissected fish). During the breeding season resident and anadromous sticklebacks co-occur at those brackish water sites and hence fish of both populations were collected. Adult sticklebacks were caught at the beginning of the breeding season, when most fish were still reproductively inactive. Fish were caught in spring 2010 (April and May) and 2011 (April) using minnow traps (green nylon mesh, 3–4 mm, in 2010–Jenzi, Plüderhausen, Germany; galvanized steel mesh, Gee's G40 M, G48 M, in 2011–Tackle Factory, Fillmore, NY, USA). In 2011, 20 nine-spined sticklebacks were caught in Loch Sanndaraigh (8SAN). Fish were transported individually in their original lake water in 1 litre boxes to a rented cottage where they were either dissected the same day or after an average period of four days.

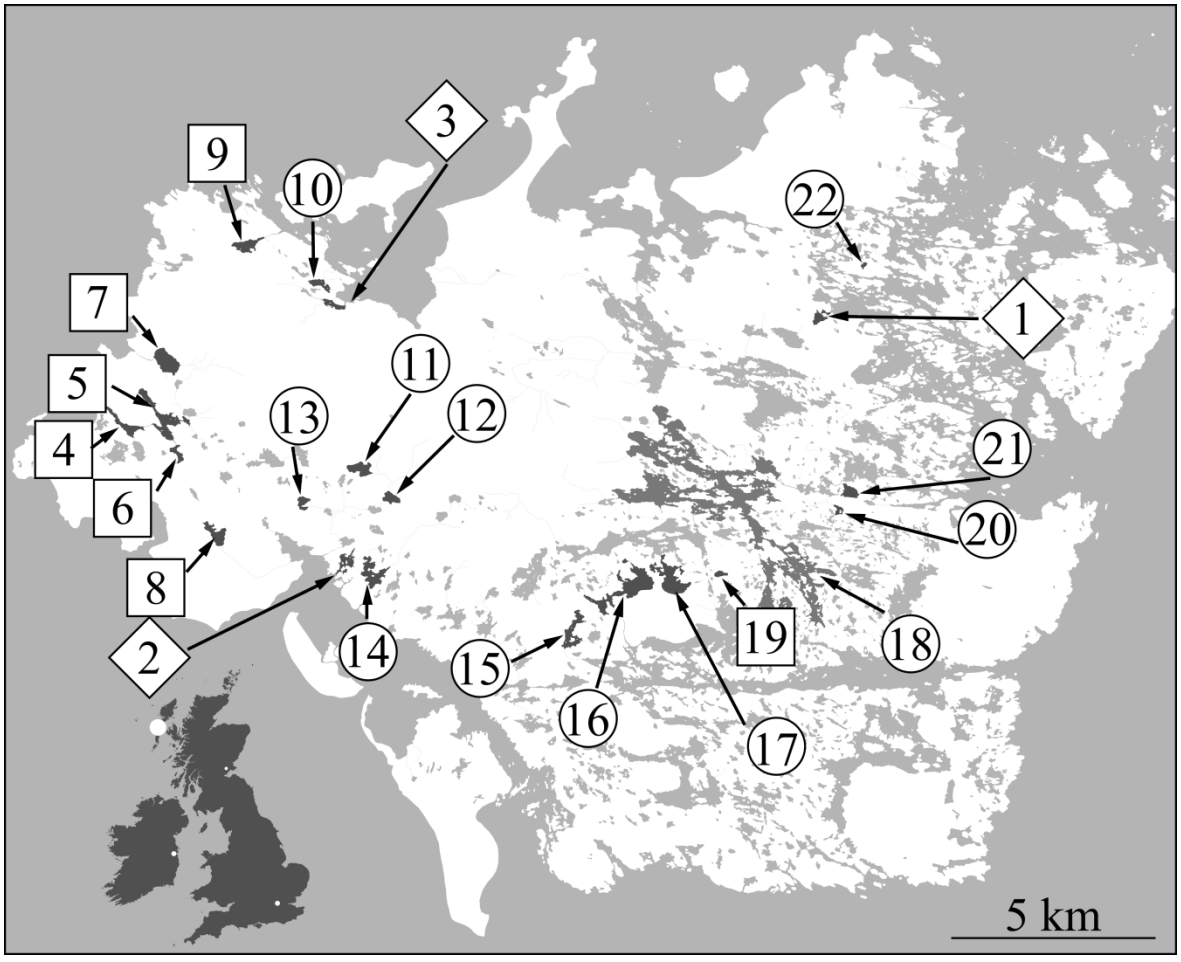

Fig. 1. Distribution of the sampling locations on North Uist. Numbers correspond to numbers in Table 1 (LocID). Squares = alkaline lakes, circles = acidic lakes, diamonds = brackish water sites.

Table 1. Sampling locations (19 freshwater lakes, 3 coastal lagoons with anadromous and resident fish) with three letter codes (LocID), surface area (Area) in km², number of dissected fish (N_{dis}), sex ratio (proportion of males), pH value (mean of three measurements), conductivity in µS, habitat type, and absorbance at 400 nm (A400). Fish used for the calculation of pairwise F_{ST} values (N_{ms}, genotyped at nine microsatellite loci, see Materials and methods and supplementary Table S1 for details) were caught in 2010 and 2011 and partly overlap with dissected fish.

Location name	Geographic coordinates	LocID	Area	Year	N_{dis}[c]	N_{ms}	Sex ratio[d]	pH	µS	Habitat	A400
Aileodair *anadromous*	57°38'7"N, 7°12'54"W	1ana	0.069	2011	21	–	0.50	8.32	–	brackish	0.01
Aileodair *resident*		1res		2010	20	–	0.10				
Aird Heisgeir *anadromous*	57°34'48"N, 7°24'48"W	2ana	0.114	2011	19	–	0.84	7.85	–	brackish	0.03
Aird Heisgeir *resident*		2res		2011	20	–	0.33				
nan Clachan *anadromous*	57°38'14"N, 7°24'45"W	3ana	0.109	2011	21	–	0.29	7.52	–	brackish	0.02
nan Clachan *resident*		3res		2011	19	–	0.39				
Croghearraidh	57°36'54"N, 7°30'40"W	4GRO	0.108	2011	21	22	0.45	7.94[e]	375[e]	alkaline	0.03
Eubhal	57°37'6"N, 7°29'42"W	5EUB	0.379	2011	20	20	0.55	7.89	408	alkaline	0.01
nam Magarlan[g]	57°36'10"N, 7°28'54"W	6MAG	0.066	2010	21	22	0.24	7.19	325	alkaline	0.03
Hosta[g]	57°37'40"N, 7°29'18"W	7HOS	0.247	2011	21	20	0.14	8.34	324	alkaline	0.01
Sanndaraigh[a]	57°35'12"N, 7°27'48"W	$8SAN_{10}$	0.157	2010	17	41	0.36	8.10[f]	384[f]	alkaline	0.02
		$8SAN_{11}$		2011	30		0.33				
		8SAN9sp		2011	20	–	0.25				
Olabhat	57°39'8"N, 7°26'48"W	9OLA	0.141	2011	21	20	0.52	7.47	231	alkaline	0.02
na Gearrachun	57°38'34"N, 7°25'18"W	10GEA	0.070	2011	24	33	0.52	6.89	236	acidic	0.02
Mhic Gille-bhride[g]	57°36'6"N, 7°24'36"W	11MGB	0.142	2010	21	21	0.29	6.77	164	acidic	0.03
a' Charra	57°35'45"N, 7°23'42"W	12ACH	0.093	2010	21	21	0.30	6.62	188	acidic	0.03
Mhic a' Roin[g]	57°35'42"N, 7°25'48"W	13MOI	0.064	2011	20	20	0.55	6.30	177	acidic	0.04
Dubhasairidh[g]	57°34'54"N, 7°24'12"W	14DUB	0.234	2011	20	25	0.50	6.67	183	acidic	0.05
Tormasad[g]	57°33'45"N, 7°19'W	15TOR	0.213	2010	18	40	0.11	6.87	181	acidic	0.04
a' Bharpa[g]	57°34'24"N, 7°17'42"W	16BHA	0.482	2011	23	20	0.52	6.10	140	acidic	0.03
na Moracha[g]	57°34'30"N, 7°16'18"W	17MOR	0.367	2011	21	30	0.05	6.53	175	acidic	0.03
Sgadabhagh[b,g]	57°35'6"N, 7°14'10"W	18SCD	5.516	2011	20	20	0.32	6.16	139	acidic	0.03
nan Ceithir Eilean	57°34'24"N, 7°15'30"W	19EIL	0.033	2011	21	21	0.05	7.37	370	alkaline	0.01
an Daimh[g]	57°35'35"N, 7°12'35"W	20DAI	0.034	2011	20	20	0.30	6.87[e]	176[e]	acidic	0.04
na Maighdein[g]	57°35'42"N, 7°12'6"W	21MAI	0.095	2011	21	24	0.35	6.30	187	acidic	0.02
na Buaile[g]	57°38'48"N, 7°11'48"W	22BUA	0.020	2010	20	20	0.65	6.29	247	acidic	0.02

[a] Three-spined sticklebacks caught in 2010 (8SAN10) and 2011 (8SAN11), and nine-spined sticklebacks (8SAN9sp).
[b] Referred to as "South Sgadabhagh" by Spence et al. (2013).
[c] 2010 samples of lakes 21MAI, 9OLA, 14DUB, 10GEA, and 17MOR were excluded due to low sample sizes (3, 4, 5, 8, and 9 fish, respectively).
[d] Sex not determined for one fish from 1ana, 3res, 4GRO, 10GEA, 12ACH, 18SCD, and 21MAI, two fish from 2res, and six fish from 8SAN10.
[e] One measurement.
[f] Average of four measurements.
[g] Lake also sampled by de Roij and MacColl (2012).

Dissection and parasite screening

For every fish, standard length (SL), measured as the distance between the tip of the mouth and the end of the caudal peduncle, was measured using graph paper covered by a plastic film. Sticklebacks were killed by decapitation immediately followed by a cut through the brain. Fish were screened for ectoparasites as well as parasites infecting the lens, vitreous chamber, and retina of the eyes under a microscope (Novex RZ-Range, 6.5–45× magnification; Euromex Microscopen, Arnhem, Netherlands) with a cold light source (Schott KL 1500; Schott AG, Mainz, Germany). Additionally, the presence of *Schistocephalus solidus*, a *G. aculeatus*-specific cestode, was recorded and the sex of the respective fish was determined by gonad inspection. Where possible, parasites were identified to species level.

Calculation of parasite indices

Prevalence (percentage of infected fish in a lake), abundance (sum of parasite individuals on/in infected fish divided by the number of dissected fish) and mean infection intensity (MI, mean number of parasite individuals on infected fish) were calculated for all parasites and locations sampled in 2010 and 2011. If less than 10 fish were caught in a lake in 2010, that lake was sampled again in 2011 and the 2010 fish were excluded from the analysis (see Table 1). Two indices for comparing the similarity of parasite communities were calculated using the program Past3 (Hammer et al., 2001): the Jaccard index, i.e. the proportion of parasite species shared between two lakes, based on presence/absence data, and the Bray–Curtis similarity index that also takes into account the mean abundance. Calculation of both indices was based on infection data of *Thersitina gasterostei*, *Gyrodactylus* spp., *Schistocephalus solidus*, *Diplostomum* spp. (non-lens), *Apatemon* spp., and *Diplostomum* spp. (lens). As for *S. solidus* only presence/absence data were available, 0 and 1 were included as mean abundance of this parasite.

Microsatellite genotyping and analysis

Pairwise F_{ST} values calculated from microsatellite data were used as a measure of neutral genetic differentiation between host populations. F_{ST} values were taken from another study (Rahn et al., unpublished data), which largely used tissue samples of the present study as raw material. F_{ST} values were calculated in Arlequin 3.5.1.3 (Excoffier and Lischer, 2010) with 1000 permutations.

In short, a minimum of 20 fish per sampling location (24.2 ± 6.8, mean ± sd; Table 1) was genotyped at nine polymorphic microsatellite loci (genotypes are available at http://dx.doi.org/10.17632/rr434xd2dm.1). Further details on sample sizes and PCR conditions can be found in Table 1 and Table A1 of the appendix for this chapter.

Abiotic habitat characteristics

For each freshwater lake, pH and conductivity were measured using a pH meter (HI 98129; Hanna Instruments, Woonsocket, RI, USA). Water samples were taken to the Institute of Cellular and Molecular Botany (IZMB, University of Bonn), where absorbance was measured with a spectrophotometer (range: 300–700 nm, UV mini 1240, program: UVProbe 2.31; Shimadzu Corp., Kyoto, Japan). Absorbance at 400 nm (A400) was used as a measure for turbidity, as differences between water bodies were most pronounced at this wavelength. This measure has proven useful in other studies as well (Reimchen, 1989; Scott, 2001). Lake surface area was used as a proxy for host population size as larger water bodies can be assumed to contain larger populations and expected heterozygosities (H_e) of the stickleback populations on North Uist are significantly positively correlated with lake surface area (Rahn et al., unpublished data). Measures of lake surface area were taken from Rahn et al. (unpublished data). They had been determined from a 1:25000 Ordnance Survey map using ImageJ 1.45 s (Rasband, 1997-2009).

Statistical analysis

Statistical tests were performed in R 3.0.1 (R-Core-Team, 2013) except for Mantel tests, which were performed in Arlequin 3.5.1.3 (Excoffier and Lischer, 2010). Significance was determined from Bonferroni-adjusted α levels. Overall sample size was low in 2010 (6 lakes, compared to 14 lakes in 2011) and different lakes were sampled in both years (except for 8SAN). Also, overall parasite abundances might have been different in the two years. We therefore analysed data of 2010 and 2011 separately. First, we tested for associations between the habitat characteristics turbidity (A400), pH, conductivity and lake surface area of all 19 freshwater lakes. Pearson correlations and Spearman rank correlations were used for normally distributed data and data significantly deviating from normal distribution (tested with a Kolmogorov–Smirnov test), respectively.

We then used generalised linear models (GLM) to test whether infections varied significantly among lakes (the only fixed factor; with SL, sex, date of capture as covariates) and generalised linear mixed models (GLMM) with lake as random factor to

analyse whether infection status could statistically be explained by host (SL, sex) or habitat characteristics (pH, lake surface area as fixed factors; date of capture as covariate). For this, two different measures of infection were used as a dependent variable in separate models: prevalence, which could take the values 'infected' (with at least one parasite of a given species) and 'uninfected' (respective parasite species not found on/in the fish), and abundance, which was defined as the number of parasites of a given species found on/in the fish. Models with prevalence data were fitted using the glm (GLMs) and glmer function (lme4 package for GLMMs; Bates et al., 2015) with binomial error distribution and logit link function. GLMs with abundance data were fitted using the glm.nb function of the MASS package (Venables and Ripley, 2002), which is specially designed for handling negative binomial data. For GLMMs with abundance data we used the glmmadmb function of the glmmADMB package (Fournier et al., 2012) with negative binomial error distribution and log link function. Changes between full and reduced models were compared to a χ^2 distribution. Model reduction was performed in order of decreasing P values until a minimum model including only terms accounting for significant ($P < 0.05$) changes in model fit was found. All models were calculated for prevalence and abundance data of *Gyrodactylus* spp., *Diplostomum* spp. found in the lens (only 2011 due to low sample sizes in 2010), *Diplostomum* spp. and *Apatemon* spp. from the non-lens region, and *T. gasterostei* as well as for prevalence data of *S. solidus* infections.

Following an approach similar to that in Karvonen et al. (2015), we estimated pairwise differences in parasitic faunas between lakes using three measures: 1-Jaccard dissimilarity, Bray–Curtis dissimilarity (1-Bray–Curtis similarity), and absolute differences in mean abundances of *Gyrodactylus* spp., *Diplostomum* spp. (non-lens), and *Apatemon* spp. We then performed Mantel tests (5000 permutations) to test for significant correlations between dissimilarity in parasitic fauna, absolute differences in pH and pairwise genetic differentiation (F_{ST}).

We also tested for associations between our prevalence and abundance data and those of de Roij and MacColl (2012) using Pearson or Spearman rank correlations. Additionally, we compared our results to the published data by applying similar statistics as used in de Roij and MacColl (2012) to our own data of the lakes sampled in the aforementioned study ($N = 12$) as well as to those sampled in 2010 ($N = 6$), and in 2011 ($N = 14$). In detail, prevalence and mean abundance per lake were regressed against pH and lake surface area.

Results

Parasite abundance

Prevalence and mean infection intensities of 11 common stickleback parasites are summarised in Appendix Table A2 and Fig. A1. The distribution of 6 freshwater parasites in relation to pH is displayed in Fig. 2. We detected the ectoparasites *Thersitina gasterostei*, a copepod, the monogenean *Gyrodactylus* spp. (probably *Gyrodactylus arcuatus*; de Roij et al., 2010) , and the peritrichs *Trichodina* spp. and *Apiosoma* spp. *Gyrodactylus* spp. was present at nearly all sampling locations, except for two acidic lakes (20DAI and 22BUA). *T. gasterostei* was present only on resident fish from the brackish water sites, on fish from alkaline freshwater lakes (except for 7HOS) and from acidic lakes 10GEA, 11MGB, and 12ACH. This is – regarding the 12 lakes sampled in both studies – nearly the same finding as in de Roij and MacColl (2012) for 2008, when *Gyrodactylus* spp. was absent from lakes 16BHA, 20DAI, and 22BUA and when *T. gasterostei* was only present in lakes 6MAG and 11MGB, but not in lake 7HOS or one of the other more acidic lakes. Metacercariae of the endoparasite *Diplostomum* spp. are notoriously difficult to identify morphologically and species diversity within the stickleback eye is considered higher than previously thought (Locke et al., 2010b; Blasco-Costa et al., 2014; Locke et al., 2015). Molecular identification using the barcode region of the cytochrome *c* oxidase subunit 1 (*cox*1) of the mitochondrial DNA indicates that at least the species *D.* lineage 6 sensu Blasco-Costa et al. (2014) and *D. baeri* 2 sensu Georgieva et al. (2013) are present on North Uist (Rahn et al., unpublished data). As species could not be identified for every metacercaria, we will speak of "*Diplostomum* spp." and only distinguish between *Diplostomum* spp. from the lens or the non-lens region of the eye. *Diplostomum* spp. (lens and non-lens) were not found in resident and anadromous fish caught at the brackish water sites (see Fig. A1) due to a lack of the mollusc intermediate host (the lymnaeid snail *Radix peregra*). Likewise, *Apatemon* spp. (probably *A. gracilis;* Blair, 1976) and *S. solidus* were, as expected, found almost exclusively in freshwater lakes with the exception of one *Apatemon*- and one *Schistocephalus*-infected fish caught at the north-western brackish water site. Trematodes causing the 'black spot disease' (probably *Cryptocotyle* spp.) and the microsporidian *Glugea anomala* were predominantly found in fish from the brackish water sites (*G. anomala* also in 17MOR, 'black spot' also in nine-spined sticklebacks from lake 8SAN; see Table A2). *Diplostomum* spp. from the lens and from the non-lens region as well as *Apatemon* spp. were also found in nine-spined sticklebacks. As we did not

identify these parasites to species level, we cannot say whether they represent the same species as found in the three-spined sticklebacks. However, *Diplostomum* species infesting the eye lens are usually not considered very host-specific (Locke et al., 2010a) and sequencing the barcode region of three *Diplostomum* metacercariae from the non-lens region indicated that at least *Diplostomum* lineage 6 (Blasco-Costa et al., 2014) is present in both stickleback species (Rahn et al., unpublished data).

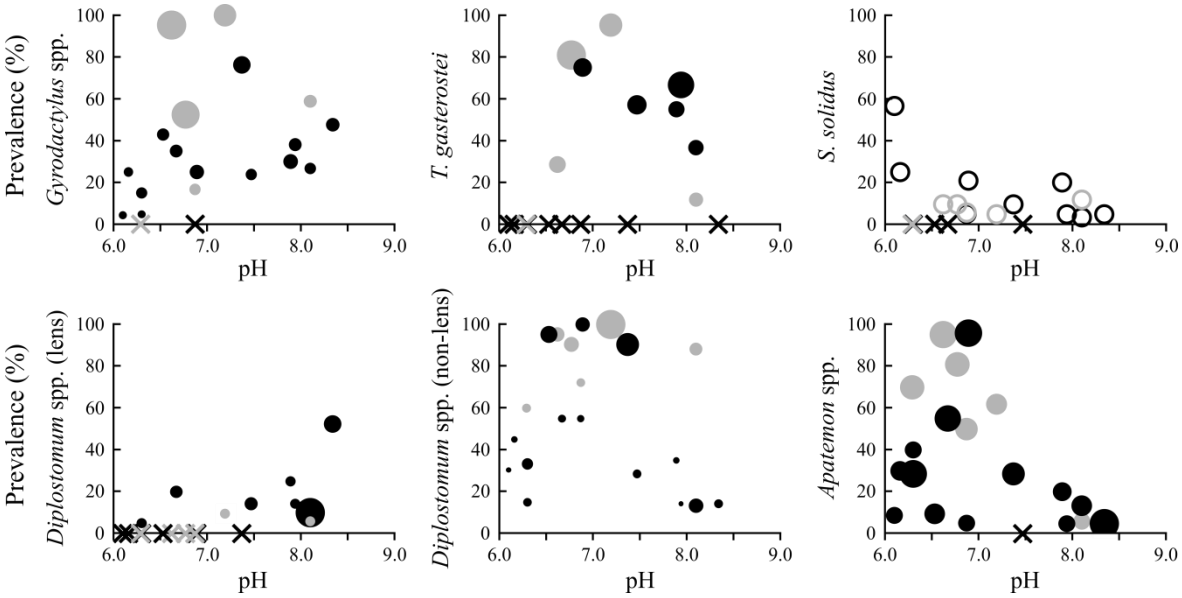

Fig. 2. Prevalence of six stickleback parasites in 19 freshwater lakes on North Uist in relation to pH. Prevalence (% infected) is given as black (2011) and grey (2010) circles or crosses (prevalence = 0%). Circle areas correspond to mean infection intensities (mean number of parasites on infected fish) and are proportional to each other within, but not among, plots. The largest circle in a plot corresponds to an average of 13.2 (*Gyrodactylus* spp.), 6.5 (*T. gasterostei*), 8.3 (*Diplostomum* spp. (lens)), 33.4 (*Diplostomum* spp. (non-lens)), and 3.0 (*Apatemon* spp.) parasites on infected fish. Only prevalence data were available for *S. solidus*.

Table 2. ANOVA results from generalised linear mixed models (GLMM) with infection status as dependent variable, lake surface area (Area), pH, standard length (SL), and sex as fixed factors (1 degree of freedom each), date of capture as covariate, and lake as random factor. Separate models were fitted for 2010 (a, 6 lakes, 111 fish) and 2011 (b, 14 lakes, 299 fish). Note that *P* values are those that resulted from model reduction, whereas significance (Sig.) was determined from Bonferroni-adjusted (B.ad.) α levels. Significant *P* values are printed in bold. Tendencies (0.1 > B.ad. $P \geq 0.05$) are printed in italics.

(a)		Area			pH			SL			Sex		
		χ^2	*P*	Sig.	χ^2	*P*	Sig.	χ^2	*P*	Sig.	χ^2	*P*	Sig.
Gyrodactylus spp.	prevalence	0.3	0.598	ns	0.5	0.476	ns	8.7	0.003	*	0.2	0.685	ns
Gyrodactylus spp.	abundance	0.1	0.803	ns	0.2	0.701	ns	30.0	**<0.001**	***	0.1	0.803	ns
T. gasterostei	prevalence	0.5	0.476	ns	0.6	0.434	ns	6.8	*0.009*	(*)	1.1	0.305	ns
T. gasterostei	abundance	0.3	0.583	ns	0.6	0.451	ns	9.4	**0.002**	*	0.3	0.617	ns
Diplostomum spp. (non-lens)	prevalence	0.1	0.754	ns	1.6	0.211	ns	17.9	**<0.001**	***	0.2	0.626	ns
Diplostomum spp. (non-lens)	abundance	5.1	0.024	ns	7.8	**0.005**	*	52.7	**<0.001**	***	0.2	0.701	ns
Apatemon spp.	prevalence	2.2	0.139	ns	9.8	**0.002**	*	2.2	0.140	ns	4.6	0.032	ns
Apatemon spp.	abundance	0.0	0.920	ns	14.8	**0.0001**	**	8.0	**0.005**	*	0.2	0.626	ns
S. solidus	prevalence	0.5	0.499	ns	0.2	0.675	ns	0.8	0.360	ns	2.4	0.126	ns
(b)		Area			pH			SL			Sex		
		χ^2	*P*	Sig.	χ^2	*P*	Sig.	χ^2	*P*	Sig.	χ^2	*P*	Sig.
Gyrodactylus spp.	prevalence	0.2	0.701	ns	1.6	0.204	ns	0.3	0.613	ns	5.7	0.017	ns
Gyrodactylus spp.	abundance	0.2	0.639	ns	3.1	0.080	ns	0.6	0.426	ns	4.7	0.030	ns
T. gasterostei	prevalence	0.2	0.699	ns	4.0	0.045	ns	0.2	0.667	ns	8.2	**0.004**	*
T. gasterostei	abundance	0.2	0.691	ns	4.0	0.046	ns	6.2	0.013	ns	1.3	0.248	ns
Diplostomum spp. (lens)	prevalence	0.1	0.791	ns	10.9	**0.001**	*	7.9	*0.005*	(*)	1.2	0.271	ns
Diplostomum spp. (lens)	abundance	0.3	0.580	ns	9.6	**0.002**	*	24.2	**<0.001**	***	1.8	0.179	ns
Diplostomum spp. (non-lens)	prevalence	0.3	0.618	ns	1.6	0.206	ns	19.5	**<0.001**	***	1.7	0.190	ns
Diplostomum spp. (non-lens)	abundance	0.5	0.479	ns	1.6	0.208	ns	78.2	**<0.001**	***	0.4	0.508	ns
Apatemon spp.	prevalence	0.1	0.768	ns	2.3	0.133	ns	4.6	0.033	ns	4.7	0.031	ns
Apatemon spp.	abundance	0.0	0.882	ns	1.6	0.201	ns	5.3	0.022	ns	0.5	0.488	ns
S. solidus	prevalence	2.0	0.158	ns	0.0	0.837	ns	1.1	0.301	ns	0.4	0.547	ns

*** B.ad. $P < 0.001$; ** B.ad. $P < 0.01$; * B.ad. $P < 0.05$; (*) 0.1 > B.ad. $P \geq 0.05$; ns B.ad. $P \geq 0.1$

Parasite abundance in relation to host and habitat characteristics

Light transmission was reduced in acidic lakes as suggested by the significant and negative correlation between absorbance at 400 nm and pH (Pearson correlation: $r_P = -0.59$, $N = 19$, $P = 0.009$; Fig. A2), which was significantly positively correlated with conductivity (Spearman rank correlation: $r_S = 0.76$, $N = 19$, $P < 0.001$). There was no significant correlation between lake surface area and any of the mentioned habitat characteristics (all $P > 0.2$). Parasitic infections significantly varied among lakes in 2010 and in 2011 ($\chi^2 > 33$, $P < 0.001$; Table A3) except for infections (prevalence) with *Diplostomum* spp. from the non-lens region in 2010 ($\chi^2 = 7.0$, $P = 0.218$; Table A3) and *S. solidus* in 2010 ($\chi^2 = 3.7$, $P = 0.597$; Table A3). Bigger fish were significantly more likely to be infected (with higher burdens) with *Gyrodactylus* spp. (abundance), *T. gasterostei* (abundance), *Diplostomum* spp. (non-lens, prevalence and abundance), and *Apatemon* spp. (abundance) in the lakes sampled in 2010, and with *Diplostomum* spp. (lens, abundance) and *Diplostomum* spp. (non-lens, prevalence and abundance) in the lakes sampled in 2011 (Table 2). For 2011, a female bias of *T. gasterostei* infections was detected ($\chi^2 = 8.2$, $P = 0.004$; Table 2). *Diplostomum* spp. (non-lens) infection (abundance) was significantly positively correlated with pH in the 2010 lake data ($\chi^2 = 7.8$, $P = 0.005$; Table 2), but not in the 2011 data. Infections with *Apatemon* spp. (prevalence and abundance) were significantly negatively associated with pH in the 2010 lake data (both $\chi^2 > 9.7$, $P < 0.003$; Fig. 2 and Table 2). Regarding the lakes sampled in 2011, only infections (prevalence and abundance) with *Diplostomum* spp. (lens) were significantly (positively) correlated with pH (both $\chi^2 > 9.5$, $P < 0.003$; Fig. 2 and Table 2). Lake surface area was never a significant predictor of infection (all $\chi^2 < 5.2$, $P > 0.02$, $\alpha = 0.0056$).

Analysis of dissimilarity in the parasite community revealed that (qualitative) differences in parasite community composition based on presence/absence data (1-Jaccard) were significantly associated with genetic differentiation, but not with the extent of differences in pH in the 2011 data set (Fig. 3 and Table A4). After correcting for multiple tests, no such correlation was found for the 2010 lakes (ibidem). Differences in parasite abundances (Bray–Curtis dissimilarity, *Gyrodactylus* spp., *Diplostomum* spp. (non-lens), *Apatemon* spp.) were not significantly correlated with genetic differentiation (Table A4).

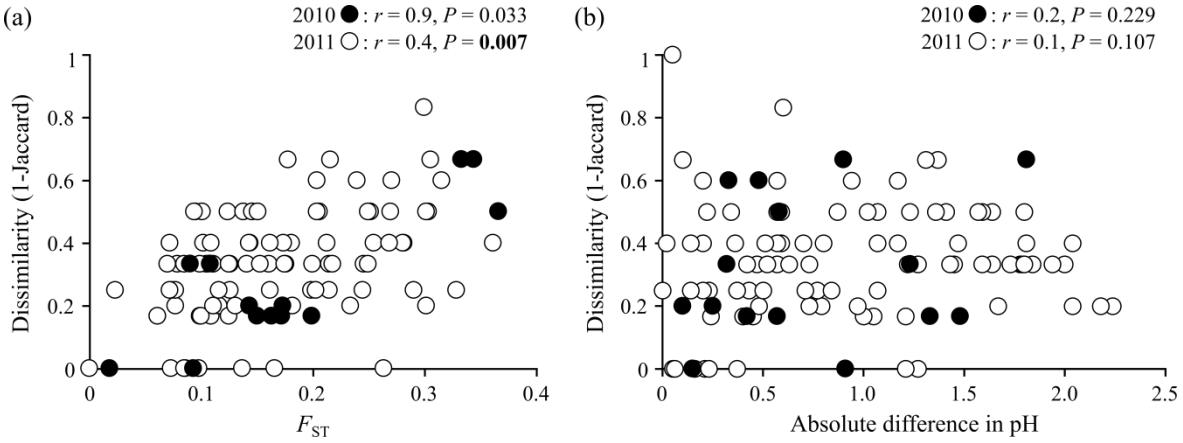

Fig. 3. Dissimilarity in parasite communities given as 1-Jaccard between lakes in relation to (a) pairwise genetic differentiation determined from microsatellite data and (b) absolute differences in pH. Data of 2011 (2010) are shown as empty (filled) circles. Correlation coefficients (*r*) and *P* values of the Mantel tests are given in each plot. The significant *P* value (after Bonferroni correction) is printed in bold.

The direct comparison of our data of the lakes sampled in de Roij and MacColl (2012) with the published data revealed positive trends in all cases, but correlations were only significant regarding infection with *Apatemon* spp. (prevalence: 2008, abundance: 2007 and 2008, Spearman rank correlations, $r_S > 0.8$, $P < 0.001$), *Diplostomum* spp. (non-lens, abundance: 2008, Spearman rank correlation, $r_S = 0.8$, $P = 0.003$), and *Gyrodactylus* spp. (abundance: 2008, Spearman rank correlation, $r_S = 0.8$, $P = 0.001$; Table A5). Applying similar statistics to analyse associations with habitat characteristics as in de Roij and MacColl (2012) to our own data yielded (qualitatively) the same result: no significant correlation of infection with pH or lake surface area (all Bonferroni-adjusted $P > 0.05$). The only (positive) trend that remained after correcting for multiple tests was between pH and *Diplostomum* spp. (lens, abundance) in 2011 (Spearman rank correlation, $r_S = 0.7$, $P = 0.005$, $\alpha = 0.0045$; Table A6).

Discussion

In accordance with the findings published by de Roij and MacColl (2012), we detected significant variation in parasite distribution among lakes. Distribution patterns found in both studies were generally similar regarding presence/absence of the parasites *T. gasterostei*, *Gyrodactylus* spp., *Diplostomum* spp. (non-lens), *Apatemon* spp., and *S. solidus*. Simple correlations revealed that relative differences in abundance data were also similar to those in the previous study, at least for the parasites *Apatemon* spp., *Diplostomum* spp. (non-lens), and *Gyrodactylus* spp. Interestingly, the *Diplostomum* species which was found in the eye lens of fish from the western and two of the more central lakes was not analysed in de Roij and MacColl (2012) due to very low abundances

in 2007 and 2008. Taken together, the results suggest that the parasites are not randomly distributed on the island and that distribution patterns have been consistent, at least over several host generations.

In the study by de Roij and MacColl (2012) only two of the twelve lakes examined were 'alkaline' (pH > 7) and situated in the western part of the island. In our study six of fourteen lakes (2011, two of six in 2010) had pH values above 7 and five of these were located in the western part of North Uist. Despite a more balanced choice of lakes in terms of pH and geographic location, we did not find convincing evidence that pH was a decisive factor in shaping parasite distribution on North Uist. *T. gasterostei* was absent from acidic lakes (except for 11MGB) and the alkaline lake 7HOS in both studies. The explanation that acidic lakes might be unsuitable for copepods in general seems unlikely given that *S. solidus*, which requires copepods as intermediate hosts, was found in several lakes with pH values below 7. Significant correlations between pH and infection with *Diplostomum* spp. (lens) indicate that pH might play a role in the distribution and/or infection success of this trematode. But, as the eye fluke seemed to be absent from most central and eastern lakes, this correlation cannot be distinguished from geographical distribution and, e.g., preference of the final (bird) host for the Atlantic coast.

It might appear counterintuitive at first that we did not find evidence for lower *Diplostomum* spp. (non-lens) or *Apatemon* spp. prevalence in more acidic lakes. Both eye flukes depend on a snail as intermediate host and therefore the distribution of these parasites might be expected to be indirectly associated with calcium availability as was indeed found for the distribution of *Diplostomum* infections originating from *Lymnaea arctica* snails in Canada (Curtis and Rau, 1980). Like *Lymnaea stagnalis*, *L. arctica* requires much higher calcium concentrations than those found on North Uist. This might be the reason why *R. peregra*, which can cope with low calcium concentrations, is the predominant species on this island while *L. stagnalis* is absent (Briers, 2003a, 2003b). As both eye flukes were present in fish from almost all lakes examined in this study, we can assume that either snail prevalence was not significantly affected by spatial differences in pH or that lower snail prevalence in more acidic lakes was compensated by higher infection rates.

Neutral genetic differentiation between host populations was significantly positively correlated with dissimilarity of parasite community composition between lakes based on presence/absence data (1-Jaccard). Considering the distribution of *Diplostomum* spp. (lens)

and *T. gasterostei*, which were both included in the calculation of the similarity indices and occurred mostly in western, alkaline lakes, this effect is likely to be driven by those two parasites. The result could suggest that parasite distribution patterns have been shaped by the connectivity among lakes. De Roij and MacColl (2012) had tested for distance decay in similarity using Jaccard similarity and the shortest geographical distance between lakes as distance measure. Their negative result was interpreted as evidence that they "could (...) rule out 'isolation by distance' as an explanation for spatial variation in parasite communities" (de Roij and MacColl, 2012). We argue that our results suggest that pairwise F_{ST} values between host populations might be a better proxy for 'geographical distance', especially since several of the numerous lakes on North Uist (>180 according to Giles, 1983) are connected by streams (also underground streams). Unlike qualitative dissimilarity in parasite communities, quantitative differences in mean abundances, like Bray–Curtis dissimilarity, or abundances of *Gyrodactylus* spp., *Apatemon* spp., and *Diplostomum* spp. (non-lens) were not significantly correlated with genetic differentiation between host populations. This might be due to the use of neutral genetic markers (microsatellites) for estimating genetic differentiation and indicate that parasite abundances are not determined by geographical position on the island, i.e. neighbouring, but isolated lakes can have very different abundances, but the result of local dynamics.

One further explanation for the different abundances among lakes could be local adaptation of either hosts or parasites or both. Several studies on *D. pseudospathaceum*, which infests the eye lens of sticklebacks, have shown that sticklebacks are able to locally adapt to parasites like eye flukes that quickly evade the immune system of the host before an adaptive response can be elicited (Kalbe and Kurtz, 2006; Rauch et al., 2006; Scharsack and Kalbe, 2014). Also, experimental infections with *G. gasterostei* have shown that the stickleback populations on North Uist differ in their resistance to this monogenean (de Roij et al., 2010). Experimental infections with a fully crossed design could help to find out whether the patterns observed on this island can be the result of host and/or parasite local (co-)-adaptation.

Although we disagree that parasite abundance is completely independent of pH (at least not for all parasites), our results generally confirm the results and conclusion of the study conducted by de Roij and MacColl (2012) that found that individual lake characteristics such as local host/parasite adaptations rather than general physicochemical variables must be responsible for the different patterns of parasite distribution across North

Uist. Further work will be necessary to disentangle the mechanisms behind the consistent parasite distribution patterns, but we conclude that connectivity among habitats, water quality, and host traits contribute to the differences in parasite abundance. It might also be possible that certain abiotic habitat characteristics indirectly affect host local adaptation by providing better or worse conditions for the parasites. Likewise, it also remains to be tested whether physical connectivity among water bodies shapes distribution patterns of hosts and of parasites, whether parasites have 'followed' their hosts during colonisation, or whether parasites have contributed to population divergence of their fish host.

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Chapter III

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

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Chapter III

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Abstract

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.

Introduction

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 and Mangel, 1986; Pitcher and 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 and 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 (Loehle, 1995; Krause and Ruxton, 2002).

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 and 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 parasites, such as biting flies on feral horses and *Argulus* spp., a crustacean sucking blood from sticklebacks (Rutberg, 1987; Poulin and FitzGerald, 1989; Rubenstein and Hohmann, 1989), 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 and Poulin, 1995; Krause and 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 and Hohmann, 1989) or for viviparous gyrodactylids on fish (Boeger et al., 2005; Johnson et al., 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 et al., 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 et al., 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 and 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 et al., 2002). Female guppies usually shoal more than males and transmission of *Gyrodactylus* is more easily facilitated among interacting conspecifics (Richards et al., 2010; Stephenson et al., 2015; but see Richards et al., 2012) . Experimental infection showed a negative effect on shoal cohesion in studies by Croft et al. (2011), and Hockley et al. (2014b), 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. Malmberg, 1970; Kalbe et al., 2002; Özer et al., 2004; Sulgostowska and Vojtkova, 2005; de Roij and MacColl, 2012) and their shoaling behaviour has been well studied (see e.g. Frommen et al., 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 non-reproductive phase (Wootton, 1976) and their shoaling decisions are known to be influenced by group composition, for example with regard to body size (Hoare et al., 2000), as well as by the nutritional state of the choosing individual (Frommen et al., 2007b). 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 et al., 1994), *Schistocephalus solidus* (see Barber et al., 1998) or *Glugea anomala* (see Ward et al., 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. Lester, 1972; de Roij et al., 2010; Konijnendijk et al., 2013) 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 and 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 hypothesised 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 and Thomson, 1995). Thus, we expected infected individuals not to show a clear preference for one of the shoals.

Materials and 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°40’ 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. Soleng and Bakke, 1998; Boeger et al., 2005). 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× 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 and Bakker, n.d.). Still, it is not unlikely that both ponds harbour a community of different *Gyrodactylus* species (Raeymaekers et al., 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× 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.

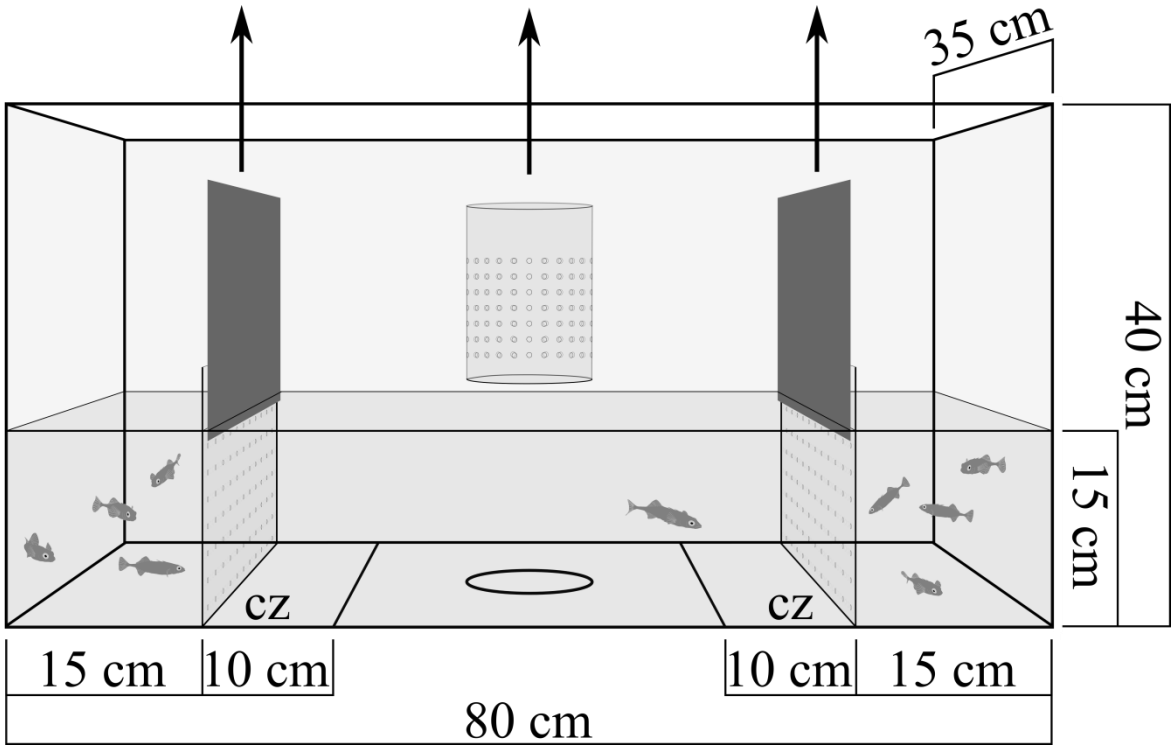

Figure 1. Schematic overview of the experimental set-up during the data recording phase with cylinder and opaque partitions raised. Transparent, perforated (hole diameter 0.5 cm) Plexiglas partitions separate the shoal compartments from the central (focal fish) area. Black felt-tip pen lines drawn onto 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.

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 video recorded 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 × 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 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 hepato-somatic index (I_H) and the splenosomatic index (I_S) according to Wootton et al. (1978) as I = 100 × 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: $t_{27.2} = -2.63$, $P = 0.014$). 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: $r_{29} = 0.63$, $P = 0.0002$).

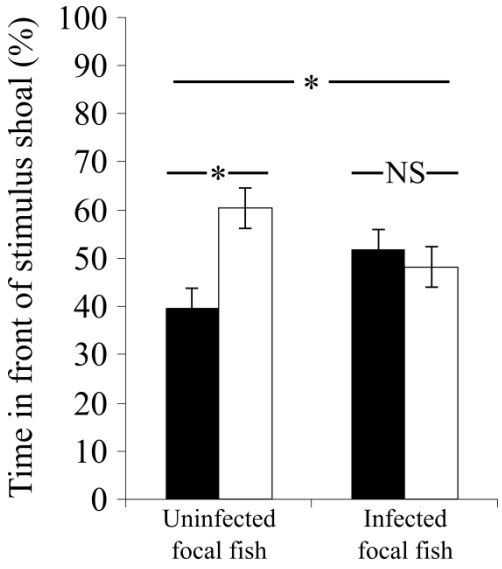

Figure 2. Mean amount of time ± SE that uninfected ($N = 14$) and infected ($N = 17$) focal fish spent in front of the infected (black bars) and uninfected (white bars) stimulus shoals relative to time spent in both choice zones, respectively. *$P < 0.05$.

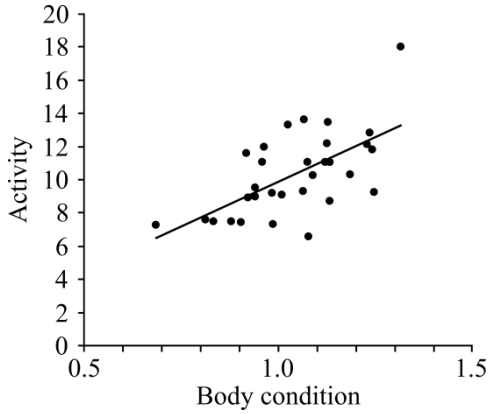

Figure 3. Relationship between activity and body condition of all 31 focal fish. Activity is given as square root-transformed number of zone switches.

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: $t_{25} = -3.47$, $N_{\text{uninfected}} = 13$, $N_{\text{infected}} = 15$, $P = 0.002$) and a lower splenosomatic index (Mann–Whitney *U* test: $U = 48$, $N_{\text{uninfected}} = 11$, $N_{\text{infected}} = 16$, $P = 0.048$). Body condition was significantly positively correlated with relative liver mass (Pearson correlation: $r_{26} = 0.52$, $N_{\text{uninfected}} = 13$, $N_{\text{infected}} = 15$, $P = 0.005$), but was not significantly correlated with relative spleen mass (Spearman rank correlation: $r_S = -0.11$, $N_{\text{uninfected}} = 11$, $N_{\text{infected}} = 16$, $P = 0.601$). Significantly more chironomid head capsules were found in the stomachs of uninfected focal fish (Mann–Whitney *U* test: $U = 67$, $N_{\text{uninfected}} = 13$, $N_{\text{infected}} = 15$, $P = 0.039$). 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 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).

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.

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 ($N_{\text{focal fish}}$ = 17, N_{June} = 31, N_{October} = 13; Kruskal–Wallis test: χ^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 and 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 (Loehle, 1995; Krause and Ruxton, 2002). 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., 2014b) 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 and Godin, 1994; Tobler and 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 hypothesising about parasitic influence on shoaling behaviour (see e.g. Côté and Poulin, 1995; Barber et al., 2000). 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 et al., 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 et al. (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. Crompton, 1984; Kyriazakis et al., 1994; Arneberg et al., 1996; van Oosterhout et al., 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 et al., 2004; Seppänen et al., 2009). Since *Gyrodactylus* spp. is known to cause an immune response by the host (Lester, 1972; Bakke et al., 2007), the higher splenosomatic indices in this study suggest an activation of the immune system caused by the infection. As some animals are able to recognise infected conspecifics by specific odours associated with infection (e.g., Kavaliers and Colwell, 1995; Hughes et al., 2014), 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 et al. (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* (Richards et al., 2010; Croft et al., 2011; Johnson et al., 2011; Richards et al., 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.

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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Chapter IV

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

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Chapter IV

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Abstract

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 pseudo-spathaceum*, 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.

Introduction

Parasitic infections can have a significant influence on grouping behaviour (reviewed by Barber et al., 2000; Krause and 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 and 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 et al., 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 and Ruxton, 2002) and is therefore systemspecific. Conspicuousness caused by an infection, such as altered coloration (Seppälä et al., 2005a; Ondrackova et al., 2006) or abnormal behaviour (Lafferty and Morris, 1996), potentially increases predator attraction (e.g. Landeau and Terborgh, 1986; Bakker et al., 2017) for ‘odd’ individuals ('oddity effect'; Ohguchi, 1978) and for their shoal mates (Landeau and 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 and Schlupp, 2008), killifish (Krause and Godin, 1996) and sticklebacks (Barber et al., 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 and 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 and Bell, 1993), or affect sensory organs (Chappell et al., 1994) or the central nervous system of their hosts (Lafferty and Morris, 1996; Shirakashi and Goater, 2001). Uninfected individuals, on the other hand, might even benefit from grouping with weak competitors (Metcalfe and 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 et al., 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 et al., 1980). In cyprinids and salmonids, infections with *Diplostomum* spp. can have severe consequences for food intake (Crowden and Broom, 1980; Voutilainen et al., 2008), predation risk (Seppälä et al., 2005b; but see Seppälä et al., 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 et al., 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 and 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 and 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 et al., 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 et al., 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.

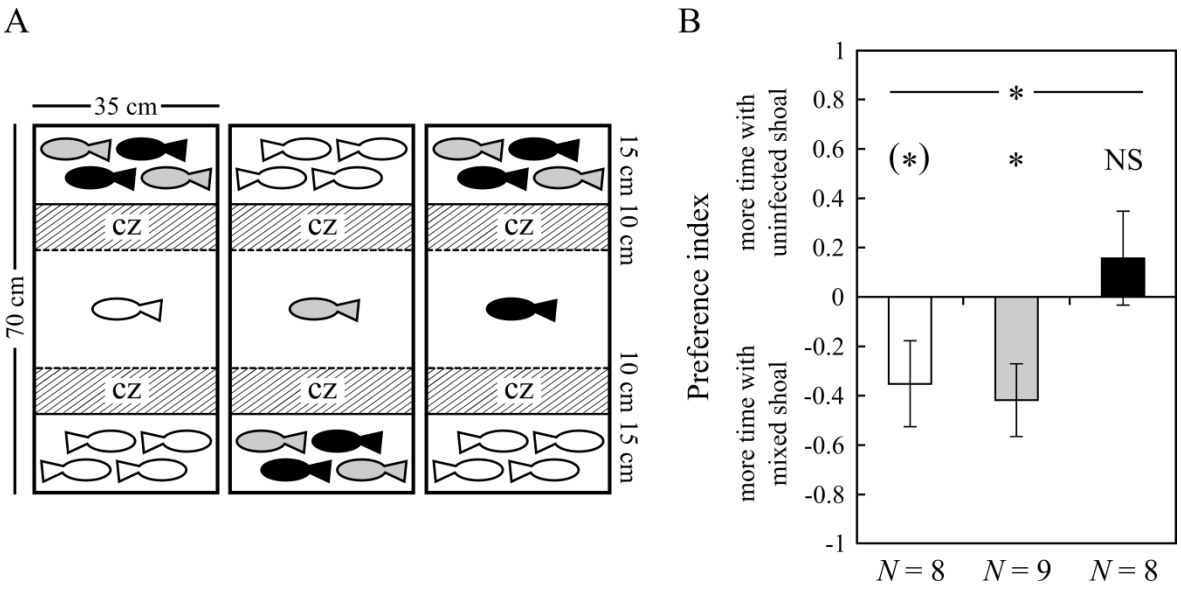

Figure 1. Set-up and main result of the shoal choice experiments. Each pair of shoals was presented to three focal fish [one of each treatment: uninfected fish from uninfected groups (white fish symbol in A and bar in B), uninfected (grey) and infected (black) fish from mixed groups]. (A) Schematic bird's 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 × (ln body $mass_{April}$ – ln body $mass_{at\ infection}$)/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 (Rauch et al., 2006; Scharsack et al., 2007; Haase et al., 2014). 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 (cm^3), 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 stepwise 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 and Uldal (1994) and Karvonen and 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, $N = 34$), Appendix Fig. A1]. 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: $N_{\text{uninfected}} = 10$, $N_{\text{mixed}} = 10$, 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).

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.

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, $N_{\text{mixed infected}} = 28$) and body size, mass and body condition at the end of the experimental period (Spearman rank correlations: all $r_S < 0.12$, all $P > 0.5$).

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 eyeflukeinfected 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 et al., 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 and 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 and 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 and 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 and 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 and 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 and 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 et al., 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 et al., 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 and 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 and Kalbe, 2014). Within the first few days after

infection, activation of the innate immune system ceases (Scharsack and 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 et al., 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 riskaverse behaviour of their host and making it more prone to predation by piscivorous birds. Eyefluke-infected dace swam closer to the surface (Crowden and Broom, 1980) and infected rainbow trout were more easily caught by human 'predators' with a dip-net (Seppälä et al., 2004, 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 theparasite'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 et al., 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 and 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. pseudo-*

spathaceum 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.

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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General discussion

General discussion

Due to their very different approach, *Chapters I* and *II*, and *III* and *IV* will be dealt with separately.

Chapters I *and* II – *Host motility and water quality as determinants of parasite distribution*

Population genetic structure of *Gasterosteus aculeatus* and *Diplostomum* lineage 6

Although population genetic research has seen the implementation of new techniques and genetic markers in the past decades (e.g. single nucleotide polymorphisms, restriction-site associated DNA sequencing), microsatellites are still valuable tools when it comes to detecting population (sub-) structuring (e.g. Lee et al., 2017; Wang et al., 2017; Bigi et al., 2018). They show high mutation rates and are easy to analyse once species-specific primers have been developed. Thanks to the three-spined stickleback being a model organism, I could choose a suitable set of primers from several hundred published sequences to analyse the population genetic structure and relationships among the sticklebacks on North Uist. Since no published microsatellite primer sequences were available for *Diplostomum* lineage 6, a *Diplostomum* species commonly found in the retina of the North Uist sticklebacks, I tested those published for *D. pseudospathaceum* and developed new *D.* lineage 6-specific primers from parasite DNA sequences that had been enriched for repeat motifs. Developing specific primers for parasites that feed from their host, generally brings about the difficulty that DNA-samples are usually contaminated with host DNA (Calvignac-Spencer et al., 2013). This circumstance required me to first omit those sequences that matched with the stickleback genome (detected by using "basic local alignment search tools", BLAST) and, of the remaining sequences, those that yielded a PCR product when stickleback DNA was used as template. Molecular species identification of five *Diplostomum* metacercariae that had failed to yield a PCR product using *cox*1 sequences revealed that the newly developed markers are suitable for *D.* lineage 6, but not for *D. baeri* 2 – a *Diplostomum* species that co-occurrs with *D.* lineage 6 (Kuhn et al., 2015). All new markers were polymorphic and able to detect weak but significant genetic differentiation between Iceland and three lakes on North Uist. For all loci, I detected a significantly higher number of homozygotes than would have been expected by chance. While I cannot rule out the presence of null alleles as underlying reason, this may be the result of inbreeding or mating with clones. I discuss this aspect further down.

I could indeed confirm that the sticklebacks on North Uist form distinct populations, as had been assumed from the post-glacial history of the island (Ballantyne, 2010). The strong population genetic structuring was supported by microsatellite as well as by mitochondrial DNA data. Population structures mostly depict physical connectivity between habitats facilitated by streams – both directly and indirectly via neighboring lakes. Deeper analysis of the results revealed different levels of population structure with some lakes being completely isolated while other lakes seem to occasionally receive gene flow from the sea. This could point to an influence of infrequent flooding events (SEPA, 2015) on the genetic diversity within those lakes that are situated near the coast. I found stickleback populations in isolated lakes with a small surface area to be significantly less heterozygous than populations in larger lakes. This indicates an effect of genetic drift in smaller lakes (with small population sizes) that adds to the population genetic differentiation between the lakes on North Uist. My findings regarding the population genetic characteristics of the North Uist sticklebacks were generally confirmed in a paper published shortly after *Chapter I* of this thesis had come out (Magalhaes et al., 2016).

Analysis of resident and anadromous sticklebacks caught at three brackish water lagoons around the island revealed that the anadromous sticklebacks from different sides of North Uist are not further separated into distinct populations. Furthermore, they appear to be reproductively isolated from the resident sticklebacks they share their breeding sites with. Numerous examples of this phenomenon have been found for resident/anadromous pairs elsewhere (reviewed e.g. in Bell and Foster, 1994) and pairwise values of genetic differentiation were similar to those obtained in a study on Irish stickleback (Ravinet et al., 2015).

I did not find evidence of any structuring into distinct populations of the *D*. lineage 6 samples collected on North Uist. This is in line with a previous study on the lens-infecting *D. pseudospathaceum* collected from several sampling locations across a geographic range of 300 km in Finland (Louhi et al., 2010). Given the motility and migratory behaviour of gulls, terns, and divers – common piscivorous birds on North Uist (Giles, 1981) –, this is not surprising but it also does not explain the consistent spatial heterogeneity in eyefluke abundances on the island (de Roij and MacColl, 2012). Contrary to the Louhi et al. (2010) study, I found relatively high (0.289 across all samples) inbreeding coefficients. Usually, bird hosts can be assumed to "collect" high numbers – and presumably a high genetic diversity – of parasites (Karvonen et al., 2006) by consuming fish from several lakes. This

should lead to a "mixing bowl" effect analogous to that in human hosts (Van den Broeck et al., 2014). Although 1–10 % prevalence in snails is enough to infect 100 % of fish (Chappell et al., 1994), low prevalence and spatial heterogeneity in distribution of infected snails could promote self-fertilisation or mating with clones inside the bird host (Anderson et al., 2000; Prugnolle et al., 2005a). Parasites that are host specific and infect only one or a few host species, tend to be locally adapted, more prone to local extinction, and therefore show higher genetic differentiation and lower genetic diversity (Lajeunesse and Forbes, 2002; Barrett et al., 2008). Yet, it seems unlikely that host specificity – at least regarding the fish host – is responsible for the high inbreeding coefficients. First, diplostomids are generally not considered very host specific. Second, although the vulnerability to inflammation of the retina gives reason to assume that retina-infecting *Diplostomum* spp. are more host-specific than lens-infecting species (Locke et al., 2010b), I could show that *D.* lineage 6 infects at least two fish species – *Gasterosteus aculeatus* and *Pungitius pungitius*.

Associations between local environmental conditions and parasite distribution

Congruent with the study by de Roij and MacColl (2012), I found significant variation in parasite distribution among lakes on North Uist. Most macroparasites did not only differ in abundance, two parasites – the copepod *Thersitina gasterostei* and lens-infecting *Diplostomum* spp. – were found almost exclusively in western, alkaline lakes, but were absent from most acidic water bodies. When I compared the data of the de Roij and MacColl study from years 2007 and 2008 with my own dissection data from 2010 and 2011, I found similar distribution patterns of the major parasite species. Further, distribution patterns seem to have been largely consistent over several years and host generations. As explained in the introduction, water quality in the lakes differs by pH. Due to the presence of tannins, acidity of the water is negatively associated with calcium availability and positively associated with light absorbance. Therefore, environmental conditions for hosts and parasites vary substantially among lakes. Congruent with the de Roij and MacColl study, I did not find convicing evidence that parasite abundances are generally significantly influenced by pH. Although, *Th. gasterostei* and *Diplostomum* spp. (lens) were mostly found in alkaline lakes, this cannot be separated from occurrence in western lakes. At least for the lens-infecting *Diplostomum* spp., it might be possible that the parasite, which was not mentioned in de Roij and MacColl (2012), has only appeared in the past two decades and was introduced to the lakes from the Atlantic side of the island.

In line with the previously published survey but against my expectations, eyefluke (*Diplostomum* spp. (non-lens) and *Apatemon* spp.) abundances were not significantly negatively affected by acidic pH values. These findings were also largely supported in a study that came out shortly after *Chapter II* of this thesis was published (Young and MacColl, 2016). Both eyefluke genera depend on the availability of snail intermediate hosts which again require a minimum concentration of dissolved calcium. Compared with the de Roij and MacColl study that had examined 10 acidic and only 2 alkaline lakes, I had expected my more balanced (with respect to pH) set of lakes (7 alkaline and 12 acidic lakes) to reveal a significant association of eyefluke abundance and acidity, but this was not the case. One possible explanation might be the first intermediate host. *Radix peregra* is the predominant lymnaeid snail host on North Uist. Compared to *Lymnaea stagnalis*, which is not found on the island, *R. peregra* can also thrive at lower calcium concentrations typical for Hebridean waters (Briers, 2003a, 2003b). Since *R. peregra* can cope with the conditions in both alkaline and acidic lakes, snail prevalence might not be pH-dependent.

Parasite distribution in relation to host population connectivity and pH

As I explained in the previous sections, abundances of macroparasites on North Uist are not randomly distributed. Patterns of parasite distribution are consistent over time, but, so far, could not be explained by water quality. My studies reveal two important main results that should contribute to the understanding of what shapes the local success of parasites in a fragmented habitat such as the lakes on North Uist.

First, the fact that low pH does not prevent eyeflukes from spreading and infecting fish hosts and that the *D.* lineage 6 on North Uist are not further separated into distinct populations, point to differences in susceptibility among the stickleback populations as the main factor underlying parasite distribution patterns. My results of the population genetic analysis are congruent with the paradigm that the most motile host in a parasite's life cycle determines its population structure and support the hypothesis that population structures of complex life-cycle parasites are generally weaker in the parasite than in the host (Mazé-Guilmo et al., 2016). In theory, higher migration rates in the parasite than in the host should favour adaptation of the parasite to the host (Gandon et al., 1996; Gandon, 2002) if gene flow is not too high (Lenormand, 2002). Yet, adaptation of *D.* lineage 6 to their stickleback host on North Uist is unlikely at a local lake-to-lake scale. Furthermore, although innate immunity against *Diplostomum* spp. can be specific (Kalbe and Kurtz,

2006; Rauch et al., 2006; Scharsack and Kalbe, 2014), resistance of stickleback populations is probably not against single *D.* lineage 6 genotypes as this would have shown in spatially non-randomly distributed *Diplostomum*-genotype frequencies (Edelaar and Bolnick, 2012).

That differences between stickleback populations are at least partly responsible for local parasite abundances, is further supported by the second finding – a significant positive correlation of neutral genetic differentiation between stickleback populations with dissimilarity in parasite communities based on presence/absence data. Although mainly driven by two parasites (*Th. gasterostei*, lens-infecting *Diplostomum* spp.) that mostly occurred in western, alkaline lakes, parasite distribution appears to be affected by habitat and host population connectivity. Habitat connectivity, which cannot completely be separated from (intermediate) host population connectivity in this case, is probably the better proxy for isolation of habitats than mere geographical distance (de Roij and MacColl, 2012). In my analysis, qualitative but not quantitative differences in parasite distribution, i.e. differences in mean abundance, were correlated with host genetic differentiation. This suggests that not only host genetic factors, such as resistance, but also water quality and (intermediate) host prevalence contribute to parasite distribution patterns. Interestingly, an experimental study, which was published after *Chapter II* of this thesis, found a significant correlation of virulence of *Gyrodactylus arcuatus* with pH indicating a significant role of this lake characteristic in shaping local parasite communities (Mahmud et al., 2017). The authors had tested for local adaptation in the stickleback–*Gyrodactylus* system using sticklebacks and parasites from selected habitats on North Uist and found *Gyrodactylus* to be adapted to their local host population as well as to the pH of its habitat of origin (Mahmud et al., 2017). Recent research on the North Uist sticklebacks also suggests the opposite effect – habitat characteristics and availability of intermediate hosts may drive the evolution of resistance in local stickleback populations (El Nagar and MacColl, 2016).

Chapters III *and* IV – *Parasitic influence on the shoaling behaviour of sticklebacks*

The effect of *Gyrodactylus* spp. on infected sticklebacks

There are only few studies that have specifically examined the effects of *Gyrodactylus*-infections on sticklebacks. The parasite is known to trigger reactions of the immune system and increase mortality (Lester, 1972; Lester and Adams, 1974). Also, a negative effect of *Gyrodactylus* spp. on weight gain has been observed previously (Eizaguirre et al., 2012; Anaya-Rojas et al., 2016). Infected sticklebacks in my experiments showed reduced (absolute and relative) body masses compared to uninfected animals. Further, infected fish had lower relative liver masses and less food in their stomachs pointing to a generally reduced nutritional state (Chellappa et al., 1995). Higher relative spleen masses in infected fish suggest an activation of the immune system – an association that has been found in other fishes (Lefebvre et al., 2004; Seppänen et al., 2009). Taken together, I could confirm that *Gyrodactylus*-infections significantly affect the health status and nutritional state of the sticklebacks in a negative way.

Gyrodactylus spp. also had significant effects on the behaviour of its host. Infected focal fish had a lower tendency to associate with the stimulus shoals and, although there was no significant difference in activity between infected and uninfected focal fish, animals with higher body condition indices were more active. These results are in line with general findings that sick individuals across a range of taxa are less active and reduce their tendency to join groups of conspecifics (Loehle, 1995), and with observations that *Gyrodactylus*-infected guppies show reduced shoal cohesion and shoaling tendency (Croft et al., 2011; Hockley et al., 2014b). When given the choice between two stimulus shoals, uninfected sticklebacks preferred to shoal with groups of uninfected conspecifics, while infected sticklebacks did not show a significant preference. Preferences differed significantly between infected and uninfected focal fish. Hence, *Gyrodactylus*-infections influenced the shoal choice behaviour of the choosing sticklebacks as well as the relative "attractiveness" of the stimulus shoals. To my knowledge, the experiments described in *Chapter III* were the first study that examined the effects of *Gyrodactylus* spp. on the behaviour of sticklebacks. Yet, the results are in line with previously published shoal choice tests with sticklebacks that found that shoals of uninfected fish were preferred over shoals infected with *Schistocephalus solidus* (Barber et al., 1998), *Glugea anomala* (Ward et al., 2005), or *Argulus canadensis* (Dugatkin et al., 1994).

The effect of *Diplostomum pseudospathaceum* on infected sticklebacks

Knowledge of significant effects of *Diplostomum* spp. on the health status and behaviour of fishes mostly stems from studies on salmonids and cyprinids (see *Introduction,* Diplostomum *spp.*). Previous studies found negative effects of lens-infecting *Diplostomum* spp. on prey detection (Crowden and Broom, 1980; Voutilainen et al., 2008), nutrition, and body condition (Shariff et al., 1980; Karvonen and Seppälä, 2008; Kuukka-Anttila et al., 2010). Research on sticklebacks has so far mainly focused on taxonomical and immunological aspects (e.g. Scharsack and Kalbe, 2014; Kuhn et al., 2015; Haase et al., 2016; but see Owen et al., 1993). Generally, effects caused by eyeflukes on the physical condition and behaviour of the host are attributed to the formation of cataracts and reduced capabilities to feed and escape from predators resulting from visual impairments. In the experiments of *Chapter IV*, only few sticklebacks that were heavily infected showed early stages of cataracts. Thus, it is not surprising that I was not able to detect significant effects of the eyefluke infections on the growth rate or body condition of the experimental fish. Yet, results are congruent with previous studies that found only heavily infected fish bearing cataracts to be severely affected by *Diplostomum* spp. (e.g. Seppälä et al., 2005b). As experiments were carried out in winter, development of metacercariae inside the eye lenses was probably retarded due to low temperatures (Sweeting, 1974) and by the end of the experimental period, most metacercariae had probably not been infective yet. In Arctic charr (*Salvelinus alpinus*), chronic *Diplostomum*-infections resulted in higher oxygen consumption and larger spleens (Voutilainen et al., 2008; Seppänen et al., 2009). *D. pseudospathaceum* escapes accessibility of the immune system within hours after penetration of the skin and, usually, innate immune reactions cease within the first few days after infection (Scharsack and Kalbe, 2014). Hence, if detected, effects on immune variables two months after infection would have been an indirect consequence of infection and visual impairment, but not a direct effect.

Diplostomum-infection did not significantly reduce shoaling tendency in sticklebacks. Apart from a study on rainbow trout that found animals infected with the lens-infecting *D. spathaceum* to form smaller shoals (Seppälä et al., 2008), specific influence of *Diplostomum* spp. on the shoaling decisions of fishes has not been tested systematically before. My results support the view that infection did not severely affect the general health status of experimental animals. Surprisingly, uninfected sticklebacks preferred to shoal with mixed groups, comprising infected as well as uninfected conspecifics, while infected

fish did not show any significant preference for either of the two shoal types. Despite the absence of significant measurable effects of *D. pseudospathaceum* on the health status and general tendency to shoal of sticklebacks in this thesis, the results indicate an effect of the eyefluke on the shoal choice of sticklebacks regarding the choosing fish as well as the appearance of the stimulus shoals. That mixed shoals were preferred does not suggest that infected fish appeared as sick or in any way unfavourable shoal mates. Instead, mixed shoals may have appeared more attractive because they were heterogenous with respect to infection status and also comprised individuals that could be seen as "weak competitors" (Metcalfe and Thomson, 1995; also see next section).

How infection-associated changes in behaviour can affect parasite transmission

Research of grouping behaviour usually accredits parasites with an exclusively negative role ("risk of infection"; e.g. Krause and Ruxton, 2002). When the impact of parasitic infections on grouping behaviour is examined, studies often concentrate on host–parasite systems with high infectiousness and pathogenicity, and/or conspicuous (visual) signs of infection (e.g. Krause and Godin, 1996; Barber et al., 1998; Ondrackova et al., 2006; Tobler and Schlupp, 2008). In theory, effects of a parasite on the shoaling behaviour of its host should depend on its route of transmission, on the damage it causes to its host, and on the recognisability of infected individuals. Therefore, parasitic influence on shoaling behaviour can vary considerably among systems. Furthermore, many parasites go through different stages of development. As a consequence, the impact of a parasite on its host and whether the parasite can benefit from transmission to the next host also vary with developmental stage. Depending on whether the pathogen has reached its infective stage or not, early transmission to the next host may even end the parasite's life cycle (e.g. Hammerschmidt et al., 2009).

Whether or not infected conspecifics should be avoided as shoal mates, does not only depend on a direct risk of infection. Depending on the type of infection, sick individuals often reduce food intake (Crompton, 1984) or require even more resources due to higher energy demands (e.g. Pascoe and Mattey, 1977). But, shoaling with them can also bring about the advantages of grouping while being seen as weak competitors. Generally, animals can benefit from being able to identify infected individuals and adjust their (social) behaviour accordingly.

As I could show, *Gyrodactylus* spp. impairs the body condition and general health status of sticklebacks. Uninfected sticklebacks would therefore benefit from being able to recognise and from avoiding infected conspecifics just as individuals already infected with the parasite would benefit from avoiding infection with even more worms. In addition to reducing a direct risk of infection, uninfected fish could also escape an increased risk of attracting predators brought about by conspicuous, or in any way "odd", behaviour of infected animals. Infected sticklebacks might not benefit from preferring to shoal with uninfected conspecifics compared to infected fish if their own competitiveness is impaired. *Gyrodactylus* spp. have short generation times and adult worms often already carry embryos that are prepared to hatch and transmit (Scott, 1982; Bakke et al., 2007). Further, the monogeneans are transmitted through body contact and are able to initiate epidemics in large and dense host groups (Bagge et al., 2004; Johnson et al., 2011). Hence, a reduced tendency to shoal and an avoidance of infected fish is in favour of the host, not the parasite.

In the experiments of *Chapter IV*, *D. pseudospathaceum* did not significantly impair its hosts' body condition or immune status. Further, the parasite cannot be transmitted directly from stickleback to stickleback. Hence, shoaling with infected conspecifics does not constitute a risk of infection to healthy sticklebacks. Metacercariae in the experiments were not fully developed yet due to low winter temperatures (Sweeting, 1974). Further, at temperatures below 10 °C, miracidia cannot infect snails (Chappell et al., 1994). Therefore, hosts as well as parasites would benefit from an absence of effects that increase transmission to the next (bird) host. In line with the predation suppression hypothesis, *Diplostomum* metacercariae that are not infective yet could benefit from a preference for their hosts, i.e. for infected sticklebacks, as shoal mates if this increases shoal size and leads to a dilution effect. Infective stages, on the other hand, can increase transmission by making their hosts easier to catch by birds and, indeed, dace (*Leuciscus leuciscus*) that had contracted lens-infecting *Diplostomum* spp. swam closer to the water surface (Crowden and Broom, 1980) and *Diplostomum*-infected rainbow trout were more easily caught by human "predators" with dip-nets (Seppälä et al., 2004, 2005b). That different developmental stages of a parasite can have opposing effects on their hosts, has been shown, e.g., in studies on rainbow trout (*Oncorhynchus mykiss*). Mikheev et al. (2010) found that pre-infective *D. spathaceum* reduced aggressiveness in trout while aggressiveness was increased (and competitiveness reduced) compared to controls in fish

that harboured infective developmental stages of the parasite. Gopko et al. (2015; 2017), who work on the same system, found that trout harbouring pre-infective stages of *D. pseudospathaceum* were less active and less easily caught by humans with dip-nets (Gopko et al., 2015), while trout harbouring infective metacercariae were more active and resumed activity earlier after a simulated avian predator attack than controls (Gopko et al., 2017). Interestingly, the effect was not correlated with the intensity of infection, i.e. with the number of eyeflukes per infected individual. This suggests that the state of being infected is more relevant than the parasite burden. In my experiments, infection intensity was also not correlated with body condition, both in *Gyrodactylus*- and in *Diplostomum*-infected sticklebacks, despite a significant impact of *Gyrodactylus* spp. on this trait in infected compared to uninfected animals. In *Gyrodactylus*-infected experimental sticklebacks, relative spleen mass was increased, but this measure was also not correlated with the actual number of worms on the fish. While a positive association of worm load on infected choosing fish with the relative time near infected stimulus shoals suggests that primarily high infection intensities affect shoaling decisions, parasite load was not correlated with any measure of stickleback behaviour in *Diplostomum*-infected fish.

As I could show, both parasites affect shoaling decisions in sticklebacks. Since neither of them causes visually conspicuous alterations of the outer appearance of the host, it is not quite clear how uninfected animals perceive their infected conspecifics. Barriers between shoal and focal fish compartments were transparent and perforated, thus enabling visual as well as olfactory communication. Previous studies have shown that animals can discriminate infected conspecifics based on olfactory cues (Kavaliers and Colwell, 1995; Hughes et al., 2014; Stephenson and Reynolds, 2016). Additionally, infected fish may have behaved differently in a subtle way, which was not obvious to the human observer. Experimental sticklebacks originated from populations that were familiar with *Gyrodactylus* spp., but probably not with *Diplostomum* spp. Thus, at least in case of the *Gyrodactylus*-infections, parasite-associated shoaling decisions may have been the result of specific adaptations.

Conclusion

Using a population genetics approach, I could show that the sticklebacks on North Uist indeed form distinct populations as had been assumed in the literature, but not examined before. Further, the *Diplostomum* lineage 6 metacercariae that infect the North Uist sticklebacks do not show any structuring into distinct populations. My results are in line with the

conception that the host with the widest geographic range determines dispersal and gene flow in a parasite with a complex life cycle. I could largely confirm previously published patterns of macroparasite distribution. Like the authors of the previous study, I failed to clearly identify pH as a significant influencing variable of parasite distribution although this variable varies substantially among lakes. Instead, I could reveal a decrease in parasite community similarity with increasing pairwise genetic differentiation between (stickleback) host populations. Based on my findings, I conclude that differences in resistance between the stickleback populations rather than physico-chemical variables or specific adaptations to single host populations are the reason for the spatial heterogeneity in *D*. lineage 6 abundances on North Uist.

With the experimental work in *Chapters III* and *IV*, I could demonstrate that the impact of parasitic infections on the social behaviour of three-spined sticklebacks is system-specific and differs between parasites. The two parasites used in the experiments had very different effects on their hosts: *Gyrodactylus*-infected sticklebacks were in a reduced nutritional state and their relative spleen masses were increased indicating activation of the immune system. Also, infected individuals showed a reduced tendency to shoal. In contrast to that, evidence of detrimental effects of infections with lens-infecting *Diplostomum* spp. that have not yet reduced vision in sticklebacks is scarce and could not be detected in this thesis. As expected, and in line with an avoidance of a risk of contracting a contagious disease, uninfected sticklebacks preferred uninfected shoals over shoals of *Gyrodactylus*-infected conspecifics, while infected animals showed no significant preference. Contrary to my expectations, uninfected sticklebacks spent significantly more time with mixed shoals comprising uninfected as well as *Diplostomum*-infected individuals than with uninfected shoals. *Diplostomum*-infected sticklebacks did not show any significant preference. This behaviour could be explained if sticklebacks perceived *Diplostomum*-infected conspecifics as less competitive but not sick or contagious shoal mates.

Summary

If one is to understand how parasitic diseases spread within and among host populations, one has to take into account characteristics of the given parasite species, the host as well as of their common environment. To what extent each of these three axes shapes the distribution of the parasite is determined by a range of different factors. These include the life cycle (generation time, reproductive potential), the virulence, and the transmissibility of the parasite, as well as the availability and motility of susceptible hosts, and also the suitability of the prevailing environmental conditions for the parasite and also for its (intermediate) host(s).

For this thesis, I resorted to the three-spined stickleback, *Gasterosteus aculeatus*, – a well-established model species – and two of its most common macroparasites – the digenean trematode *Diplostomum* spp. and the monogenean *Gyrodactylus* spp. The main aims of the first part of this work were 1) to identify potentially different barriers of migration for the stickleback host and one of its parasites with a complex life cycle – indirectly measured as population genetic structure of host and parasite – and 2) to examine water quality (particularly pH) as a potential cause of the consistently different parasite distribution in 19 natural freshwater lakes on the Scottish island of North Uist (*Chapters I* and *II*). In the second part of my thesis, I investigated host behavioural aspects of parasite distribution since grouping with infected conspecifics can favour parasite transmission to new host individuals. *Chapters III* and *IV* examine whether and in what way *Gyrodactylus* spp. and *Diplostomum pseudospathaceum* affect the shoaling behaviour of their hosts.

For the population genetic analysis in *Chapter I* I developed new microsatellite primers specially designed for the parasite *Diplostomum* lineage 6. Fish-eating birds like sea gulls, whose ranges expand far across the boundaries of individual lakes, are final hosts of *D.* lineage 6, a common complex life cycle parasite of the North Uist sticklebacks. Confirming the paradigm that the most motile host in a parasite's life cycle significantly influences its dispersal and gene flow, no population genetic structure of *D.* lineage 6 was found on North Uist. For the population genetic analysis of the sticklebacks, I used previously published microsatellite primers and additionally analysed mitochondrial sequences (cytochrome *b* and control region) to obtain a more precise picture of the relationships among the populations. My results confirm that the sticklebacks of the individual lakes have been isolated from each other for many generations. Using field data

in *Chapter II* I could reveal an association between the differences in parasite communities among individual lakes and the genetic differentiation between host populations (measured as pairwise F_{ST} values of the microsatellite analysis). However, I could not detect a clear influence of abiotic factors like pH on the distribution of several stickleback macroparasites. Taken together, the results of *Chapters I* and *II* suggest different levels of resistance of the stickleback populations rather than an influence of abiotic factors as potential cause underlying the consistent differences in parasite distribtion on North Uist.

In *Chapters III* and *IV* I used experimental infections to examine a potential influence of the ectoparasite *Gyrodactylus* spp. and the endoparasite *Diplostomum pseudospathaceum* on the shoaling behaviour of sticklebacks. In both shoal choice experiments, infected and uninfected test fish behaved significantly differently from each other. Uninfected test fish preferred uninfected shoals compared to shoals infected with the directly transmitted *Gyrodactylus* spp. while *Gyrodactylus*-infected test fish did not show any significant preference. Surprisingly, uninfected sticklebacks preferred shoals comprising uninfected as well as infected animals over uninfected shoals while *Diplostomum*-infected test fish, again, did not show any significant preference. The avoidance of shoals infected with *Gyrodactylus* spp. might be based in a poorer physical condition. Infected animals had a lower absolute and relative body weight. To maintain an infection within a host population, *Gyrodactylus* spp. depends on the frequent introduction of new host individuals. To reject conspecifics infected with *Gyrodactylus* spp. would therefore reduce the risk of infection for uninfected sticklebacks and favour the host. *D. pseudospathaceum* settles in the eye lens of the stickleback and infective stages can impair the vision of its host. The preference for partially infected shoals might therefore reduce competition for healthy individuals. However, eyeflukes in my experiments were not yet infective for the bird host and had not yet cause any damage in the sticklebacks. Therefore, the results do not provide a disadvantage for the parasite.

Zusammenfassung

Will man verstehen, wie sich parasitäre Erkrankungen innerhalb und zwischen Wirtspopulationen ausbreiten, muss man Eigenschaften des Parasiten, des Wirtes sowie ihrer gemeinsamen Umwelt betrachten. Auf welche Weise jede dieser drei Achsen die Verbreitung des Parasiten beeinflusst, wird jeweils durch eine ganze Reihe von Faktoren bestimmt. Hierzu gehört der Lebenszyklus (Generationszeit, Reproduktionspotenzial), die Virulenz sowie die Übertragbarkeit des Parasiten, die Verfügbarkeit und die Mobilität empfänglicher Wirte und auch die Eignung der vorherrschenden Umweltbedingungen sowohl für den Parasiten selbst als auch für seine (Zwischen-) Wirte.

Für diese Arbeit griff ich auf den Dreistachligen Stichling, *Gasterosteus aculeatus*, – einen gut etablierten Modellorganismus – und zwei seiner häufigsten Makroparasiten – den digenen Trematoden *Diplostomum* spp. und den Monogenen *Gyrodactylus* spp. – zurück. Die Hauptziele des ersten Teils dieser Arbeit waren 1. die Identifizierung potenziell unterschiedlicher Migrationsbarrieren für den Stichlingswirt und einen seiner Parasiten mit komplexem Lebenszyklus – indirekt gemessen als genetische Populationsstruktur von Wirt und Parasit – sowie 2. eine Untersuchung der Wasserqualität (insbesondere des pH-Wertes) als mögliche Ursache der konstant unterschiedlichen Parasitenverteilungen in neunzehn natürlichen Süßwasserseen auf der schottischen Insel North Uist (*Kapitel I* und *II*). Im zweiten Teil meiner Arbeit untersuchte ich das Wirtsverhalten betreffende Aspekte der Parasitenverbreitung, da das Schwärmen mit infizierten Artgenossen die Übertragung von Parasiten auf neue Wirte begünstigen kann. *Kapitel III* und *IV* untersuchten, ob und wie *Gyrodactylus* spp. und *Diplostomum pseudospathaceum* das Schwarmverhalten ihrer Wirte beeinflussen.

Für die populationsgenetische Analyse in *Kapitel I* entwickelte ich neue Mikrosatellitenprimer speziell für den Parasiten *Diplostomum* lineage 6. Endwirte von *D.* lineage 6, einem auf North Uist häufigen Sichlingsparasiten mit komplexem Lebenszyklus, sind fischfressende Vögel wie z. B. Möwen deren Bewegungsradius weit über die Grenzen einzelner Seen hinausgeht. Das Paradigma bestätigend, dass der Wirt mit der höchsten Motilität im Lebenszyklus eines Parasiten dessen Verteilung und damit Genfluss maßgeblich beeinflusst, ließ sich erwartungsgemäß keine Populationsstruktur bei *D.* lineage 6 auf North Uist finden. Für die populationsgenetische Analyse der Stichlinge nutzte ich bereits publizierte Mikrosatellitenprimer und analysierte zusätzlich mitochondriale Sequenzen (Cytochrom *b* und Kontrollregion) um ein genaueres Bild der Verwandschaftsverhältnisse

zwischen den Populationen zu erhalten. Durch meine Ergebnisse konnte ich bestätigen, dass die Stichlinge in den einzelnen Seen bereits seit vielen Generationen voneinander isoliert sind. In *Kapitel II* konnte ich mit Hilfe im Freiland aufgenommener Daten einen Zusammenhang zwischen der Unterschiedlichkeit der Parasitengemeinschaften der einzelnen Seen und der genetischen Trennung zwischen den Wirtspopulationen (gemessen als paarweise F_{ST}-Werte der Mikrosatellitenanalyse) aufdecken. Einen klaren Einfluss abiotischer Faktoren wie des pH-Wertes auf die Verbreitung verschiedener Stichlingsmakroparasiten konnte ich hingegen nicht bestätigen. Zusammengenommen deuten die Ergebnisse der *Kapitel I* und *II* darauf hin, dass die konstant unterschiedlichen Parasitenverteilungen in den Seen auf North Uist weniger Folge des Einflusses abiotischer Faktoren als vermutlich in verschieden stark ausgeprägten Resistenzen der einzelnen Stichlingspopulationen begründet sind.

In *Kapitel III* und *IV* untersuchte ich mittels experimenteller Infetionen einen möglichen Einfluss des Ektoparasiten *Gyrodactylus* spp. und des Endoparasiten *Diplostomum pseudospathaceum* auf das Schwarmverhalten von Stichlingen. In beiden Schwarmwahlversuchen verhielten sich die infizierten und uninfizierten Testfische signifikant unterschiedlich voneinander. Uninfizierte Testfische bevorzugten uninfizierte gegenüber mit dem direkt von Fisch zu Fisch übertragbaren *Gyrodactylus* spp. infizierten Schwärmen während *Gyrodactylus*-infizierte Tiere keine signifikante Präferenz zeigten. Überraschenderweise bevorzugten uninfizierte Stichlinge teilweise mit *D. pseudospathaceum* infizierte Schwärme gegenüber uninfizierten Schwärmen während *Diplostomum*-infizierte Fische auch hier keine signifikante Präferenz zeigten. Die Ablehnung mit *Gyrodactylus* spp. infizierter Schwärme mag in einem schlechteren allgemeinen Gesundheitszustand begründet liegen. Infizierte Tiere hatten ein geringeres absolutes und relatives Körpergewicht. *Gyrodactylus* spp. bedarf zur Aufrechterhaltung einer Infektion innerhalb einer Wirtspopulation regelmäßig neuer Wirte. Eine Vermeidung mit *Gyrodactylus* spp. infizierter Artgenossen würde demnach für uninfizierte Stichlinge das Ansteckungsrisiko verringern und den Wirt begünstigen. *D. pseudospathaceum* siedelt sich im Stichling in der Augenlinse an und kann im späteren Stadium die Sehfähigkeit beeinträchtigen. Die Bevorzugung teilweise mit *D. pseudospathaceum* infizierter Schwärme geht daher möglicherweise mit einer geringeren Konkurrenz für die gesunden Fische einher. Die Augenparasiten in meinen Versuchen waren jedoch noch nicht bereit den Vogelwirt zu infizieren und hatten in den Stichlingen keine messbaren Schäden angerichtet. Die Ergebnisse stellen somit keinen Nachteil für den Parasiten dar.

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Appendices

Appendix Chapter I

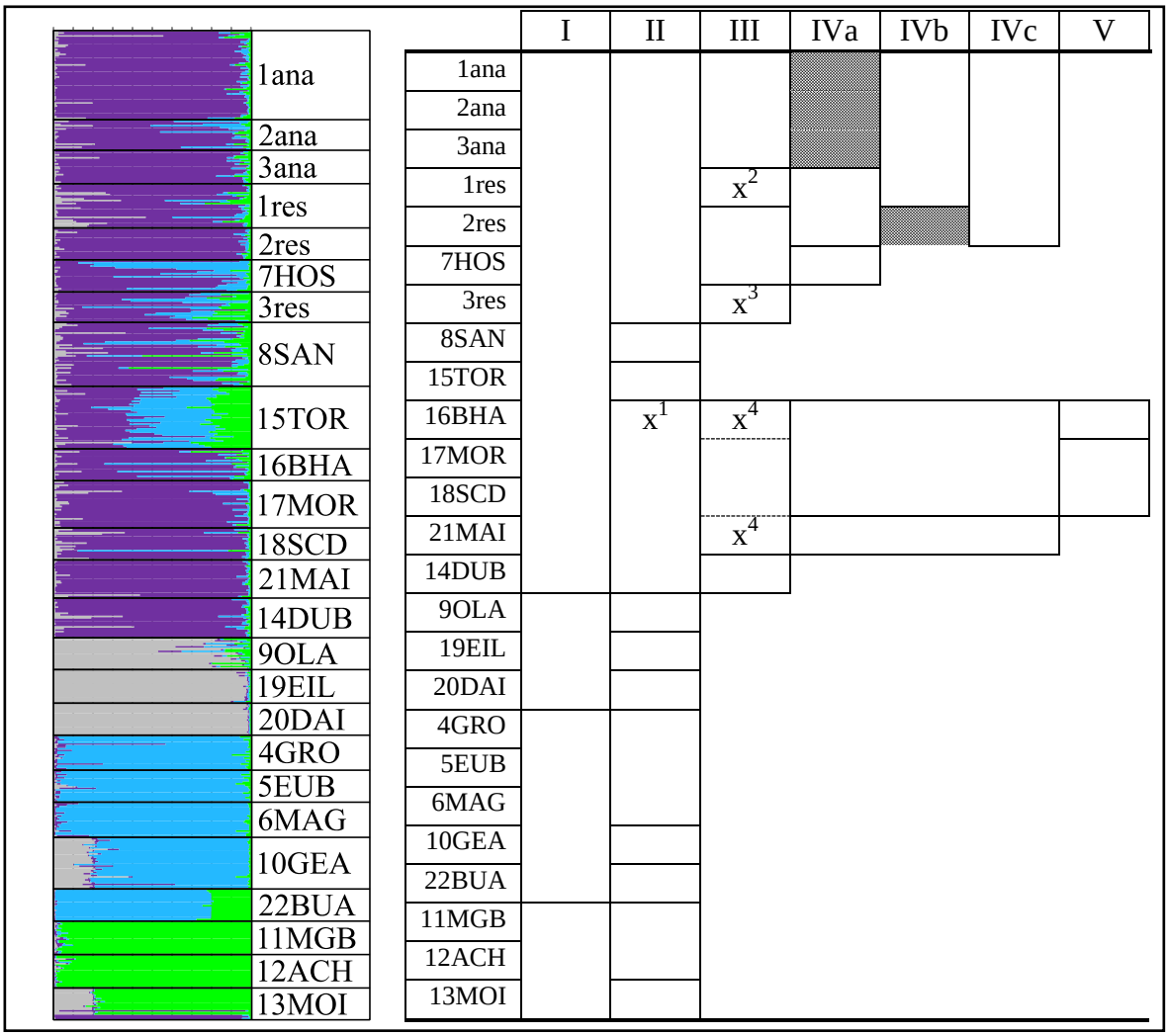
Supplementary methods – Development of microsatellite primers for Diplostomum *spp.*

To obtain suitable primer sequences, a total of 139 metacercariae taken from 25 three-spined sticklebacks caught in Loch Tormasad (15TOR) were pooled and conserved in 98 % EtOH. Extraction of the genomic DNA, enrichment for simple sequence repeats (SSRs), and sequencing were carried out by a commercial service (Ecogenics, Zürich-Schlieren, Switzerland). In short, magnetic streptavidin beads and biotin-labelled CT and GT repeat oligonucleotides were used to enrich size selected fragments from genomic DNA for sequences containing SSRs. This SSR enriched library was analysed on a Roche 454 platform using the GS FLX titanium reagents. The sequencing resulted in a total of 10,852 reads with an average length of 192 base pairs. Of these reads, 1,040 contained a microsatellite insert with a tetra- or a trinucleotide of at least six repeat units or a dinucleotide of at least ten repeat units. To avert the risk of developing markers for stickleback DNA present in the worms' intestines, the 334 reads suitable for primer design were checked against the published stickleback genome on Ensembl! (www.ensembl.org) and fish and vertebrate sequences published on GenBank (http://www.ncbi.nlm.nih.gov/). Primers were chosen based on the remaining 24 sequences with the help of NetPrimer (http://www.premierbiosoft.com/netprimer/) and tested together with the six primer pairs published for *Diplostomum pseudospathaceum* (Reusch et al., 2004) on a CEQTM 8800 capillary sequencer (Beckman Coulter, software GenomeLabTM GeXP (version 10.2)) using the tailed-primer method (Schuelke, 2000). All markers were tested with *Diplostomum* DNA as well as with eight different stickleback DNA samples which had worked well in the stickleback microsatellite genotyping.

Supplementary methods – Molecular Diplostomum *species identification*

PCRs had a volume of 20 µl and included 0.6 pmol of forward and reverse primer respectively, 10 µl Multiplex mix, 6.8 µl H_2O, and 2 µl DNA. The PCR-programme began with a 15 min denaturation step at 94 °C followed by five cycles of 60 s at 94 °C, 60 s at 50 °C and 90 s at 72 °C, and 30 cycles of 60 s at 94 °C, 60 s at 55 °C and 90 s at 72 °C, and a final elongation step of 30 min at 72 °C. PCR products were sequenced by a commercial sequencing service (LGC Genomics GmbH, Berlin). The resulting sequences

had a length of 407 bp and were compared to published sequences on BOLD (http://boldsystems.org; Ratnasingham and Hebert, 2007) and GenBank.



x^1 15 of 20 16BHA fish were assigned to the 8SAN cluster (average proportion = 62 %) and to the 18SCD cluster (27 %). In an additional analysis 16BHA and 8SAN fish were clearly assigned to separate clusters.

x^2 1res: 10 of 28 fish were assigned to the "anadromous" cluster (average proportion = 72 %), 10 of 28 to the 2res-7HOS cluster (76 %).

x^3 3res: 9 of 19 fish were assigned to 15TOR (79 %), 8 of 19 to the "anadromous" cluster (82 %). In an additional analysis 3res and 15TOR fish were clearly assigned to separate clusters.

x^4 ΔK2 = 266, ΔK3 = 14, ΔK4 = 250, ΔK5 = 1.

Figure A1. Bayesian cluster assignment for K = 4 and hierarchical STRUCTURE analysis. Each of the four clusters was tested independently with 1 to ($N_{\text{sampling locations}}$ + 1) K and five runs per K. This procedure was repeated for the resulting clusters until the most likely number of clusters was 1 according to Ln probabilities. Roman numbers indicate the different levels of population structure – I first level according to first highest Delta K, IVa 1res-2res-7HOS tested as one cluster, IVb 1ana- 2ana-3ana-1res tesed as one cluster, IVc 1ana-2ana-3ana-1res-2res tested as one cluster.

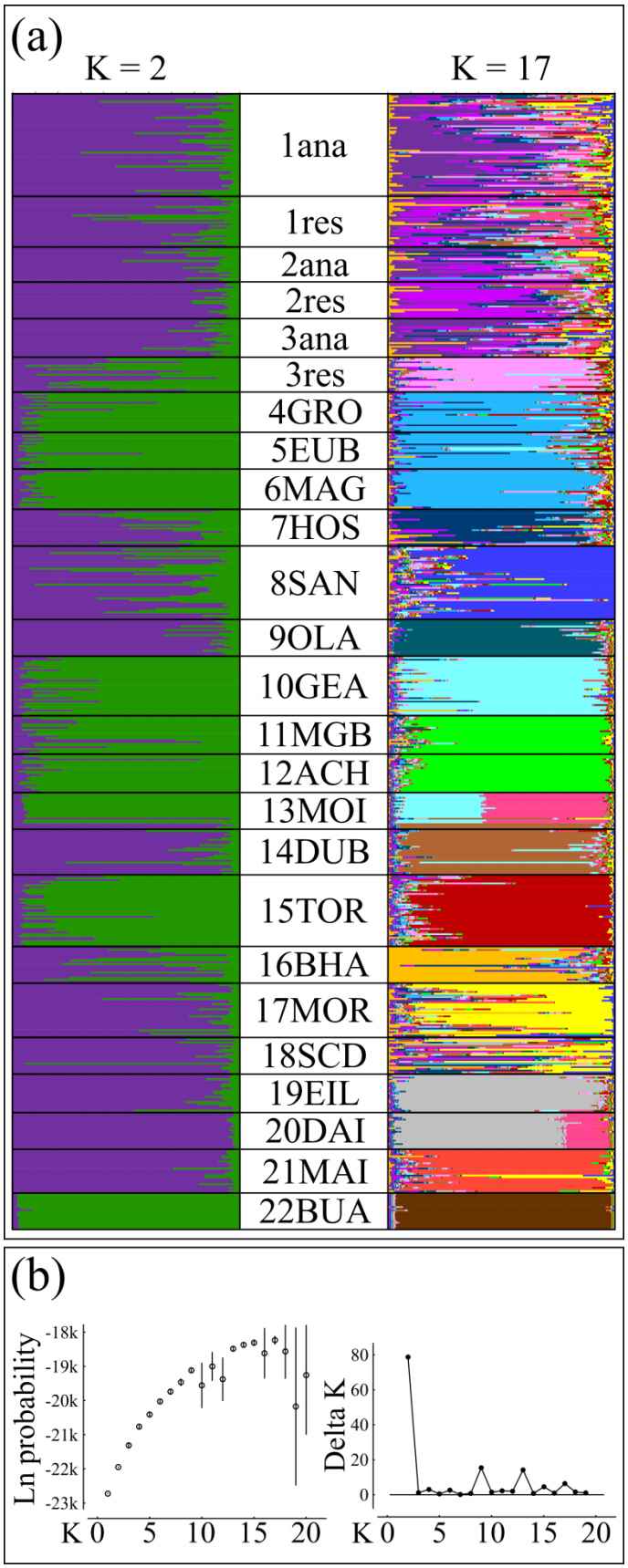


Figure A2. Results of the Bayesian cluster analysis (STRUCTURE) without the two loci suspected to be linked to plate morphology. (a) Cluster membership proportions for K = 2 and K = 17. Colours in the right column correspond to colours used in Figures 3 and 4. (b) Mean estimated Ln posterior probabilities for each K (1–20, 5 runs per K) with standard deviations and Delta K values calculated from posterior probabilities.

Table A1. Stickleback primers for microsatellites and mitochondrial DNA with GenBank accession numbers and PCR conditions: annealing temperature (T_A, °C), combination of markers within a single PCR reaction (Mix), amount of primer molecules (Reverse/Labelled/Forward, pmol)

Locus	Tail (dye)	Primer sequences 5'→3'	T_A[a]	Mix[b]	Reverse	Labelled	Forward
Gac1116PBBE	T7	for GGTGTCATGTGGGGGCGAGCAG	60/56	A	4	4	2
AJ010353	(D3)	rev CCCGAAGCATTGTGGCATCATC					
Gac7033PBBE	M13	for AGGTGGATTGGTTTTCTG	60/56	A	0.6/1	0.6/1	0.3/0.5
AJ010360	(D4)	rev GGACGCTCGCTCTTTC					
Gac3133PBBE	SP6	for CGCCCAGTTCCTGAACTTGAACTG	56	B	1	1	0.5
AJ010356	(D4)	rev CATGGTGGGCTGACTGAC					
Gac4174PBBE	T7	for CCGCGATGATGAGAGTG	56	B	2	2	1
AJ010358	(D3)	rev GTGAAATGCGACAGATGATG					
Gac7010PBBE	M13	for CGAGTAAAGACACGGAGTAG	56	B	1.6	1.6	0.8
AJ311863	(D2)	rev CTGTAGGGAGGGTTGACT					
Gac1097PBBE	M13	for AGGAACTCTCTTCTTCTCTG	58	C	3/2.5	3/2.5	1.5/1.25
AJ010352	(D2)	rev CCCGGGTTAGTCACT					
Gac1125PBBE	M13	for CATCACACCCAGCCTCTC	58	C	0.7/0.6	0.7/0.6	0.35/0.3
AJ010354	(D2)	rev CCTCCCTCCAACTCTTATCA					
Gac4170PBBE	SP6	for GCCGAGCCACATAGAGA	58	C	1/1.5	1/1.5	0.5/0.75
AJ010357	(D4)	rev CCAATATAACAGCCGAGCAG					
Gac5196PBBE	T7	for ACTTCTCCCCTCATTATGCT	58	C	4	4	2
AJ010359	(D3)	rev GGGGTCTGATGGATACAAA					
Cytochrome *b*	-	for ATGAAACTTTGGTTCCCTCC	52	D	5	-	5
		rev CGCTGAGCTACTTTTGCATGT					
Control region	-	for CCTTTAGTCCTATAATGCATG	52	E	5	-	5
		rev CCGTAGCCCATTAGAAAGAA					

[a] PCR programme microsatellites: 15 min at 94 °C, 60 s at 94 °C, 45 s at T_A, 60 s at 72 °C (30 cycles), 60 s at 94 °C, 45 s at 53 °C and 60 s at 72 °C (8 cycles), 30 min at 72 °C. PCR programme mitochondrial DNA fragments: 15 min at 94 °C, 30 s at 94 °C, 45 s at 52 °C, 60 s at 72 °C (32 cycles), 30 min at 72 °C.

[b] PCR mixes A, B, and C included primers, 5 µl Multiplexmix (Qiagen), 40 ng DNA and H_2O to adjust reaction volume to 10 µl. PCR mixes D and E included primers, 10 µl Multiplexmix (Qiagen), 20 ng DNA and H_2O to adjust reaction volume to 20 µl.

Table A2. *Diplostomum* spp. microsatellite primers with GenBank accession numbers and PCR conditions, T_A annealing temperature (°C), Mix combination of markers within a single PCR reaction, Reverse/Labelled/Forward amount of primer molecules (pmol) within a single PCR reaction

Locus	Tail (dye)	Primer sequences 5'→3'	T_A[a]	Mix[b]	Reverse	Labelled	Forward
Diga1	T7	for TTGAGCAGTGGATGAGGGTG	56	A	0.2	0.2	0.1
KT971126	(D3)	rev TGAACCCCTCTTGTGATGGC					
Diga3	SP6	for ACTGGCATCTCAAACCTGGG	56	B	0.1	0.1	0.05
KT971128	(D4)	rev TCATGTTTCATCTTTGCGG					
Diga2	SP6	for GGATTCCAGCAATTGTCCCG	64-60-56	C	0.2	0.1	0.1
KT971127	(D4)	rev ACAAATAGGGTACAGTTTGAGCG					
Diga4	T7	for TGGCAGTTAGTCTCGTATTTGG	64-60-56	C	0.1	0.1	0.05
KT971129	(D3)	rev ATACCTGGTTCAATTTCTCGC					
Diga5	SP6	for TTGATTTTTGGTTGACTAAG	64-60-56	D	0.1	0.1	0.05
KT971130	(D4)	rev GAGTAAACAGTGTGACAGAGGG					
Diplo23[c]	T7	for TTTCGAGTGTCTGTGTGCAA	56	E	0.2	0.2	0.1
AJ629253	(D3)	rev AGAACAAATGCCGTTTTCAA					

[a] PCR programme: 15 min at 94 °C, 60 s at 94 °C, 60 s at T_A (annealing temperature, either all 30 cycles at 56 °C or ten cycles at 64 °C, ten at 60 °C and ten cycles at 56 °C), 60 s at 72 °C (30 cycles), 60 s at 94 °C, 60 s at 53 °C and 60 s at 72 °C (8 cycles), 30 min at 72 °C.

[b] PCR mixes include primers, 5 µl Multiplexmix (Qiagen), 2 µl DNA (3 µl for Diga1) and H_2O to adjust reaction volume to 10 µl.

[c] Published by Reusch et al. (2004).

Table A3. Genotyping results for *Diplostomum* spp., repeat motif and product length (bp) without primers as determined from sequencing results, *N* number of individuals successfully genotyped, A number of alleles, PIC polymorphism information content (Botstein et al., 1980), H_e (expected heterozygosity), H_o (observed heterozygosity)

	Repeat motif	Product length	*N*	A	PIC	H_e	H_o
Diga1	$(ATC)_n$	190	247	9	0.75	0.783	0.389
Diga2	$(CA)_n$	191	251	14	0.76	0.787	0.438
Diga3	$(CA)_n$	95	249	7	0.48	0.532	0.474
Diga4	$(TTGG)_n$	97	244	6	0.61	0.671	0.504
Diga5	$(GT)_n$	78	248	15	0.77	0.790	0.536
Diplo23	$(GA)_n$[a]	86-140[a]	250	5	0.40	0.437	0.384

[a] Reusch et al. (2004)

Table A4. Pairwise F_{ST} values (stickleback: lower matrix, *Diplostomum* spp.: upper matrix) calculated from microsatellite data, sample sizes are given next to three-letter codes (first column for sticklebacks, first row for *Diplostomum* spp.)

		1ana		2ana		3ana		1res		2res		3res		4GRO	19	5EUB	15	6MAG	20	7HOS	22	8SAN	18	9OLA	6	10GEA	20
1ana	58			-		-		-		-		-		-		-		-		-		-		-		-	
2ana	19	**0.010**	***			-		-		-		-		-		-		-		-		-		-		-	
3ana	21	0.001		0.000				-		-		-		-		-		-		-		-		-		-	
1res	28	**0.029**	***	**0.019**	***	**0.016**	***			-		-		-		-		-		-		-		-		-	
2res	20	**0.049**	***	**0.032**	***	**0.032**	***	**0.033**	***			-		-		-		-		-		-		-		-	
3res	19	**0.054**	***	**0.040**	***	**0.051**	***	**0.053**	***	**0.063**	***			-		-		-		-		-		-		-	
4GRO	22	**0.078**	***	**0.044**	***	**0.063**	***	**0.068**	***	**0.081**	***	**0.096**	***			0.006		0.020		0.010		0.015		0.015		0.013	
5EUB	20	**0.086**	***	**0.050**	***	**0.069**	***	**0.070**	***	**0.083**	***	**0.095**	***	-0.001				0.024		0.015		0.013		0.002		0.009	
6MAG	22	**0.086**	***	**0.056**	***	**0.073**	***	**0.076**	***	**0.079**	***	**0.096**	***	0.007		0.003				0.005		0.011		0.013		0.011	
7HOS	20	**0.066**	***	**0.046**	***	**0.060**	***	**0.058**	***	**0.062**	***	**0.086**	***	**0.060**	***	**0.060**	***	**0.059**	***			0.017		0.008		0.009	
8SAN	41	**0.076**	***	**0.049**	***	**0.054**	***	**0.070**	***	**0.071**	***	**0.092**	***	**0.097**	***	**0.085**	***	**0.092**	***	**0.099**	***			0.006		0.013	
9OLA	20	**0.167**	***	**0.164**	***	**0.161**	***	**0.144**	***	**0.175**	***	**0.194**	***	**0.214**	***	**0.199**	***	**0.210**	***	**0.205**	***	**0.174**	***			-0.012	
10GEA	33	**0.126**	***	**0.097**	***	**0.117**	***	**0.112**	***	**0.113**	***	**0.137**	***	**0.108**	***	**0.098**	***	**0.117**	***	**0.140**	***	**0.124**	***	**0.249**	***		
11MGB	21	**0.131**	***	**0.110**	***	**0.121**	***	**0.114**	***	**0.138**	***	**0.148**	***	**0.147**	***	**0.149**	***	**0.162**	***	**0.164**	***	**0.149**	***	**0.230**	***	**0.184**	***
12ACH	21	**0.155**	***	**0.135**	***	**0.142**	***	**0.142**	***	**0.169**	***	**0.165**	***	**0.176**	***	**0.180**	***	**0.198**	***	**0.202**	***	**0.171**	***	**0.268**	***	**0.211**	***
13MOI	20	**0.161**	***	**0.151**	***	**0.154**	***	**0.138**	***	**0.148**	***	**0.181**	***	**0.175**	***	**0.160**	***	**0.170**	***	**0.181**	***	**0.152**	***	**0.254**	***	**0.145**	***
14DUB	25	**0.094**	***	**0.084**	***	**0.093**	***	**0.081**	***	**0.067**	***	**0.119**	***	**0.125**	***	**0.110**	***	**0.116**	***	**0.115**	***	**0.124**	***	**0.212**	***	**0.150**	***
15TOR	40	**0.086**	***	**0.073**	***	**0.068**	***	**0.073**	***	**0.081**	***	**0.084**	***	**0.087**	***	**0.092**	***	**0.089**	***	**0.123**	***	**0.107**	***	**0.216**	***	**0.137**	***
16BHA	20	**0.079**	***	**0.058**	***	**0.063**	***	**0.062**	***	**0.060**	***	**0.092**	***	**0.104**	***	**0.105**	***	**0.098**	***	**0.110**	***	**0.098**	***	**0.215**	***	**0.142**	***
17MOR	30	**0.069**	***	**0.055**	***	**0.062**	***	**0.058**	***	**0.050**	***	**0.092**	***	**0.094**	***	**0.100**	***	**0.087**	***	**0.101**	***	**0.093**	***	**0.203**	***	**0.143**	***
18SCD	20	**0.044**	***	**0.028**	***	**0.036**	***	**0.038**	***	**0.032**	***	**0.066**	***	**0.078**	***	**0.084**	***	**0.083**	***	**0.076**	***	**0.069**	***	**0.177**	***	**0.130**	***
19EIL	21	**0.202**	***	**0.187**	***	**0.203**	***	**0.166**	***	**0.208**	***	**0.207**	***	**0.245**	***	**0.249**	***	**0.261**	***	**0.233**	***	**0.217**	***	**0.305**	***	**0.301**	***
20DAI	20	**0.205**	***	**0.237**	***	**0.232**	***	**0.215**	***	**0.238**	***	**0.272**	***	**0.303**	***	**0.301**	***	**0.300**	***	**0.281**	***	**0.251**	***	**0.299**	***	**0.280**	***
21MAI	24	**0.099**	***	**0.089**	***	**0.097**	***	**0.079**	***	**0.091**	***	**0.133**	***	**0.123**	***	**0.137**	***	**0.145**	***	**0.108**	***	**0.145**	***	**0.239**	***	**0.180**	***
22BUA	20	**0.311**	***	**0.338**	***	**0.344**	***	**0.333**	***	**0.346**	***	**0.335**	***	**0.320**	***	**0.329**	***	**0.343**	***	**0.311**	***	**0.332**	***	**0.482**	***	**0.370**	***

*P < 0.05; **P < 0.01; ***P < 0.001

Values that remained significant after Boferroni correction are printed in bold.

F_{ST} < 0.05 little genetic differentiation, 0.05 < F_{ST} < 0.15 moderate genetic differentiation (light blue), 0.15 < F_{ST} < 0.25 great genetic differentiation (medium light blue), F_{ST} > 0.25 very great genetic differentiation (dark blue)

Table A4 continued

		11MGB	19	12ACH	17	13MOI	6	14DUB	7	15TOR	11	16BHA	5	17MOR	22	18SCD	9	19EIL	20	20DAI	6	21MAI	6	22BUA	5	ICE	26
1ana	58	-		-		-		-		-		-		-		-		-		-		-		-		-	
2ana	19	-		-		-		-		-		-		-		-		-		-		-		-		-	
3ana	21	-		-		-		-		-		-		-		-		-		-		-		-		-	
1res	28	-		-		-		-		-		-		-		-		-		-		-		-		-	
2res	20	-		-		-		-		-		-		-		-		-		-		-		-		-	
3res	19	-		-		-		-		-		-		-		-		-		-		-		-		-	
4GRO	22	0.009		0.030	*	0.018		0.063	*	0.037		0.033		-0.008		-0.007		0.008		0.077	**	0.010		0.068		0.046	***
5EUB	20	0.012		0.008		0.012		0.041		0.039		0.031		0.012		0.012		0.007		0.071	**	0.023		0.057		0.046	**
6MAG	22	0.011		0.042	*	0.066		0.054		0.024		0.010		0.015		0.019		0.011		0.105	**	0.082	*	0.104	*	0.035	*
7HOS	20	0.006		0.009		0.034		0.029		0.032		0.025		0.011		-0.001		0.021		0.072	*	0.032		0.095	**	0.025	
8SAN	41	0.008		0.023		0.032		0.068	*	-0.003		0.009		0.003		0.009		0.008		0.096	**	0.073	*	0.059		**0.062**	***
9OLA	20	0.039		0.038		0.077		-0.013		0.018		0.020		0.025		0.018		0.010		0.038		0.048		0.007		0.011	
10GEA	33	0.008		0.025		0.037		0.032		0.015		0.019		0.009		0.017		0.015		0.064	*	0.050		0.071	*	0.024	
11MGB	21			0.019		0.014		0.074	*	0.014		0.011		-0.007		-0.002		0.011		0.087	**	0.047		0.101	*	0.047	**
12ACH	21	0.017	*			-0.002		0.055	*	0.044	*	0.064	*	0.023		0.011		0.019		0.086	**	0.044		0.107	**	0.056	**
13MOI	20	**0.207**	***	**0.236**	***			0.113	*	0.052		0.100		0.007		0.005		0.032		0.127	*	0.023		0.104		0.094	**
14DUB	25	**0.192**	***	**0.227**	***	**0.136**	***			0.093	*	0.088		0.076	*	0.034		0.064	*	0.082	*	0.058		0.110		0.025	
15TOR	40	**0.142**	***	**0.172**	***	**0.180**	***	**0.129**	***			0.021		0.020		0.029		0.020		0.127	**	0.114	*	0.063		**0.078**	***
16BHA	20	**0.146**	***	**0.179**	***	**0.173**	***	**0.142**	***	**0.087**	***			0.018		0.014		0.018		0.107	*	0.090		0.131		0.067	*
17MOR	30	**0.177**	***	**0.214**	***	**0.161**	***	**0.073**	***	**0.078**	***	**0.076**	***			-0.016		0.011		0.074	*	0.022		0.073	*	0.043	**
18SCD	20	**0.148**	***	**0.181**	***	**0.161**	***	**0.071**	***	**0.074**	***	**0.072**	***	**0.022**	***			0.017		0.066	*	0.010		0.072		0.036	
19EIL	21	**0.315**	***	**0.345**	***	**0.361**	***	**0.268**	***	**0.232**	***	**0.263**	***	**0.198**	***	**0.165**	***			0.107	**	0.055		0.069		**0.073**	***
20DAI	20	**0.339**	***	**0.380**	***	**0.315**	***	**0.270**	***	**0.262**	***	**0.290**	***	**0.203**	***	**0.202**	***	**0.328**	***			0.058		0.144	*	0.067	*
21MAI	24	**0.199**	***	**0.229**	***	**0.214**	***	**0.125**	***	**0.134**	***	**0.115**	***	**0.084**	***	**0.071**	***	**0.244**	***	**0.269**	***			0.089		0.084	**
22BUA	20	**0.441**	***	**0.471**	***	**0.439**	***	**0.388**	***	**0.365**	***	**0.393**	***	**0.358**	***	**0.354**	***	**0.474**	***	**0.556**	***	**0.395**	***			0.112	*

Table A5. List of the stickleback composite mtDNA haplotypes and their distribution across North Uist. The last three columns show the haplotype names together with corresponding haplotypes published in Mäkinen and MErilä (2008) and Ravinet er atl. (2014) in parentheses, as well as the GenBank accession numbers for the cytochrome *b* and control region sequences from this study. Table was split in two parts for this print version.

		Cytochrome *b* (1-1014)																																			
	Fish	14	32	122	218	235	236	239	240	242	251	263	293	299	305	324	461	564	584	612	623	651	686	728	737	779	783	784	807	812	847	866	879	945	965	971	977
anadromous	2ana12	T	C	T	G	T	C	A	A	A	C	C	A	G	T	G	T	G	T	C	T	T	C	A	C	C	A	T	G	T	C	T	A	T	C	A	A
	2ana2	.	.	.	A	.	.	G	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
	2ana5	.	.	.	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
	2ana10	.	.	.	A	.	.	G	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
	2ana17	.	.	.	A	.	.	G	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
	2ana19*	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
	1ana27	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	1ana32	.	.	.	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
	1ana33	.	.	.	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
	1ana40	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
	1ana44	.	.	.	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
	1ana57*	.	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
	1ana58*	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
	3ana3	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	3ana6	.	.	.	A	.	.	G	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
	3ana8	.	.	.	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
	3ana14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	T	.	.
	3ana18	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
resident	2res4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	2res7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	2res12	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
	2res16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	2res17	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	1res7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	1res12	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	1res16	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	1res18	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
	1res3	.	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
	3res3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	3res5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	3res9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	3res15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	3res19	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table A5 part 1 continued

		Cytochrome *b* (1-1014)																																		
	Fish	14	32	122	218	235	236	239	240	242	251	263	293	299	305	324	461	564	584	612	623	651	686	728	737	779	783	784	807	812	847	866	879	945	965	971	977
freshwater	4GRO22	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	4GRO4	.	.	.	A	G	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
	4GRO9	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	4GRO12	C	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	4GRO18	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	5EUB5	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	5EUB10	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	5EUB12	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	5EUB18	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	5EUB20	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
	6MAG3	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	6MAG6	C	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	6MAG8	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	6MAG15	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	6MAG21	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	7HOS1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	7HOS7	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	7HOS11	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	7HOS17	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	7HOS18	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.
	8SAN1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	8SAN6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	8SAN13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	8SAN17	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	8SAN25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	9OLA3	.	.	.	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
	9OLA9	.	.	.	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.	.	.
	9OLA14	.	.	.	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.	.	.
	9OLA16	.	.	.	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.	.	.
	9OLA18	.	.	.	A	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
	10GEA5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C
	10GEA7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.
	10GEA12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	10GEA13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C
	10GEA20	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table A5 part 1 continued

		Cytochrome *b* (1-1014)																																		
	Fish	14	32	122	218	235	236	239	240	242	251	263	293	299	305	324	461	564	584	612	623	651	686	728	737	779	783	784	807	812	847	866	879	945	965	971	977
	11MGB7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	G	.
	11MGB11	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	11MGB19	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	G	.
	11MGB13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	G	.
	11MGB17	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	G	.
	12ACH4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
	12ACH6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	G	.
	12ACH11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
	12ACH14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	G	.
	12ACH20	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
	13MOI3	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
	13MOI7	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
	13MOI12	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
	13MOI17	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
	13MOI20	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	14DUB3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
freshwater	14DUB8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	14DUB11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	14DUB13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	14DUB18	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	15TOR4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.
	15TOR9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	15TOR15	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	15TOR22	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	15TOR26	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	16BHA12	.	.	.	.	.	.	.	G	.	.	.	.	A	C	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	16BHA4	.	.	.	.	.	.	.	G	.	.	.	.	A	C	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.
	16BHA6	.	.	.	.	.	.	.	G	.	.	.	.	A	C	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	16BHA17	.	.	.	.	.	.	.	G	.	.	.	.	A	C	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	16BHA20	.	.	.	.	.	.	.	G	.	.	.	.	A	C	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	17MOR1	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	17MOR4	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	17MOR10	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	17MOR16	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	17MOR21	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table A5 part 1 continued

		Cytochrome *b* (1-1014)																																		
	Fish	14	32	122	218	235	236	239	240	242	251	263	293	299	305	324	461	564	584	612	623	651	686	728	737	779	783	784	807	812	847	866	879	945	965	971	977
freshwater	18SCD2	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	18SCD15	C	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	18SCD17	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	18SCD20	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	19EIL1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	19EIL3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
	19EIL8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	19EIL14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.
	19EIL18	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	20DAI7	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	20DAI8	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	20DAI14	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	20DAI18	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	C	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	21MAI02	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	21MAI10	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	21MAI15	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	21MAI19	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	21MAI22	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	22BUA5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	22BUA11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	22BUA16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	22BUA19	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	22BUA1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table A5 part 2

	Fish	Control region (1015-1467)																		Haplotype	Accession number cytochrome *b*	Accession number control region
		1116	1212	1248	1259	1295	1296	1297	1304	1305	1309	1312	1314	1362	1377	1378	1385	1419	1420			
anadromous	2ana12	C	C	T	T	C	T	T	A	C	A	G	A	T	T	T	T	A	A	NU1 (Eu1)	KT971020	KT971073
	2ana2	.	T	.	.	.	.	.	.	T	.	.	.	.	.	A	.	.	G	NU12 (At20)	KT971031	KT971084
	2ana5	.	T	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	G	NU3	KT971022	KT971075
	2ana10	.	T	.	.	.	.	.	.	T	.	.	.	.	.	A	.	.	G	NU12 (At20)	KT971031	KT971084
	2ana17	.	T	.	.	.	.	.	.	T	.	.	.	.	.	A	.	.	G	NU12 (At20)	KT971031	KT971084
	2ana19*	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	NU13 (Eu91)	KT971032	KT971085
	1ana27	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	NU2	KT971021	KT971074
	1ana32	.	T	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	G	NU3	KT971022	KT971075
	1ana33	.	T	.	.	T	.	.	.	.	.	.	.	.	.	A	.	.	G	NU4	KT971023	KT971076
	1ana40	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	NU5 (Eu62)	KT971024	KT971077
	1ana44	.	T	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	G	NU3	KT971022	KT971075
	1ana57*	.	.	.	.	.	A	.	.	T	.	T	.	.	.	C	.	.	.	NU6	KT971025	KT971078
	1ana58*	.	.	.	.	.	A	.	.	T	.	T	.	.	.	C	.	.	.	NU7	KT971026	KT971079
	3ana3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU15	KT971034	KT971087
	3ana6	.	T	.	.	.	.	.	.	T	.	.	.	.	.	A	.	.	G	NU12 (At20)	KT971031	KT971084
	3ana8	.	T	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	G	NU3	KT971022	KT971075
	3ana14	.	.	C	G	.	A	.	.	T	.	T	.	.	.	C	.	.	.	NU16	KT971035	KT971088
	3ana18	.	.	.	.	G	A	C	.	T	.	T	.	.	.	.	.	.	.	NU17 (Eu85)	KT971036	KT971089
resident	2res4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU1 (Eu1)	KT971020	KT971073
	2res7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU1 (Eu1)	KT971020	KT971073
	2res12	A	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	NU14	KT971033	KT971086
	2res16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU1 (Eu1)	KT971020	KT971073
	2res17	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU1 (Eu1)	KT971020	KT971073
	1res3	.	.	.	.	.	A	.	.	T	.	T	.	.	.	C	.	.	.	NU6	KT971025	KT971078
	1res7	.	.	.	.	G	A	.	.	T	.	T	.	.	.	C	.	.	.	NU8	KT971027	KT971080
	1res12	.	.	.	.	.	A	.	.	T	.	T	.	.	.	C	.	.	.	NU9 (Eu60)	KT971028	KT971081
	1res16	.	.	.	.	.	A	.	.	T	.	T	.	.	.	C	.	.	.	NU10	KT971029	KT971082
	1res18	.	.	.	.	.	A	.	.	T	.	T	.	C	.	C	.	.	.	NU11	KT971030	KT971083
	3res3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU1 (Eu1)	KT971020	KT971073
	3res5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU1 (Eu1)	KT971020	KT971073
	3res9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU1 (Eu1)	KT971020	KT971073
	3res15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU1 (Eu1)	KT971020	KT971073
	3res19	.	.	.	.	.	A	.	.	T	.	.	.	.	.	C	.	.	.	NU18 (Ir4)	KT971037	KT971090

Table A5 part 2 continued

		Control region (1015-1467)																					
	Fish	1116	1212	1248	1259	1295	1296	1297	1304	1305	1309	1312	1314	1362	1377	1378	1385	1419	1420	Haplotype	Accession number cytochrome *b*	Accession number control region
freshwater	4GRO22	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	NU19	KT971038	KT971091
	4GRO4	.	T	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	G	NU20	KT971039	KT971092
	4GRO9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU21	KT971040	KT971093
	4GRO12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU22	KT971041	KT971094
	4GRO18	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU21	KT971040	KT971093
	5EUB5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU21	KT971040	KT971093
	5EUB10	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	NU23	KT971042	KT971095
	5EUB12	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	NU23	KT971042	KT971095
	5EUB18	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU21	KT971040	KT971093
	5EUB20	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	NU24	KT971043	KT971096
	6MAG3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU21	KT971040	KT971093
	6MAG6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU22	KT971041	KT971094
	6MAG8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU21	KT971040	KT971093
	6MAG15	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU25	KT971044	KT971097
	6MAG21	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	NU23	KT971042	KT971095
	7HOS1	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU26	KT971045	KT971098
	7HOS7	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU25	KT971044	KT971097
	7HOS11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU21	KT971040	KT971093
	7HOS17	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU25	KT971044	KT971097
	7HOS18	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU27	KT971046	KT971099
	8SAN1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	NU28	KT971047	KT971100
	8SAN6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU1 (Eu1)	KT971020	KT971073
	8SAN13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU1 (Eu1)	KT971020	KT971073
	8SAN17	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	NU29	KT971048	KT971101
	8SAN25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU1 (Eu1)	KT971020	KT971073
	9OLA3	.	T	.	.	.	.	.	.	.	.	.	.	.	.	A	C	.	G	NU30	KT971049	KT971102
	9OLA9	.	T	.	.	.	.	.	.	.	.	.	.	.	.	A	C	.	G	NU31	KT971050	KT971103
	9OLA14	.	T	.	.	.	.	.	.	.	.	.	.	.	.	A	C	.	G	NU31	KT971050	KT971103
	9OLA16	.	T	.	.	.	.	.	.	.	.	.	.	.	.	A	C	.	G	NU31	KT971050	KT971103
	9OLA18	.	T	.	.	.	.	.	.	.	.	.	.	.	.	A	C	.	G	NU30	KT971049	KT971102
	10GEA5	.	.	.	.	.	A	.	.	.	.	.	.	.	.	C	.	.	.	NU32	KT971051	KT971104
	10GEA7	.	.	.	.	.	A	.	.	.	.	.	.	.	.	C	.	.	.	NU33	KT971052	KT971105
	10GEA12	.	.	.	.	.	A	.	.	.	.	.	.	.	.	C	.	.	.	NU34	KT971053	KT971106
	10GEA13	.	.	.	.	.	A	.	.	.	.	.	.	.	.	C	.	.	.	NU32	KT971051	KT971104
	10GEA20	.	.	.	.	.	A	.	.	T	.	.	.	.	.	C	.	.	.	NU18 (Ir4)	KT971037	KT971090

Table A5 part 2 continued

		Control region (1015-1467)																					
	Fish	1116	1212	1248	1259	1295	1296	1297	1304	1305	1309	1312	1314	1362	1377	1378	1385	1419	1420	Haplotype	Accession number cytochrome *b*	Accession number control region
freshwater	11MGB7	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	NU35	KT971054	KT971107
	11MGB11	.	.	.	.	.	.	.	.	T	.	.	G	.	.	.	.	.	.	NU36	KT971055	KT971108
	11MGB19	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	NU35	KT971054	KT971107
	11MGB13	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	NU35	KT971054	KT971107
	11MGB17	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	NU35	KT971054	KT971107
	12ACH4	.	T	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	NU37	KT971056	KT971109
	12ACH6	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	NU35	KT971054	KT971107
	12ACH11	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	NU5 (Eu62)	KT971024	KT971077
	12ACH14	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	NU35	KT971054	KT971107
	12ACH20	.	T	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	NU37	KT971056	KT971109
	13MOI3	.	T	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	NU38	KT971057	KT971110
	13MOI7	.	T	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	NU38	KT971057	KT971110
	13MOI12	.	T	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	NU38	KT971057	KT971110
	13MOI17	.	T	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	NU38	KT971057	KT971110
	13MOI20	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU1 (Eu1)	KT971020	KT971073
	14DUB3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU1 (Eu1)	KT971020	KT971073
	14DUB8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU39	KT971058	KT971111
	14DUB11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU1 (Eu1)	KT971020	KT971073
	14DUB13	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	NU40 (Eu4)	KT971059	KT971112
	14DUB18	.	.	.	.	.	A	.	.	T	.	.	.	.	.	C	.	.	.	NU41 (Ir13)	KT971060	KT971113
	15TOR4	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU42	KT971061	KT971114
	15TOR9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU1 (Eu1)	KT971020	KT971073
	15TOR15	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU43	KT971062	KT971115
	15TOR22	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU44 (Eu2)	KT971063	KT971116
	15TOR26	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU44 (Eu2)	KT971063	KT971116
	16BHA12	.	.	.	.	.	A	.	.	T	.	.	.	.	.	C	.	.	.	NU45	KT971064	KT971117
	16BHA4	.	.	.	.	.	A	.	.	T	.	.	.	.	.	C	.	.	.	NU46	KT971065	KT971118
	16BHA6	.	.	.	.	.	A	.	.	T	.	.	.	.	.	C	.	.	.	NU45	KT971064	KT971117
	16BHA17	.	.	.	.	.	A	.	.	T	.	.	.	.	.	C	.	.	.	NU45	KT971064	KT971117
	16BHA20	.	.	.	.	.	A	.	.	T	.	.	.	.	.	C	.	.	.	NU45	KT971064	KT971117

Table A5 part 2 continued

		Control region (1015-1467)																					
	Fish	1116	1212	1248	1259	1295	1296	1297	1304	1305	1309	1312	1314	1362	1377	1378	1385	1419	1420	Haplotype	Accession number cytochrome *b*	Accession number control region
freshwater	17MOR1	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	NU47	KT971066	KT971119
	17MOR4	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	NU48	KT971067	KT971120
	17MOR10	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	NU48	KT971067	KT971120
	17MOR16	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	NU48	KT971067	KT971120
	17MOR21	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	NU48	KT971067	KT971120
	18SCD2	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	NU48	KT971067	KT971120
	18SCD15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	NU22	KT971041	KT971094
	18SCD17	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	NU48	KT971067	KT971120
	18SCD20	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	.	.	.	NU49	KT971068	KT971121
	19EIL1	.	.	.	.	.	A	.	.	T	.	.	.	.	.	C	.	.	.	NU18 (Ir4)	KT971037	KT971090
	19EIL3	.	.	.	.	.	A	.	.	T	.	T	.	.	.	C	.	.	.	NU50	KT971069	KT971122
	19EIL8	.	.	.	.	.	A	.	.	T	.	.	.	.	.	C	.	.	.	NU18 (Ir4)	KT971037	KT971090
	19EIL14	.	.	.	.	.	A	.	.	T	.	T	.	.	.	C	.	.	.	NU50	KT971069	KT971122
	19EIL18	.	.	.	.	.	A	.	.	T	.	.	.	.	.	C	.	.	.	NU18 (Ir4)	KT971037	KT971090
	20DAI7	.	.	.	.	.	A	.	.	T	.	.	.	.	.	C	.	.	.	NU51	KT971070	KT971123
	20DAI8	.	.	.	.	.	A	.	.	T	.	.	.	.	.	C	.	.	.	NU51	KT971070	KT971123
	20DAI14	.	.	.	.	.	A	.	.	T	.	.	.	.	.	C	.	.	.	NU51	KT971070	KT971123
	20DAI18	.	.	.	.	T	A	.	.	T	.	.	.	.	.	C	.	.	.	NU52	KT971071	KT971124
	21MAI02	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	NU48	KT971067	KT971120
	21MAI10	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	NU48	KT971067	KT971120
	21MAI15	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	NU48	KT971067	KT971120
	21MAI19	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	NU48	KT971067	KT971120
	21MAI22	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	NU48	KT971067	KT971120
	22BUA5	.	.	.	.	.	A	.	.	T	.	T	.	.	.	C	.	G	.	NU53	KT971072	KT971125
	22BUA11	.	.	.	.	.	A	.	.	T	.	T	.	.	.	C	.	G	.	NU53	KT971072	KT971125
	22BUA16	.	.	.	.	.	A	.	.	T	.	T	.	.	.	C	.	G	.	NU53	KT971072	KT971125
	22BUA19	.	.	.	.	.	A	.	.	T	.	T	.	.	.	C	.	G	.	NU53	KT971072	KT971125
	22BUA1	.	.	.	.	.	A	.	.	T	.	T	.	.	.	C	.	G	.	NU53	KT971072	KT971125

Appendix Chapter II

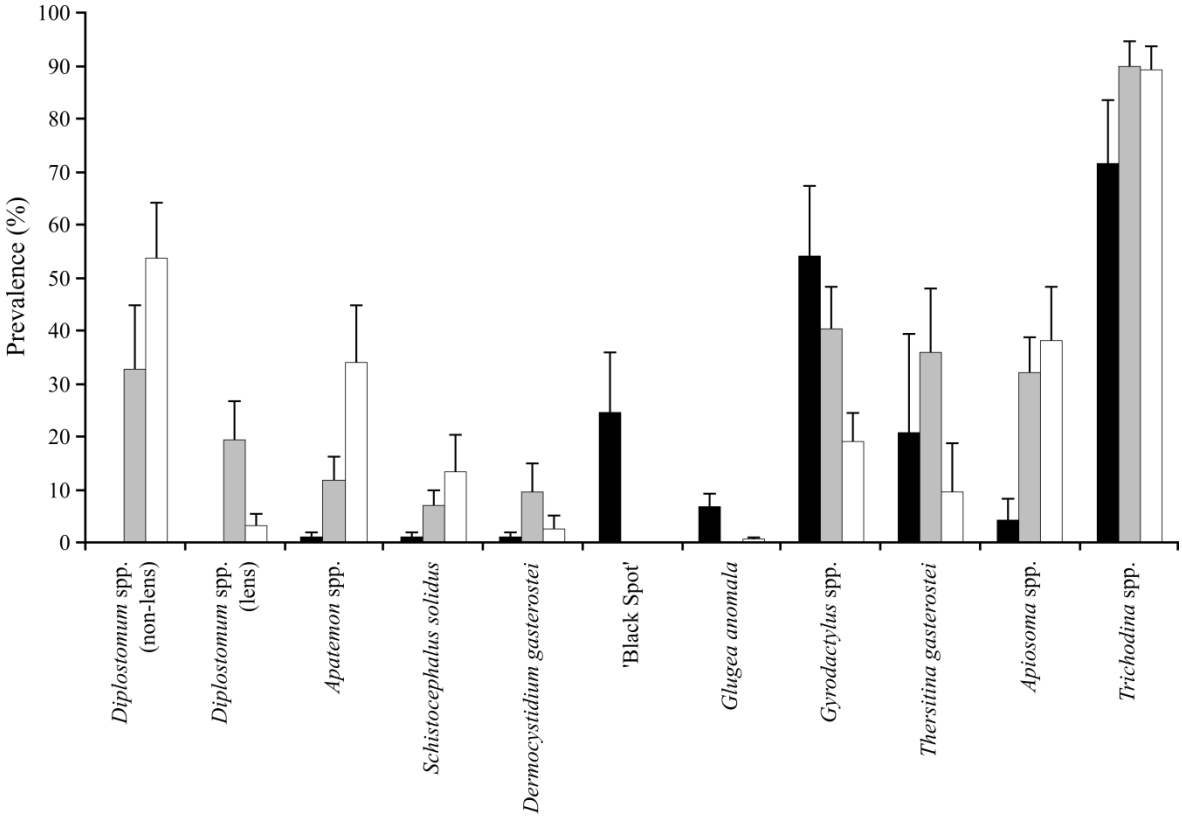


Fig. A1. Mean prevalence (+ standard error) of eleven common stickleback parasites on fish from freshwater lakes with pH > 7 ("alkaline", grey, N = 7), pH < 7 ("acidic", white, N = 12), and three brackish water sites with anadromous and resident fish ("brackish", black, N = 6). Data of fish caught in 2010 and 2011 were combined. Mean values of the years 2010 and 2011 were calculated for lake 8SAN (Sanndaraigh).

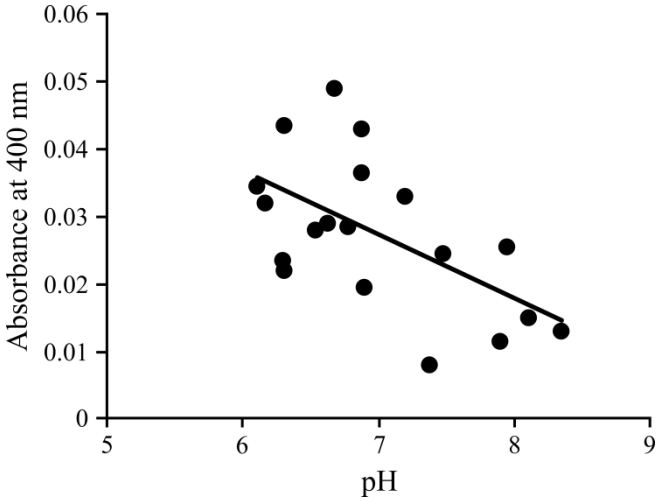


Fig. A2. Relationship between light absorbance and pH in 19 freshwater lakes on North Uist. Light absorbance was significantly higher in less alkaline lakes (Spearman rank correlation: r_S = –0.59, N = 19, P = 0.009).

Table A1. Primer sequences published by Heckel et al. (2002) and Largiadèr et al. (1999) with GenBank accession numbers and PCR conditions used for microsatellite genotyping. The tailed primer method (Schuelke, 2000) was applied. Fragments were analysed on a CEQ™ 8800 capillary sequencer (Beckman Coulter, Brea, CA, USA) with GenomeLab™ GeXP 181 (version 10.2) software. T_A = annealing temperature, Mix = combination of markers within a single PCR reaction, Reverse/Labeled/Forward = amount of primer molecules within a single PCR reaction.

Locus	Tail (dye)	Primer sequences 5'→3'	T_A (°C)[a]	Mix[b]	Reverse (pmol)	Labeled (pmol)	Forward (pmol)
Gac1116PBBE	T7 (D3)	for GGTGTCATGTGGGGGCGAGCAG	60/56	A	4	4	2
AJ010353		rev CCCGAAGCATTGTGGCATCATC					
Gac7033PBBE	M13 (D4)	for AGGTGGATTGGTTTTCTG	60/56	A	0.6/1	0.6/1	0.3/0.5
AJ010360		rev GGACGCTCGCTCTTTC					
Gac3133PBBE	SP6 (D4)	for CGCCCAGTTCCTGAACTTGAACTG	56	B	1	1	0.5
AJ010356		rev CATGGTGGGCTGACTGAC					
Gac4174PBBE	T7 (D3)	for CCGCGATGATGAGAGTG	56	B	2	2	1
AJ010358		rev GTGAAATGCGACAGATGATG					
Gac7010PBBE	M13 (D2)	for CGAGTAAAGACACGGAGTAG	56	B	1.6	1.6	0.8
AJ311863		rev CTGTAGGGAGGGTTGACT					
Gac1097PBBE	M13 (D2)	for AGGAACTCTCTTCTTCTCTG	58	C	3/2.5	3/2.5	1.5/1.25
AJ010352		rev CCCGGGTTAGTCACT					
Gac1125PBBE	M13 (D2)	for CATCACACCCAGCCTCTC	58	C	0.7/0.6	0.7/0.6	0.35/0.3
AJ010354		rev CCTCCCTCCAACTCTTATCA					
Gac4170PBBE	SP6 (D4)	for GCCGAGCCACATAGAGA	58	C	1/1.5	1/1.5	0.5/0.75
AJ010357		rev CCAATATAACAGCCGAGCAG					
Gac5196PBBE	T7 (D3)	for ACTTCTCCCCTCATTATGCT	58	C	4	4	2
AJ010359		rev GGGGTCTGATGGATACAAA					

[a] PCR programme: 15 min at 94 °C, 60 s at 94 °C, 45 s at T_A, 60 s at 72 °C (30 cycles), 60 s at 94 °C, 45 s at 53 °C and 60 s at 72 °C (8 cycles), 30 min at 72 °C.
[b] PCR mixes A, B, and C included primers, 5 µl Multiplex mix (Qiagen), 40 ng DNA and H_2O to adjust reaction volume to 10 µl.

Table A2. Distribution of common stickleback parasites on North Uist given as prevalence (Prev, percentage of infected fish) and mean infection intensity (MI, mean number of parasites per infected fish, rounded to the nearest integer). For full names of the sampling locations see Table 1 of the main article. ’10 = 2010, ’11 = 2011.

Parasite		1ana ‘11	1res ‘10	2ana ‘11	2res ‘11	3ana ‘11	3res ‘11	4GRO ‘11	5EUB ‘11	6MAG ‘10	7HOS ‘11	8SAN10 ‘10	8SAN11 ‘11	8SAN9sp ‘11	9OLA ‘11	10GEA ‘11	11MGB ‘10	12ACH ‘10	13MOI ‘11	14DUB ‘11	15TOR ‘10	16BHA ‘11	17MOR ‘11	18SCD ‘11	19EIL ‘11	20DAI ‘11	21MAI ‘11	22BUA ‘10
Gyrodactylus spp.	Prev	95	100	63	40	57	16	38	30	100	48	59	27	15	24	25	52	95	15	35	17	4	43	25	76	0	5	0
	MI	33	18	3	2	11	3	3	3	8	3	3	2	2	2	3	12	13	2	3	2	1	2	1	5	–	1	–
Diplostomum spp. (non-lens)	Prev	0	0	0	0	0	0	14	35	100	14	88	13	55	29	100	90	95	15	55	72	30	95	45	90	55	33	60
	MI	–	–	–	–	–	–	1	2	33	3	6	8	7	3	8	9	8	3	3	3	1	11	2	21	2	5	3
Diplostomum spp. (lens)	Prev	0	0	0	0	0	0	14	25	10	52	6	10	30	14	0	0	0	5	20	0	0	0	0	0	0	0	0
	MI	–	–	–	–	–	–	1	1	1	3	1	8	2	2	–	–	–	1	2	–	–	–	–	–	–	–	–
Apatemon spp.	Prev	0	0	0	0	0	5	5	20	62	5	6	13	20	0	96	81	95	40	55	50	9	10	30	29	5	29	70
	MI	–	–	–	–	–	1	1	1	2	3	1	2	2	–	3	2	3	1	2	2	1	2	1	2	1	3	2
Schistocephalus solidus	Prev	0	0	0	0	0	5	5	20	5	5	12	3	0	0	21	10	10	0	0	6	57	0	25	10	5	0	0
Dermocystidium gasterostei[a]	Prev	0	0	5	0	0	0	5	0	14	0	47	33	0	19	21	0	0	0	0	0	0	0	0	0	0	0	100
	MI	–	–	I	–	–	–	I	–	I	–	II	II	–	I	I	–	–	–	–	–	–	–	–	–	–	–	III
Thersitina gasterostei	Prev	0	5	0	10	0	95	67	55	95	0	12	37	35	57	75	81	29	0	0	0	0	0	0	0	0	0	0
	MI	–	3	–	1	–	4	5	2	4	–	2	2	5	3	3	6	2	–	–	–	–	–	–	–	–	–	–
"Black Spot"	Prev	48	90	16	55	0	5	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Glugea anomala	Prev	10	0	5	15	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0
Apiosoma spp.[a]	Prev	0	60	0	0	0	21	48	15	81	48	41	43	0	29	29	67	62	25	35	50	9	76	55	10	0	76	5
	MI	–	IV	–	–	–	IV	III	III	IV	IV	IV	III	–	III	III	IV	IV	III	III	III	II	IV	III	IV	–	III	II
Trichodina spp.[a]	Prev	95	95	32	75	62	95	95	75	100	95	100	73	100	100	83	100	100	100	95	100	70	95	70	100	100	100	95
	MI	III	IV	II	II	II	IV	III	II	III	III	III	II	II	III	III	III	III	III	III	III	II	III	III	III	II	III	II

[a] 0 = not infected, I = 1–10, II = 11–50, III = 51–100, IV = more than 100 parasites

Table A3. ANOVA results from generalised linear models (GLM) with infection status as dependent variable, lake as explaining variable, and standard length (SL), sex, and date of capture as covariates. In the separate models for the two sampling years, lake was associated with 5 (13) degrees of freedom for 2010 (2011). Note that *P* values are those that resulted from model reduction, whereas significance (Sig.) was determined from Bonferroni-adjusted (B.ad.) α levels. Significant *P* values are given in bold.

		2010			2011		
		χ^2	*P*	Sig.	χ^2	*P*	Sig.
Gyrodactylus spp.	prevalence	43.4	**<0.001**	***	53.3	**<0.001**	***
Gyrodactylus spp.	abundance	44.7	**<0.001**	***	57.3	**<0001**	***
T. gasterostei	prevalence	55.6	**<0.001**	***	143.4	**<0001**	***
T. gasterostei	abundance	70.1	**<0.001**	***	175.6	**<0.001**	***
Diplostomum spp. (lens)	prevalence	–	–		41.7	**<0.001**	***
Diplostomum spp. (lens)	abundance	–	–		39.3	**0.0002**	**
Diplostomum spp. (non-lens)	prevalence	7.0	0.218	ns	125.8	**<0.001**	***
Diplostomum spp. (non-lens)	abundance	48.0	**<0.001**	***	214.4	**<0.001**	***
Apatemon spp.	prevalence	36.0	**<0.001**	***	103.0	**<0.001**	***
Apatemon spp.	abundance	33.5	**<0.001**	***	96.0	**<0.001**	***
S. solidus	prevalence	3.7	0.597	ns	65.2	**<0.001**	***

*** B.ad. $P < 0.001$; ** B.ad. $P < 0.01$; ns B.ad. $P \geq 0.1$

Table A4. Relationship between dissimilarity of parasite communities, genetic differentiation (pairwise F_{ST} based on microsatellite data), and absolute differences in pH between sampling locations. Dissimilarity of parasite communities is given as 1-Jaccard and 1-Bray–Curtis, and absolute differences in mean abundance for single parasite groups. Separate Mantel tests (5000 permutations) were run for the data of (a) 2011 (14 lakes) and (b) 2010 (6 lakes). Note that *P* values are those from the Mantel tests, but that significance (Sig.) was determined from Bonferroni-adjusted (B.ad.) α values. The significant *P* value is printed in bold.

(a)	F_{ST}			pH				
	r	*P*	Sig.	*r*	*P*	Sig.	% explained by F_{ST}	% explained by pH
1-Jaccard	0.43	**0.007**	*	0.14	0.107	ns	19.8	3.0
1-Bray-Curtis	0.20	0.111	ns	0.12	0.145	ns	4.6	1.8
Gyrodactylus spp.	0.48	0.070	ns	0.02	0.418	ns	23.8	0.1
Apatemon spp.	0.00	0.392	ns	-0.16	0.950	ns	0.0	2.5
Diplostomum spp. (non-lens)	0.30	0.160	ns	-0.16	0.953	ns	8.4	1.8

(b)	F_{ST}			pH				
	r	*P*	Sig.	*r*	*P*	Sig.	% explained by F_{ST}	% explained by pH
1-Jaccard	0.86	0.033	ns	0.20	0.229	ns	73.4	3.0
1-Bray-Curtis	0.35	0.072	ns	-0.05	0.518	ns	12.4	0.4
Gyrodactylus spp.	0.10	0.215	ns	-0.27	0.910	ns	1.1	7.6
Apatemon spp.	-0.14	0.433	ns	0.50	0.092	ns	2.3	25.7
Diplostomum spp. (non-lens)	-0.14	0.410	ns	-0.16	0.480	ns	1.9	2.5

* B.ad. $P < 0.05$; ns B.ad. $P \geq 0.1$

Table A5. Correlation of infection data published in de Roij and MacColl (2012) and infection data obtained in the present study of those lakes that were sampled in both studies (N = 12 lakes). Given are correlation coefficients and P values as resulting from Pearson correlations (r_P) and Spearman rank correlations (r_S). Significance (Sig.) was determined from Bonferroni-adjusted (B.ad.) P values. Significant P values are printed in bold.

		All 12 lakes sampled in both studies							
		2008				2007			
			r	P	Sig.		r	P	Sig.
Diplostomum spp. (non-lens)	prevalence	r_P	0.68	0.015	ns	r_P	0.61	0.037	ns
Diplostomum spp. (non-lens)	abundance	r_S	0.78	**0.003**	*	r_S	0.55	0.064	ns
Apatemon spp.	prevalence	r_S	0.94	**<0.001**	***	r_P	0.73	*0.007*	(*)
Apatemon spp.	abundance	r_S	0.88	**0.0001**	***	r_P	0.83	**0.0008**	**
Gyrodactylus spp.	prevalence	r_P	0.54	0.071	ns	r_S	0.51	0.088	ns
Gyrodactylus spp.	abundance	r_S	0.82	**0.001**	**	r_S	0.30	0.341	ns
S. solidus	prevalence	r_S	0.43	0.168	ns	r_S	0.34	0.283	ns

*** B.ad. $P < 0.001$; ** B.ad. $P < 0.01$; * B.ad. $P < 0.05$; (*) 0.1 > B.ad. $P \geq 0.05$; ns B.ad. $P \geq 0.1$.

Table A6. Results of the regression analyses (Pearson correlations (r_P) or Spearman rank correlations (r_S)) based on infection data from the present study of the lakes sampled in de Roij and MacColl (2012), in 2010 and in 2011. Prevalence (% infected) or mean abundance (number of parasites divided by the number of dissected fish) per lake were correlated with either pH or lake surface area (Area). No significant correlation was found after Bonferroni correction. Only tendency (0.1 > Bonferroni-adjusted $P \geq 0.05$) printed in italics (*Diplostomum* spp. (lens) abundance with pH, 2011).

		pH									Area								
		de Roij and MacColl lakes			2010			2011			de Roij and MacColl lakes			2010			2011		
			r	*P*		*r*	*P*		*r*	*P*		*r*	*P*		*r*	*P*		*r*	*P*
Diplostomum spp. (non-lens)	prevalence	r_P	–0.02	0.959	r_P	0.43	0.393	r_P	–0.31	0.281	r_S	–0.18	0.586	r_P	0.08	0.874	r_S	–0.14	0.642
Diplostomum spp. (non-lens)	abundance	r_S	0.34	0.284	r_S	0.37	0.497	r_S	–0.12	0.675	r_S	–0.27	0.391	r_S	–0.09	0.919	r_S	–0.39	0.170
Apatemon spp.	prevalence	r_P	–0.15	0.641	r_P	–0.86	0.030	r_P	–0.32	0.263	r_S	–0.36	0.246	r_P	–0.42	0.408	r_S	–0.13	0.658
Apatemon spp.	abundance	r_P	–0.10	0.750	r_P	–0.79	0.063	r_S	–0.27	0.346	r_S	–0.31	0.329	r_P	–0.37	0.477	r_S	–0.17	0.573
Diplostomum spp. (lens)[a]	prevalence	r_S	0.50	0.095		–	–	r_S	0.67	0.009	r_S	–0.01	0.980		–	–	r_S	0.23	0.428
Diplostomum spp. (lens)[a]	abundance	r_S	0.50	0.095		–	–	r_S	0.70	*0.006*	r_S	–0.01	0.980		–	–	r_S	0.21	0.474
T. gasterostei[a]	prevalence	r_S	0.40	0.192	r_P	0.06	0.916	r_S	0.55	0.043	r_S	–0.20	0.524	r_P	–0.18	0.729	r_S	–0.09	0.761
T. gasterostei[a]	abundance	r_S	0.37	0.234	r_S	0.17	0.742	r_S	0.57	0.035	r_S	–0.18	0.570	r_S	–0.12	0.827	r_S	–0.08	0.787
Gyrodactylus spp.	prevalence	r_P	0.51	0.088	r_P	0.33	0.521	r_P	0.48	0.086	r_S	0.35	0.270	r_P	–0.11	0.837	r_S	0.10	0.725
Gyrodactylus spp.	abundance	r_S	0.54	0.072	r_P	–0.15	0.777	r_S	0.62	0.017	r_S	0.35	0.270	r_P	–0.23	0.664	r_S	0.05	0.863
S. solidus	prevalence	r_S	–0.05	0.875	r_P	0.63	0.177	r_S	–0.09	0.754	r_S	0.45	0.145	r_P	0.57	0.233	r_S	0.26	0.372

[a] Not analysed in de Roij and MacColl (2012)

Appendix Chapter III

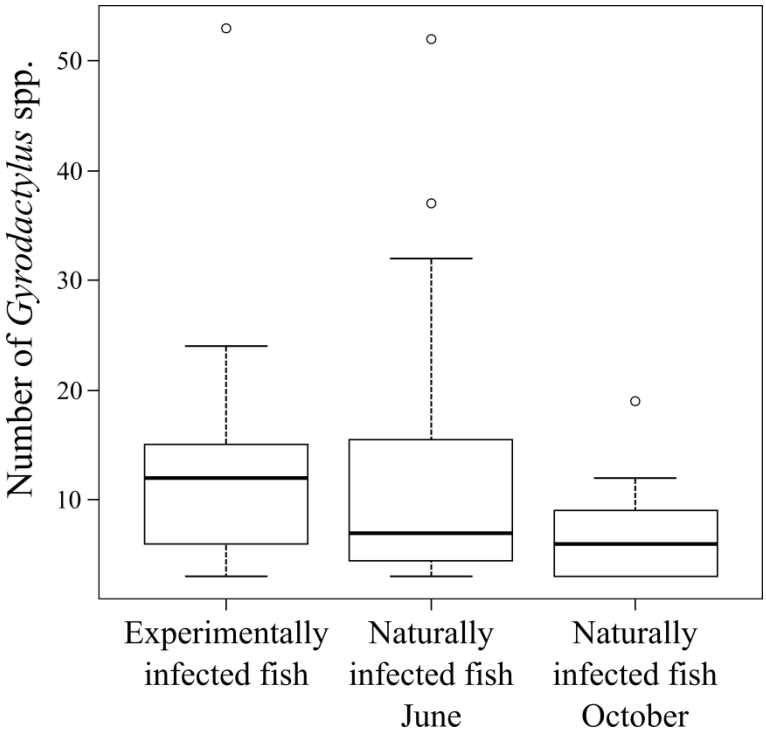


Figure A1. Intensities of *Gyrodactylus* infections on experimentally infected focal fish (*N* = 17) and naturally infected sticklebacks caught in June (*N* = 31) and October (*N* = 13) 2010 shown as median, quartiles, 1.5× interquartile range and outliers. Only fish harbouring at least three worms were considered. See text for details and statistics.

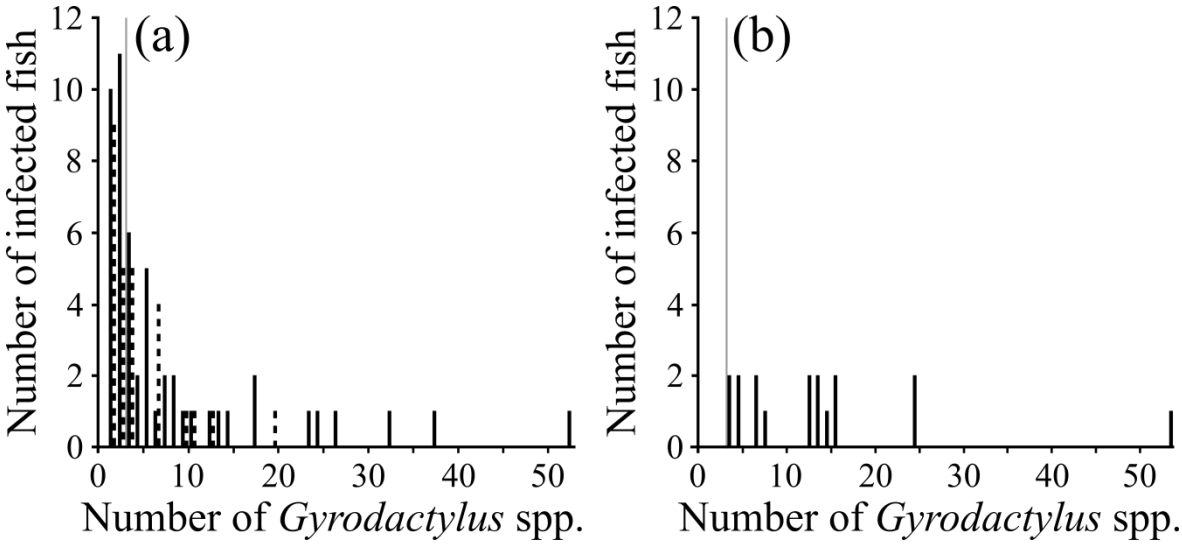


Figure A2. Distribution of *Gyrodactylus* infection intensities among (a) naturally (solid bars June, interrupted bars October) and (b) experimentally infected sticklebacks. Grey line depicts 'three-worms threshold' (see text for details). Note that only data of infected focal, not infected stimulus 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

Appendix Chapter IV

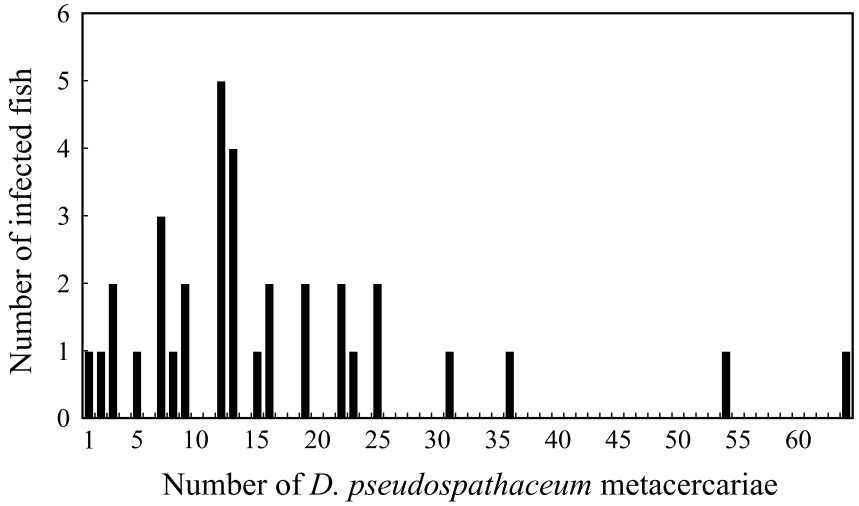


Fig. A1. Distribution of infection intensities (number of eyeflukes per infected individual) of 34 of the 36 fish of the mixed treatment groups. One fish was not dissected and one was free of parasites.

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Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Zitate wurden als solche kenntlich gemacht. Diese oder ähnliche Arbeiten habe ich bei keiner anderen Stelle zur Prüfung vorgelegt.

Bonn, den

_____

Anna Rahn