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DISTINCTIVE SITE PREFERENCE OF THE FISH PARASITE *MYXOBOLUS CEREBRALIS* (CNIDARIA, MYXOZOA) DURING HOST INVASION

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Here, we experimentally studied the site preference of *Myxobolus cerebralis*, one of the most pathogenic myxozoan (Cnidaria, Myxozoa) fish parasites, which causes whirling disease in salmonids. Parasite invasion was examined in three fish species with various susceptibility levels: the type host brown trout, the highly susceptible rainbow trout, and the non-susceptible gibel carp, in which parasite spores do not develop. We investigated the first two hours of fish invasion, and measured the site preference of triactinomyxons (TAMs) during attachment and penetration of fish in three body parts (gills, fins, skin). Infection prevalence and intensity were estimated using a species-specific nested PCR, optimised in the present study. The highest infection prevalence was detected in the most susceptible fish species, rainbow trout. Interestingly, higher prevalence was observed in gibel carp than in the type host, brown trout (95.2% vs. 85.7%). Considering body locations, remarkable differences were detected in infection intensities. The highest intensity was observed in fins, whereas skin was the least infected body part in every fish species examined. Infection prevalence and intensity did not differ significantly among fish species. Thus, we confirmed that *M. cerebralis* TAMs cannot discern fish species. Furthermore, we proved experimentally that fish fin is significantly more attractive to fish-invading parasite TAMs than gills or skin.

Key words: Myxozoans, TAM, host–parasite interaction, gibel carp, rainbow trout, brown trout, whirling disease

Myxozoa represents a diverse group of microscopic-size endoparasites belonging to phylum Cnidaria (Jiménez-Guri et al., 2007; Fiala et al., 2015; Atkinson et al., 2018). *Myxobolus cerebralis* Höfer, 1903 is one of the most pathogenic members of the taxon, responsible for high mortality among salmonids by causing whirling disease (O’Grodnick, 1979; Hoffman, 1990). The complex, two-host life cycle of the parasite includes the definitive host oligochaete *Tubifex tu-*

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bifex (Annelida: Clitellata) invaded by the parasites' myxospore stage, and salmonid fishes (intermediate hosts) that are infected by the actinospore stage of the parasite (Markiw and Wolf, 1983). The triactinomyxon-type actinospores (triac-tinomyxons, TAMs) are a water-borne form of *Myxobolus cerebralis* that leave the oligochaete host along with faeces, and float in the water column waiting for an encounter with the fish host.

Myxobolus cerebralis has a relatively wide host range. The parasite primarily infects salmonids, and causes severe disease in rainbow trout *Oncorhynchus mykiss*, cutthroat trout *O. clarki*, Yellowstone cutthroat trout *O. c. bouvieri*, sockeye salmon *O. nerka* and Danube salmon *Hucho hucho* (Steinbach Elwell et al., 2009). Mild infection is described in brown trout *Salmo trutta* m. *fario*, whereas closely related salmonid species may show different levels of clinical signs (Höfer, 1903; Hoffman and Putz, 1969; El-Matbouli et al., 1992; Hedrick et al., 2001; MacConnell and Vincent, 2002). There are also differences in susceptibility, prevalence, infection intensity and mortality among salmonid species or even strains (Steinbach Elwell et al., 2009). Brown trout, which are native to Europe, are the original host of the parasite, and as a result of the shared evolutionary origin of the parasite and host, brown trout developed an effective immune strategy against *M. cerebralis* (Hoffman, 1970). Whirling disease was not observed in Europe before the introduction of the highly susceptible, non-native rainbow trout from North America (Hedrick et al., 1999; Bartholomew and Reno, 2002). It is known from previous studies that host recognition of *M. cerebralis* is not a fish species-specific reaction (Kallert et al., 2009, 2015). Although cyprinid fishes are not susceptible to *M. cerebralis*, fish-invasive TAMs are capable of penetrating their skin, but mature spores of *M. cerebralis* do not develop (El-Matbouli et al., 1999; Kallert et al., 2009; Sipos et al., 2018).

During host invasion, TAMs attach to the skin of fish and the parasite sporoplasm, which contains the infective germ cells (sporozoites), leaves the TAM with active movement and penetrates the skin of fish through the mucous layer. Fish mucus represents a first barrier against parasites from the environment; however, it plays a significant role in the host recognition process as well. Initially, TAMs require a fish mucus-derived chemical and a mechanical (i.e. active movement of fish) stimulus for host recognition (Kallert et al., 2005, 2011). Early studies suggested that TAMs mainly target the epithelium of buccal cavity, fins, gills, and skin of the host during penetration (El-Matbouli et al., 1992; Yokoyama and Urawa, 1997; Antonio et al., 1999). While available information about the site preference of *M. cerebralis* TAMs to different body parts of fish is rather scarce, it is known that there is no significant difference in the number of actively emerging *M. cerebralis* sporoplasms in the skin of highly and less susceptible strains of rainbow trout and the non-susceptible host common carp, *Cyprinus carpio* (Kallert et al., 2009; Steinbach Elwell et al., 2009).

In the present study, we experimentally studied the early stage of *M. cerebralis* invasion in three fish species with various susceptibility levels (i.e. the original host, brown trout, the highly susceptible rainbow trout and a non-susceptible cyprinid species, gibel carp, *Carassius gibelio*). We used *in vivo* laboratory experiments, and estimated parasite intensity in fish using a semi-quantitative PCR assay, to explore if there was a difference in the site preference of the parasite among the fish species.

Materials and methods

Source and maintenance of parasite and fish hosts

The *M. cerebralis* used for experimental exposure originated from the life cycle maintained in the laboratory of the Institute for Veterinary Medical Research, Budapest, Hungary since 2007, as described by Eszterbauer et al. (2015). The parasite spores were regularly checked by microscopy and DNA sequencing for contamination by other myxozoans (which were never detected). Rainbow trout (Kamloops strain) and brown trout were obtained from the Lillafüred Trout Hatchery at Lillafüred, Hungary. Trout fry were kept in a parasite-free environment in the hatchery, and transported to the laboratory about a week after hatching, in 'yolk-sac stage'. Prior to exposure, fish were kept in aerated, water flow-through aquaria at 15 °C. The fertilised eggs of gibel carp were sourced from a cyprinid fish farm at Hortobágy, Hungary, and they were transported to the laboratory before hatching. Fish were reared under parasite-free laboratory conditions in aerated aquaria at 15 °C for salmonids and at 20 ± 2 °C for gibel carp. All fish species were fed with commercial fish pellets (Aller Aqua, Denmark) *ad libitum*. For the experimental exposure, 7–8 weeks old brown and rainbow trout (7–7.5 cm long), and 1-year-old gibel carp specimens (5.5–7 cm long) were used.

Experimental exposure

Parasite TAMs were harvested by filtering the water from the culture containers through a 20-µm nylon mesh as described previously (Forró and Eszterbauer, 2016). TAMs used for the infection trials were < 2 days old. Naïve fish were exposed individually to 10,000 freshly filtered TAMs/fish in 500 ml dechlorinated tap water for 2 h at 15 °C. Twenty-one fish of each species were exposed, and the same number of non-exposed control fish were kept under identical conditions without the addition of TAMs. Samples were taken 2 h post exposure (p.e.). During sample collection, fish were first anaesthetised with 200 mg/l tricaine methanesulphonate (MS222, Sigma, Germany), and killed by cranial cut, per a protocol approved by the National Scientific Ethical Committee on Animal Experimentation (Hungary), which provided full approval for the animal experi-

ment (No. XIV-I-001/1326-4/2012 and PEI/001/4087-4/2015). From every fish, two whole gill arches and approximately 1 cm² of skin (without muscle tissue) and 1 cm² of caudal fin were sampled. The weight of samples was measured and recorded. The mean weight of sampled gills, skin and fin was 69.10 ± 12.57, 50.54 ± 13.05 and 67.83 ± 15.98, respectively (mean weight ± SD). The samples were stored at -20 °C until further molecular use.

Isolation of DNA and nested PCR analysis

Skin, gill and fin samples of all examined fish species were homogenised manually with a polypropylene micropestle (ThermoFisher Scientific, USA) in a 1.5-ml microcentrifuge tube. DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. The amount and integrity of extracted genomic DNA were checked with 1% agarose gel electrophoresis in 0.5× TAE buffer, and with a NanoDrop 2000c spectrophotometer (ThermoFisher Scientific, USA).

An *M. cerebralis*-specific nested PCR assay was used to amplify a fragment of the parasite's 18S rDNA. The primer combination was chosen and optimised in the present study. The nested PCR profile comprised an initial denaturation at 94 °C for 10 min, followed by 35 cycles of denaturation (30 s at 94 °C), annealing (30 s at 56 °C), and elongation (50 s at 72 °C), then a final elongation step at 72 °C for 5 min. The total volume of PCR mixture was 25 µl, which contained 1× *Taq* buffer with KCl (ThermoFisher Scientific, USA), 0.25 µM of both forward and reverse primers (IDT, Belgium), 0.2 µM dNTPs (Sigma, Germany), 1.5 mM MgCl₂ (Thermo Scientific, USA), 1.25 U recombinant *Taq* DNA polymerase (ThermoFisher Scientific, USA), nuclease-free water, and approximately 50 ng template DNA (all DNA samples were diluted to the same concentration prior to PCR). In the first round of the nested PCR, primers were Tr5-17 (5'-GCC CTA TTA ACT AGT TGG TAG TAT AGA AGC-3'; Andree et al., 1998) and Myx18-996R (5'-GCG GTC TGG GCA AAT GC-3'; Kelley et al., 2004). In the second round, primers Tr3-17 (5'-GGC ACA CTA CTC CAA CAC TGA ATT TG-3'; Andree et al., 1998) and Myx18-909f-R (5'-TGC TGT AAC TGA ATA ACA TTC AGT CAA AG-3', reverse complement of the one designed by Kelley et al., 2004) were used. Amplicon size was 783 bp and 245 bp, respectively. PCR products were visualised on 2.0% agarose gels in 0.5× TAE buffer.

Semi-quantification of parasite amount was based on fluorescence intensity of parasite PCR amplicon compared with a known amount of DNA ladder (GeneRuler 100 bp Plus DNA ladder, ThermoFisher Scientific, USA). We scored samples as one of four categories: (-) no visible product either in the first- or in the second-round PCR; (+) no visible product in the first round, second round weak positive; (++) first round weak positive, second round strong positive, and (+++) strong positivity in both rounds.

Statistical analysis

The prevalence and intensity of infection were calculated from molecular data, and the obtained values were statistically analysed with R (version 3.4.4; R Core Team, 2018). Fisher's exact test was used to analyse differences in parasite occurrence in different body locations. The Kruskal–Wallis H test was applied to compare differences in infection intensity among host species. If a significant difference was detected, pairwise comparisons were implemented with the Mann–Whitney U test. For different body locations (i.e. skin, gill, and fin), the Friedman test was used, and the Mann–Whitney U test applied for pairwise comparisons. The P values were corrected by the Bonferroni–Holm method. The level of significance was determined at $P < 0.05$, and marginal significance was identified in cases when the P value was between 0.05 and 0.1.

Results

The prevalence of *M. cerebralis* infection was between 85.7–100%, which confirmed the high efficiency of the experimental infection regime. The highest prevalence was detected in the most susceptible fish species, rainbow trout. Interestingly, gibel carp had 95.2% prevalence, a higher value than in the type host, brown trout (85.7%) (Table 1). The occurrence of parasite was significantly different among body locations for every fish species; highest occurrence was found in fins, and lowest in skin (Table 1, Fig. 1). We found no significant difference between fish species; however, occurrence in skin was borderline significant ($P = 0.059$), with the lowest parasite occurrence in brown trout. Control fish were all PCR negative.

The infection intensity varied slightly among fish species (Fig. 2), and the intensity differences among species correlated with the trend of prevalence (regarding fish species). The highest intensity was detected in rainbow trout, and the lowest in brown trout (Fig. 1 sum). Most of these relations were, however, not significantly different. Using pairwise comparison, we found significant disparity in one case, in the infection intensity in fin samples between brown trout and rainbow trout ($P = 0.002$). Furthermore, marginal significance was observed between the skin samples of brown trout and rainbow trout ($P = 0.059$) and between the fin samples of gibel carp and rainbow trout ($P = 0.061$) (Table 2). Considering body locations, significant differences in infection intensities were detected in most cases (Table 3). The highest intensity was detected in fins, while skin was the least infected body part in every fish species examined (Fig. 1). Gill infection showed average intensity among the three examined body parts (Fig. 2 sum). Infection intensity in fin was significantly higher than that in skin, for every fish species. Fin infection was significantly higher than that of the gills in

rainbow trout and gibel carp, whereas gill was significantly more intensively infected than skin in brown trout (Table 3).

Table 1

Prevalence, parasite occurrence and intensity parameters of the early stage of *Myxobolus cerebralis* infection among the examined fish species 2 h post exposure. Intensity categories: (+) first round of nested PCR negative, second round weak positive; (++) first round weak positive, second round strong positive, and (+++) strong positivity in both rounds

Fish species	Body location	No. of infected/ exposed fish	Prevalence (%)	Occurrence per body part (%)	Infection intensity per category (%)		
					+	++	+++
Brown trout	fin	18/21	85.7	76.2	37.5	56.3	6.3
	gill			61.9	23.1	69.2	7.7
	skin			14.3	33.3	66.7	0.0
Rainbow trout	fin	21/21	100	95.0	5.3	52.6	42.1
	gill			61.9	61.5	38.5	0.0
	skin			47.6	20.0	80.0	0.0
Gibel carp	fin	20/21	95.2	90.5	26.3	57.9	15.8
	gill			52.4	63.6	36.4	0.0
	skin			38.1	62.5	37.5	0.0
All fish	fin	59/63	93.6	87.1	22.2	55.6	22.2
	gill			58.7	48.6	48.6	2.7
	skin			30.2	38.1	61.9	0.0

Discussion

The severity of whirling disease in salmonids depends on the age and size of fish when first exposed to *M. cerebralis* (Steinbach Elwell et al., 2009). The susceptibility of salmonids declines considerably after the age of 9 weeks (Ryce et al., 2004). Water temperature and infection dose are also important factors (O'Grodnick, 1979; Ryce et al., 2004; Steinbach Elwell et al., 2009). The viability of TAMs is strongly water temperature dependent, and above 20 °C, there is a remarkable drop in the viability and infectivity of TAMs (Steinbach Elwell et al., 2009; Kallert et al., 2015). Therefore all of these factors were considered in the experimental setup.

In the present study, we investigated the first two hours of fish invasion, i.e. the early phase of *M. cerebralis* infection, and measured the site preference of TAMs during attachment and penetration. Up to 100% infection prevalence was observed in the experimental exposure, which was likely due to the optimised exposure protocol developed in the Fish Parasitology Laboratory of the

Institute for Veterinary Medical Research, Hungary (Eszterbauer et al., 2009, 2015; Forró and Eszterbauer, 2016; Sipos et al., 2018), and the relatively high dose of parasite used for fish exposure compared to natural systems, where the prevalence of myxozoan infection is usually lower (Beauchamp et al., 2002; DuBey and Caldwell, 2004; Hallett et al., 2012). Hedrick et al. (1999) examined the susceptibility of brown trout and rainbow trout to whirling disease in a dose-dependent context, by exposing age-matched groups of different trout species in parallel with 10, 100, 1,000 and 10,000 TAMs per fish for 2 hours. In our study, we used a high dose of parasite (i.e. 10,000 TAMs per fish) to be able to examine the earliest stage of parasite development in fish, where microscopic detection cannot be used for evaluation. In this case, the only sufficient method is DNA-based molecular detection that requires a higher dose of exposure for proper comparison of samples (Sipos et al., 2018).

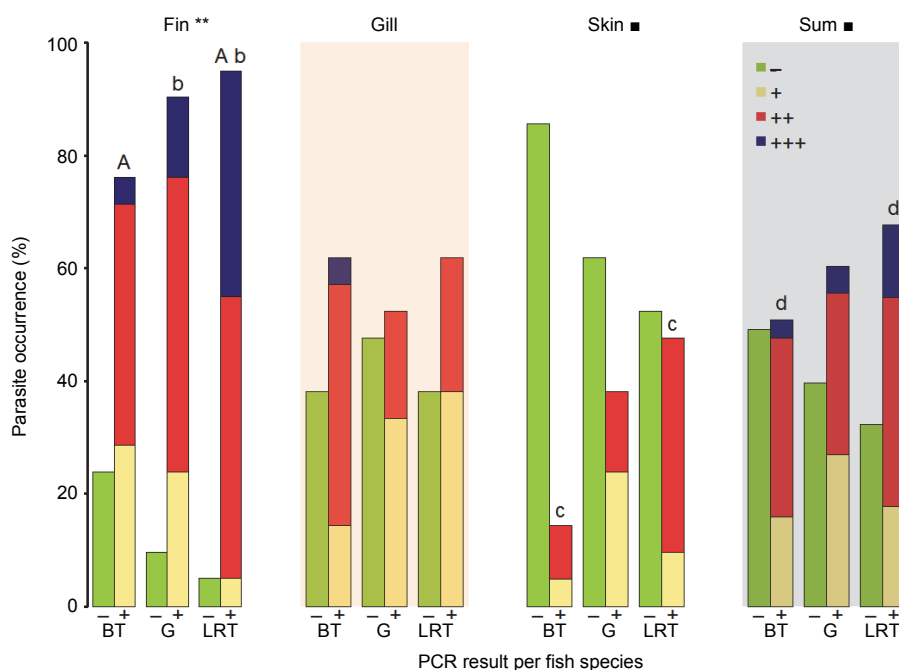


Fig. 1. Comparison of *Myxobolus cerebralis* infection intensity among fish species examined. Semi-quantification of infection intensity amount was performed on the basis of the intensity of parasite-specific PCR product. The following four categories were determined: (-/green) negative; (+/yellow) first round negative, second round weak positive; (+/red) first round weak positive, second round strong positive, and (+++/blue) strong positivity in both rounds. BT = brown trout; G = gibel carp; LRT = rainbow trout. Level of significance with Kruskal–Wallis test: *corrected P value between 0.05–0.01; **0.01–0.001; ***< 0.001. Marginal significance: ■ P value between 0.1–0.05. Significant differences between species (obtained with pairwise comparison) are indicated with upper-case letters, marginally significant differences are indicated with lower-case letters

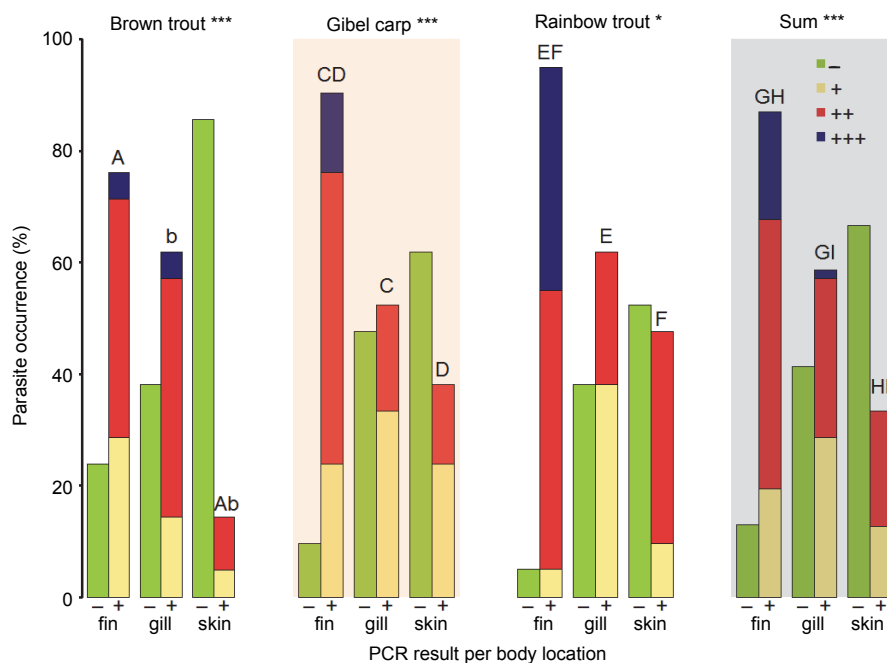


Fig. 2. Comparison of *Myxobolus cerebralis* infection intensity among body locations examined. Semi-quantification of *M. cerebralis* infection intensity amount was performed on the basis of the intensity of parasite-specific PCR product. The following four categories were determined: (-/green) negative; (+/yellow) first round negative, second round weak positive; (+/+/red) first round weak positive, second round strong positive, and (+++/blue) strong positivity in both rounds. Level of significance with Friedman test: *corrected P value between 0.05–0.01; **0.01–0.001; *** < 0.001. Marginal significance: ▣ P value between 0.1–0.05. Significant differences between body locations (obtained with pairwise comparison) are indicated with upper-case letters, marginally significant differences are indicated with lower-case letters

Table 2

Corrected P values of pairwise comparison of *Myxobolus cerebralis* infection intensity among fish species obtained with Mann–Whitney U test. Significant difference is highlighted in bold. Marginal significance is labelled in italics

	Brown trout – Gibel carp	Brown trout – Rainbow trout	Gibel carp – Rainbow trout
Fin	0.130	0.002	<i>0.061</i>
Gill	0.500	0.715	0.715
Skin	0.226	<i>0.059</i>	0.288
Sum (all sites)	0.536	0.081	0.173

Quantitative real-time PCR (qPCR) methods have become a valuable tool to estimate infection and parasite load of *M. cerebralis* (Cavender et al., 2004, Kelley et al., 2004; Sipos et al., 2018). Nonetheless, we applied an *M. cerebralis*-

specific nested PCR assay to amplify a fragment of the 18S rDNA instead of using qPCR, based on pilot comparisons. We tested both detection methods (i.e. the species-specific qPCR by Sipos et al., 2018, and the nested PCR assay developed in the present study), and we found that the nested PCR had similar sensitivity of parasite detection compared to qPCR (supplementary data are available from the corresponding author upon request). Importantly, PCR had an advantage over qPCR as it did not require normalisation using reference genes (i.e. internal controls), which are different between the three fish hosts, thus qPCR normalisation is not equally sensitive to salmonids and cyprinids, therefore the relative quantification using qPCR would have distorted data. In order to compare parasite DNA amounts in different organs from various fish species, sample normalisation was done in two steps. First, similar-sized tissue samples were collected, then an equal amount of extracted DNA was used for the nested PCR. Therefore, nested PCR was an appropriate method for a semi-quantitative comparison of *M. cerebralis* invasion in salmonid and cyprinid hosts.

Table 3

Corrected P values of pairwise comparison of *Myxobolus cerebralis* infection intensity among body parts of fish obtained with Mann–Whitney U test. Significant difference is highlighted in bold

	Fin – Gill	Fin – Skin	Fin – Skin
Brown trout	0.656	0.002	0.018
Rainbow trout	0.001	0.002	0.935
Gibel carp	0.006	0.001	0.463
Sum (all fish)	< 0.001	< 0.001	0.031

Kallert et al. (2009) examined the penetration of *M. cerebralis* TAMs into susceptible (rainbow trout) and non-susceptible (common carp) species. They proved that there is no significant difference in the amount of penetrating *M. cerebralis* sporozoites between the highly susceptible rainbow trout strains and the non-susceptible common carp. Their study focused on the first three minutes of host invasion (as fish were exposed to *M. cerebralis* for 3 min, then samples were taken immediately). In our study, the aim was to examine the putative difference in case of a prolonged parasite exposure (up to 2 h), when the activity of fish does not count, and the parasite may have time to reach all body parts of fish. Kallert et al. (2009) found that the gills of common carp were more infected (i.e. higher number of parasite stages were detected) than those of rainbow trout, but the difference was not significant. Although gibel carp in our study had almost the same age as common carp in the experiment by Kallert et al. (2009) (12 months vs. 11 months old), we found the opposite relation in gibel carp, a close relative of common carp. However, the difference observed by us was not signif-

icant either. On the other hand, we found that fin was a significantly more attractive target to fish-invading TAMs than skin or gill. Interestingly, skin was the least-attacked body location, although previous studies based on histology found that skin is the most favoured target of entry by the parasite (El-Matbouli et al., 1995). Kallert et al. (2009) also compared different body parts and found that gill was the most attractive organ, followed by fins, with fewest TAMs were detected in the mucous layer of skin. Here, we studied the host invasion 2 h p.e. (i.e. up to 2 h after penetration), and we detected a significantly higher amount of parasite in fins than in skin or even gills. This might be due to the timing of infection, and the physiological differences among the examined body parts. Fish fins, especially the caudal fin, are usually in motion, and thereby they create more intensive mechanical stimuli for the parasite, with a higher chance to get into contact with water-floating TAMs. The constant water flow across in the gills also produces a strong mechanical stimulus that makes them an attractive target for TAMs. By these criteria, skin, including the mucous outer layer of fish, however, has less active movement, and it has a relative large surface compared to fins or gills (thereby the relative number of parasites per unit area is likely lower). During host invasion, it is likely that in the first few minutes of infection, body parts able to 'produce' more intensive mechanical stimuli (i.e. gills and fins), and those able to contact the parasite more easily and in higher number are preferred. For extended exposure (i.e. 2 h in our study vs. 3 min for Kallert et al., 2009), there may be more time for the parasite to reach 'less active' body parts as well and equilibrate the dose across multiple surfaces.

In the present study, we confirmed the findings of previous studies (e.g., Kallert et al., 2009; Sipos et al., 2018) that *M. cerebralis* TAMs cannot discern fish species and strains, as infection prevalence and intensity did not differ significantly among the examined fish species, no matter how susceptible they were to the parasite. However, we showed that fish fin is significantly more attractive to fish-invading parasite TAMs than gills or skin. We found evidence for the site selection of TAM sporozoites, which is thereby the first experimental proof for the distinctive site preference of *M. cerebralis*.

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