

University of South Bohemia in České Budějovice

Faculty of Science

Department of Parasitology



Master thesis

**The effect of direct predation by fish
on cercarial populations of selected
trematode species (Digenea)**

Bc. Pavla Heinclová

Supervisor: Miroslava Soldánová, Ph.D.

Consultant: Ana Born-Torrijos, Ph.D.

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Annotation

The predation effects of four fish species on the consumption of free-living cercariae of three trematode species (*Plagiorchis elegans*, *Echinoparyphium aconiatum* and *Trichobilharzia szidati*) were experimentally investigated with regard to specific life-history traits of both predator and parasite. The study provides evidence of a considerable consumption of trematode cercariae by fish and confirms that cercariae play an important ecological role in the energy flow in aquatic food webs.

Declaration

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1. INTRODUCTION

1. 1. The role of parasites in food webs

Parasites are equally or even more diverse group as free-living organisms. Estimations for parasite species range from one third to over half the diversity on the planet, making parasitism the most successful lifestyle on Earth (Poulin and Morand 2004, Hechinger and Lafferty 2005, Dobson et al. 2008). For a long time, parasites were viewed primarily as important pathogens of men and livestock. However, they are actually important and integral elements in food webs of ecosystems because of their complex life cycles involving different hosts connected through various trophic relationships. Thus, they are more than just ‘blind passengers’ travelling between hosts as they can strongly regulate hosts’ populations and communities at different trophic levels. For example, trophically transmitted parasites that require transmission via predation often manipulate their hosts and modulate predation so that hosts become an easy prey (e.g., Moore 2002, Poulin 2010).

Yet, parasites have been generally largely overlooked in traditional food web ecology. The general assumption was that they are small and thus negligible regarding contribution to the energy flow in ecosystems’ food networks (Marcogliese and Cone 1997, Hudson et al. 2006, Lafferty et al. 2008, Sukhdeo 2010). Recent calls for the inclusion of parasites into food web studies (e.g. Marcogliese and Cone 1997) have led to increased interest in integrating parasites into food web matrix topologies (i.e. the pattern how nodes in a network are linked together) to understand how parasites affect ecosystem properties and species interactions. The parasite inclusion into predator-prey food webs of several ecosystems showed a significant increase of the important network properties, such as linkage density (number of links per species), connectance (total links possible) and complexity, which are pivotal to system stability (e.g. Lafferty et al. 2006a, b, 2008, Hernandez and Sukhdeo 2008, Amundsen et al. 2009, Dunne et al. 2013, Thielges 2013). These studies demonstrated the important role of parasites because they are involved in 75% of the trophic links between species in a food web and have a high potential to affect the structure and functioning of ecological food webs (Lafferty et al. 2006a). Hence, inclusion of parasites is essential to detect the true topological structure of a food web as the large proportion of biodiversity is not underestimated and many trophic links are incorporated. Therefore, there is no doubt that no food web could effectively represent the trophic dynamics of an ecosystem without looking at parasites (Lafferty et al. 2008).

Moreover, one important aspect, previously largely ignored and highlighted by studies mentioned above, suggests that parasites commonly function as a prey within ecosystems. Predation on parasites is common and widespread in food webs of natural aquatic communities, mainly because many parasites are usually transmitted to their host via predation (Johnson et al. 2010). The models on food-web topologies imply that predation of parasites comprise up to 44% of observed links, making predator-prey-parasite interactions one of the most important linkage pathway in food webs (Lafferty et al. 2008, Thieltges et al. 2013). However, these interactions are based on topological food webs in which the energetic aspect in terms of actual strength of trophic interactions is not included.

There are two main routes by which parasites end up as prey. Parasites or their free-living stages can be consumed either directly by a variety of non-host vertebrate and invertebrate organisms, or indirectly (so-called concomitant predation), when parasites are eaten along with their hosts. In both cases, the consumption does not lead to transmission because parasite dies (Johnson et al. 2010, Goedknecht et al. 2012, Thieltges et al. 2013). Predation on parasites may have a profound effect on food web structure, as well as on parasites and predators, with strong ecological and epidemiological consequences. It is considered an important mechanism of so-called dilution effect (the effect of biodiversity – Keesing et al. 2010) leading to the substantial reduction of parasite population, infection levels in the next hosts, transmission and disease risk (Thieltges et al. 2008a, Johnson and Thieltges 2010, Johnson et al. 2010, Goedknecht et al. 2012).

Parasites usually generate tremendous numbers of infective stages, thus creating a great biomass in aquatic ecosystems. Calculations of previously unnoticed parasite biomass provide the first step towards assessing the importance of parasites on energy flow and thus their role in the ecosystems' energetics (Lafferty 2008). Recent studies have shown that total parasite biomass is equivalent or even exceeding the total biomass of the most abundant free-living animal groups in both marine (Kuris et al. 2008) and freshwater ecosystems (Preston et al. 2013). Since parasites occupy a large amount of biomass and are involved in the productivity, thereby energy flow in aquatic ecosystems, they have the potential to affect ecosystem energetics via trophic interactions (Kuris et al. 2008). A small fraction of their biomass may contribute to the diet of higher-level consumers as foraging on parasites can provide significant energetic and nutritional resources (Johnson et al. 2010). Therefore, the considerable biomass may be transferred by consumption of parasites. Accordingly, predation on parasites has been suggested as a substantial path of energy flow throughout ecological networks (Thieltges et al. 2008b, Johnson et al. 2010). A large part of parasite biomass contribution is due to high production of free-living infectious stages of digenetic trematode

cercariae in both marine and freshwater ecosystems (Kuris et al. 2008, Preston et al. 2012, 2013). Furthermore, a number of experimental studies indicate that several organisms can interfere and/or reduce such trematode biomass, thereby decreasing parasite transmission success in aquatic habitats (e.g. Johnson et al. 2010, Goedknecht et al. 2012; see more examples in chapter 1.2.).

1. 2. The life cycle and role of trematode cercariae as prey

Trematodes (Digenea) represent a large cosmopolitan class of parasites within the phylum Platyhelminthes. They are common and abundant in aquatic ecosystems and often significant pathogens of humans and animals. The life cycle of a typical digenean trematode is complex, usually involving two or three different hosts (Galaktionov and Dobrovolskij 2003) (Fig. 1).

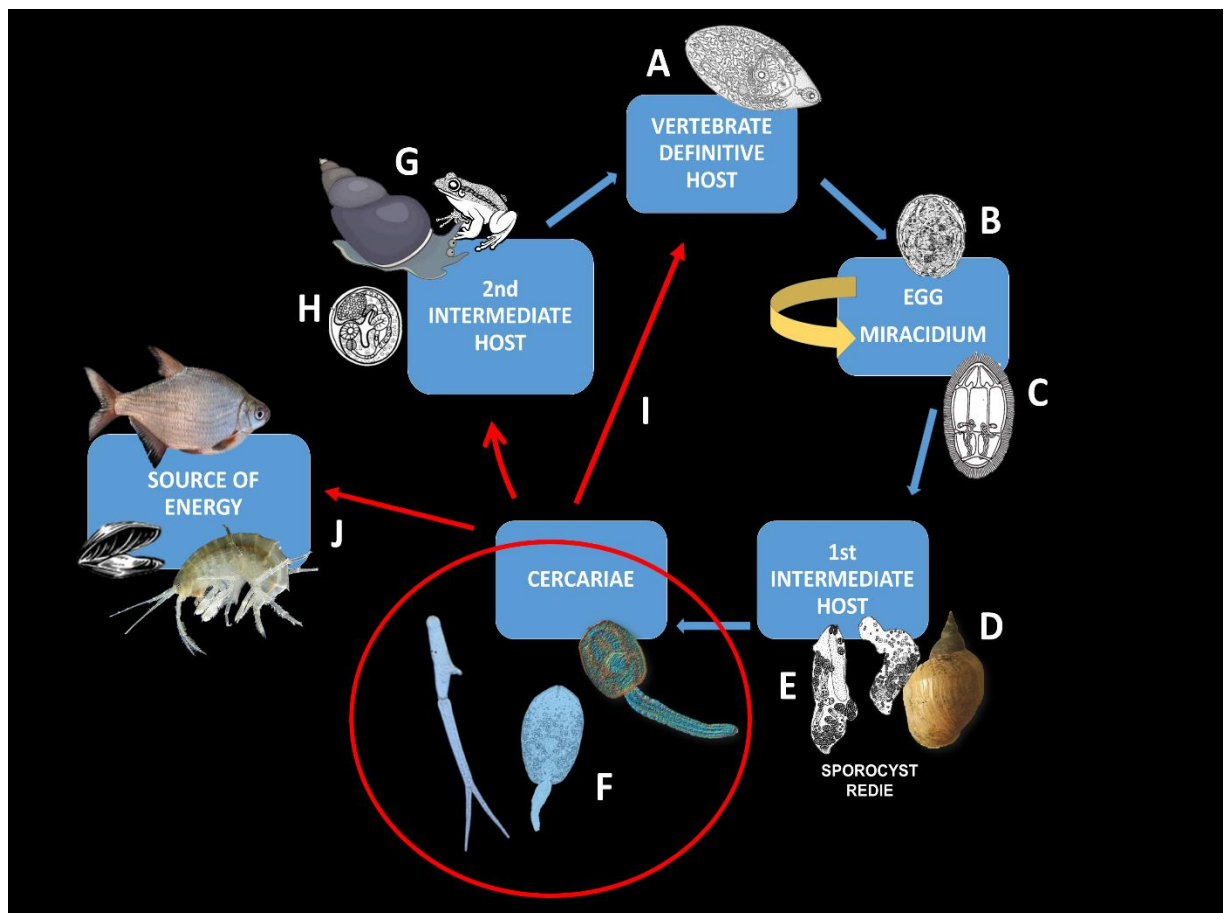


Fig. 1. Life cycle of digenetic trematodes showing primary (A–I) and secondary role (J) of free-living cercariae. A–definitive host. **B**–egg. **C**–miracidium. **D**–first intermediate host (usually molluscs). **E**–sporocyst and/or redia. **F**–cercaria. **G**–second intermediate host. **H**–metacercaria. **I**–direct transmission of cercariae to the definitive host (e.g. family Schistosomatidae; furcocercaria), **J**–direct predation of cercariae by wide range of organisms representing an important source of energy. Drawings of larval stages taken from Našincová (1992) and Galaktionov and Dobrovolskij (2003).

Digenetic trematodes are mainly endoparasites of vertebrates, mostly birds and fish, which act as definitive hosts, i.e. in which trematodes reach sexual maturity (Fig. 1A). Adult trematodes live usually in the host intestine, but they can be found also in other organs depending on the group or species. Adults produce eggs (Fig. 1B), which are released to the external, usually aquatic, environment along with faeces of the definitive host. The first free-swimming larva called miracidium emerges from the egg (Fig. 1C). The first intermediate host is usually gastropod mollusc (Fig. 1D) which becomes infected after eating eggs or, more commonly, being penetrated by miracidia. Within the molluscan host, trematodes undergo asexual reproduction via sporocyst and/or rediae depending on the trematode group (Fig. 1E). The final product of this multiplication is the stage of cercaria (Fig. 1F). Cercariae that are released from molluscs to the external environment must find second intermediate host, which can be both vertebrate and invertebrate hosts, including molluscs (Fig. 1G), in which they encyst as metacercariae (Fig. 1H). However, in some species the life cycle is reduced from three-host cycle to two or even one host (e.g. Combes 2001). In such case, the second intermediate host is bypassed and cercariae either encyst in the external environment (passive infection; e.g. trematodes of the families Fasciolidae or Philophthalmidae) or penetrate a definitive host directly (active infection; e.g. Schistosomatidae, such trematodes of the genus *Trichobilharzia*; furcocercaria in Fig. 1I). Usually, definitive hosts become infected after ingestion of encysted metacercariae in the external environment or the second intermediate host harbouring the metacercariae. In the intestine of the definitive host, several processes are involved such as metacercariae excystment, establishment, habitat location, adult development, mating, and producing and releasing eggs.

Trematode cercariae are planktonic, highly motile stages that actively swim through the water column using a muscular tail in a search for the next host. These free-living infective stages are also non-feeding and therefore short-lived. Their activity is high, but short because they live from accumulated reserves, typically lasting between 24 to 72 hours (Combes 2001, Morely 2012). Energy source for motion of cercariae is glycogen and lipids and resources to each cercaria are portioned between searching for host and longevity (Combes et al. 1994). Cercariae are a crucial stage in the trematodes' life cycle as their principal functional role is to disperse in the external environment and infect as many hosts as possible in order to enhance the probability of the life cycle completion (Morley 2012) (Fig. 1G, I). However, because they are affected by various biotic and abiotic environmental factors, only a small fraction of cercarial population reaches its target hosts (e.g. Pietrock and Marcogliese 2003, Thieltges et al. 2008a, Morley 2012). Part of remaining cercarial population play their secondary ecological role within aquatic ecosystems as an important

source of energy flow through food webs by predation (Fig. 1J) (Thieltges et al. 2008 a, b, Johnson et al. 2010, Goedknecht et al. 2012, Morley 2012).

Due to the glycogen and lipid content along with soft tissues of cercariae, cercariae embody a highly nutritious source of food for a wide variety of vertebrate and invertebrate predators (e.g. bivalves, crustaceans, juvenile fish or insects) (Ginetsinskaya 1960, Johnson et al. 2010). As any other regular members of zooplankton community, such as small crustaceans, cercariae are of a similarly small size (typically 0.2–2 mm), move actively and form abundant populations in aquatic ecosystems (Morely 2012), thereby being similarly vulnerable to predation.

Infected snails can shed thousands to millions of cercariae each day (e.g. Hass 2003, Karvonen et al. 2004, Kaplan 2009, Morley 2012, Soldánová et al. 2016). Recent studies demonstrate the importance of ecological role of cercariae despite their small size, because such productivity represents a considerable fraction of biomass in marine, estuarine and freshwater ecosystems (Kuris et al. 2008, Thieltges et al. 2008b, Preston et al. 2012, 2013). At the host level, cercarial biomass equals and often exceeds the host's own body weight during the lifetime of an infected snail (Soldánová et al. 2016). The typically high numbers of cercariae emerging from infected first intermediate hosts into the aquatic environment indicate that the energetic benefit for predators may be substantial. For example, it has been shown in laboratory experiments that estuarine fishes readily consume trematode cercariae and the annual production of cercariae could provide up to 2–3% of their energetic needs (Kaplan et al. 2009).

Transmission processes often depend on the presence of non-host organisms, such predators and decoy organisms that impede free-living stages in many ways (in both cases parasites are lost as they die), but also alternative hosts, in which parasites successfully develop (Thieltges et al. 2008a, Johnson and Thieltges 2010). All types of hosts act as effective diluters by reducing parasite population, transmission rates in the next host and infection risk. Several organisms in free-living communities have been reported to interfere or successfully consume cercariae as a regular food item, either directly (active consumption) or indirectly (passive accidental consumption) (e.g. Thieltges et al. 2008a, Johnson and Thieltges 2010, Lagrue and Poulin 2015). The passive interference may be caused by filter feeders, e.g. bivalves such as mussels and oysters (e.g. Prinz et al. 2009, Orlofske et al. 2012, Gopko et al. 2017, Vielma et al. 2018) or biotic obstacles acting as physical barriers (e.g. vegetation, shell surface), when cercariae are simply trapped and die (Prinz et al. 2009, Welsh et al. 2014).

Various organisms prey on cercariae directly, such as small fish (Siau et al. 1992, Kaplan et al. 2009, Orlofske 2012, 2015), larval insects (Schotthoefer et al. 2007, Catania et

al. 2016), sea anemones (Mouritsen and Poulin 2003, Hopper et al. 2008, Vielma et al. 2018), large crustaceans (Welsh et al. 2017, 2018) or benthic arthropods (Welsh et al. 2014, Orlofske et al. 2015). Even much smaller zooplanktonic organisms such as rotifers, cladocerans and copepods also frequently consume cercariae (Mironova et al. 2018). The effect of focal biodiversity to dilute infection risk resulted in exclusion of up to 100% cercariae in many of these aquatic host-parasite/prey systems. This suggests that cercariae may be a significant energy source for some predators. Direct predation on cercariae, thereby parasite removal, is known to be affected by body size of both hosts and trematodes (Siau et al. 1992, Kaplan et al. 2009, Orlofske et al. 2012, 2015, Catania et al. 2016, Welsh et al. 2018), light availability (Orlofske et al. 2015), presence of alternative hosts (Orlofske et al. 2015, Shoteoffer et al. 2017, Welsh et al. 2017) or temperature (Goedknecht et al. 2015). Furthermore, it has been shown that cercarial consumption rates can increase with increasing cercarial densities in both marine (Welsh et al. 2017) and freshwater predator-prey systems (Orlofske et al. 2012, 2015). Other species-specific properties of trematode cercariae, i.e. swimming behaviour or patterns in cercarial emergence from their first intermediate snail hosts, probably make some trematodes more vulnerable to predation. However, since cercarial emergence rhythms are usually synchronised with periods of host activity and behaviour, predation would also depend on parasite traits that would have to overlap with specific characteristic of their hosts and predators in space and time (Combes et al. 1994, Lagrue and Poulin 2015).

The increasing evidence of frequent consumption of cercariae indicates that cercariae provide energy for consumers/predators and thereby contribute to energy flow in ecosystems. Nevertheless, the energetic contribution of trematode cercariae to predators' diet remains unknown for most aquatic systems (Goedknecht 2012, Thielges et al. 2008a). The effect of direct predation has been tested experimentally mostly for marine predator-parasite-prey systems (Kaplan et al. 2009, Welsh et al. 2014, 2017, 2018). Some studies consider freshwater system (Siau et al. 1992, Orlofske et al. 2012, 2015, Catania et al. 2016, Gopko et al. 2017, Mironova et al. 2018), but they are limited to Africa, North America and northern Finland, and no studies from central European freshwater systems are available. Furthermore, experimental studies on predation of trematode cercariae by freshwater fish are scarce (Siau et al. 1992, Kaplan et al. 2009), especially when exposed to different cercarial densities (Orlofske et al. 2012, 2015). Most importantly, the functional response of fish predators towards its prey has been assessed for various fish species and benthic invertebrates or zooplankton such as copepods (e.g. Soluk 1993, Murray et al. 2013). However, the effect of increasing densities of trematode cercariae on the consumption of fish has never been tested and remains largely unexplored. Therefore, despite the increasing number of studies on the

effect of non-host organisms on the population of trematode cercariae, more studies are needed from other aquatic ecosystems to unravel and better understand the ecological role of trematode cercariae in natural communities, epidemiological consequences for food web functioning and disease risk.

2. AIMS

- 1) To obtain data on the ability of different fish species to consume free-living stages, the cercariae, of selected trematode species.**

- 2) To evaluate fish ability to predate upon cercariae with respect to changing prey density and determine the type of functional response of fish towards cercariae of selected trematode species.**

- 3) To evaluate the effect of direct predation by different fish species on cercarial populations.**

3. MATERIALS AND METHODS

3. 1. Experimental organisms

3. 1. 1. Fish species

Four species of freshwater fish were used in this study. They were selected based on their activity and selectivity for food items, and common abundance in their natural habitats. The four fish species do not serve as hosts for the trematode species used in this study. Basic aspects of the biology and ecology of these fishes are briefly described below.

Rainbow trout (*Oncorhynchus mykiss* [Walbaum, 1792]) (Fig. 2A)

Family: Salmonidae

Short description: Rainbow trout is usually 60 cm long (maximum 122 cm) with average weight of 0.5–2.3 kg (freshwater adults) and maximum of cc. 25 kg (lake and anadromous forms) (Bristow 1992). It has a characteristic salmon-like shape. Dark spots are clearly visible on the tail fin which is slightly forked. The back is usually a dark olive colour, shading to silvery white on the underside. The body is heavily speckled and there is a pink to red stripe running lengthwise along the fish's sides. Coloration varies with habitat, size, and sexual condition (Bristow 1992).

Distribution: It is native to Kamchatka, Pacific basin of North America to northern Mexico. Rainbow trout was widely introduced for food or sport fishing to cold waters of lacustrine and river systems elsewhere in North America and rest of the world except Antarctica (Page et al. 1991). As a result, rainbow trout is one of the most widely introduced and invasive fish species in the world affecting native fish species by introducing pathogenic diseases, hybridization or competition for food (Stankovic et al. 2015). Rainbow trout is cultured in many countries and stocked intentionally into rivers and lakes mainly for recreational fishery activities. It is the most important freshwater fish in European aquaculture produced in cold-water streams (Baruš and Oliva 1995).

Habitat and biology: Rainbow trout is an anadromous (sea-run), cool- to cold-water fish species that usually returns to fresh water to spawn after living two to three years in the ocean. Anadromous and lake forms may migrate long distances to spawning streams (Kottelat and Freyhof 2007). It occurs in all water bodies but usually not in the water with the temperature above 25°C or ponds with lower oxygen levels (Kottelat and Freyhof 2007). Rainbow trout are benthopelagic predators and carnivores but not exclusively piscivorous.

They feed on a wide variety of aquatic and terrestrial prey including insects, crustaceans, molluscs and small fish. Usually they will eat nearly anything they can capture. They are generally surface feeders but the fish living in large lakes and oceans are bottom feeders and eat molluscs, other fish, squid or amphipods. Juvenile fish in fresh water feed predominately on larvae of aquatic insects but also fish eggs. Rainbow trout feeds predominately during light period of the day with peak at dawn (Mizusawa et al. 2007). It spawns from November until May in the Northern hemisphere and from August to November on the Southern hemisphere (Gall et al. 1992).

In the Czech Republic, rainbow trout is one of the most used salmonid fish in intensive aquaculture. In the second half of the 20th century, many hybrids of rainbow trout with various behaviour and sensitivity to conditions were introduced to Czech Republic (Hanel and Lusk 2005).



Fig. 2. Experimental fish species. A–rainbow trout (*Oncorhynchus mykiss*) (mean length 81 mm). B–roach (*Rutilus rutilus*) (mean length 82 mm). C–white bream (*Blicca bjoerkna*) (mean length 60 mm). D–stone moroko (*Pseudorasbora parva*) (mean length 35 mm). Source: Pavla Heinclová (A–C), Lubomír Hlášek, www.hlasek.com (D).

Roach (*Rutilus rutilus* [Linnaeus, 1758]) (Fig. 2B)

Family: Cyprinidae

Short description: Roach is a small fish with length of 15–35 cm (maximum 45–50 cm) and weight of 0.3–2.1 kg (Hanel and Lusk 2005). Body has a silvery colour with blue and green tones and becomes pale at the belly. The fins are grey and green except the pectoral fins with red colour. Roach can often be recognised by the big red spot in the iris around the pupil (Freyhof and Kottelat 2007).

Distribution: Roach is native to most Europe and western Asia. It is found throughout Europe except for the area around the Mediterranean, and its distribution reaches eastward to Siberia. It is naturally absent in the Iberian Peninsula, Adriatic basin, Italy, Great Britain north of 56° N and Scandinavia north of 69° N. It has several subspecies in eastern Europe and Asia, some with an anadromous lifecycle living around the Caspian and Black Seas (e.g. Freyhof and Kottelat 2007). Roach has been introduced to many countries within and outside Europe mainly for sport fishing (Hanel and Lusk 2005).

Habitat and biology: Roach is a potamodromous fish often found in a wide variety of freshwater habitats, mainly in lowland areas, ranging from stationary to moving waters, and small ponds to large rivers and lakes (Freyhof and Kottelat 2007). It is the most abundant in nutrient-rich waters. It is also known from lowland streams and brackish coastal lagoons. Roach mostly inhabits habitats with rich vegetation (Baruš and Oliva 1995). It swims in shallow waters in schools which forage in the vicinity of vegetation (reed) but also in deeper open water. Juveniles and larvae live in a wide variety of littoral habitats. They are true omnivores, predominately preying on benthic invertebrates, zooplankton, plant materials and detritus. Juvenile roach preys mostly on zooplankton such as cladocerans and copepods, whereas older fish eat wide range of food from plant material (algae and detritus) to benthic invertebrates (small snails, zebra mussels, insect larvae and crustaceans). Roach may shift from littoral to pelagic habitats and between zooplankton and benthic food when abundance of a specific food item is high or for avoidance of predation and/or competition. The feeding cycle of roach is the highest early in the morning or afternoon with decreasing tendency in the evening and night (Pyka 1999). It spawns once a year from April to May (Baruš and Oliva 1995).

Roach belongs to the most widespread fish species in the Czech Republic. It can be found in all types of stagnate and running waters and typically dominates fish stock in water supply reservoirs (Pivnička 1992). It prefers calm rivers in the basins of Labe, Odra and Morava.

White bream (*Blicca bjoerkna* [Linnaeus, 1758]) (Fig. 2C)

Family: Cyprinidae

Short description: White bream is usually 30–55 cm long with usual weight less than 1 kg (maximum up to 2 kg). Body is laterally flattened and high-backed with a slightly undershot mouth. It is a silvery grey coloured but older fish can be bronze-coloured in clear waters. The fins are greyish to black without reddish colour, but with reddish base of paired fins (Giles 1994, Kottelat and Freyhof 2007).

Distribution: The distribution area of white bream includes North, Black and Caspian Sea basins, Mediterranean basin, Ail and Anatolic Black Sea. It is distributed across most of Europe except for Iberian Peninsula, Italy, Crimea, Great Britain (except southeast) and Scandinavia north of 65° N (Kottelat and Freyhof 2007).

Habitat and biology: White bream is potamodromous fish occurring in shallow, warmer lowland lakes and slow-flowing larger rivers. It is very abundant on bottom of sandy rivers and prefers shores with submerged vegetation, roots and gravel bottom where it lives in schools. White bream can feed close to the shore on benthic invertebrates using its mouth to dig for chironomid larvae, *Tubifex* worms, bivalves and gastropods. It feeds also on water plants and plankton in open water column, pelagic crustaceans and cladocerans being the main prey. Juvenile fish feed on larvae as well. In very turbid waters, white bream can occur in large numbers, which may result in a shortage of bottom-living prey. It is then forced to live by filter feeding with their gill rakers (Kottelat and Freyhof 2007, Giles 1994). White bream is mainly diurnal, feeding during the day with resting during the night (Říha et al. 2015). It spawns once a year from April to May (Baruš and Oliva 1995).

In the Czech Republic, the occurrence of white bream is abundant and common in rivers (typically Podyjí, Labe, Odra and Morava), ponds, artificial lakes, disconnected river branches and pools. Even it is a common fish with high abundance, its output is negligible (Baruš and Oliva 1995).

Stone moroko (*Pseudorasbora parva* [Temminck & Schlegel, 1846]) (Fig. 2D)

Family: Cyprinidae

Short description: Stone moroko is a small fish with common size of 3–7 cm in length (maximum of 11 cm) and weight 17–19 g. Life span is around 3-4 years. The head is more flattened in its anterior part. The mouth has top position. The dorsal and anal fins are short with big and deeply incised caudal fin; both parts have similar size. The ventral fins are positioned more anterior to the dorsal fin. Lateral line is complete, running in the middle of sides. The colouration is not different between sexes, with grey back, lighter colours on sides

and belly, passing from greenish to silver. Young individuals are typical with a dark stripe which disappears with age.

Distribution: The native range was East Asia including the basins of the rivers Amur, Yang-tze, Huang-ho, Japanese islands, western and southern parts of the Korean Peninsula and Taiwan. Stone moroko was introduced to Europe with stocking material of herbivorous fishes (*Ctenopharynx godonidella*, *Aristichthys nobilis*, *Hypophthalmichthys molitrix*) imported from China (Witkowski 2011). The first report of stone moroko in Europe comes from Romania in 1960 and the fish has spread quickly and colonised almost entirely colonized Europe over last decades.

Habitat and biology: Stone moroko is benthopelagic fish, which inhabits wide variety of habitats, most abundantly still waters such as ponds with carps, canals, ditches and shallow lakes (Kottelat and Freyhof 2007). It prefers littoral areas with abundant submerged vegetation (e.g. Kapusta et al. 2008). Stone moroko is typical plankton-eater but feeds also on fish, fish eggs, small insects and plant material. Juvenile fish feed predominately on zooplankton (Boltachev et al. 2006). It feeds in both the daytime and nighttime with highest feeding in the morning, decreasing in the middle of the day and increasing in the afternoon (Sunardi et al. 2005).

Stone moroko represents potential threat to aquatic biodiversity. Due its high reproductive rate (3–4 times in a season), it is considered as pest competing with the fry of other species (Welcomme 1988). It becomes numerically dominant in a very short time and in high densities can modify the structure of the communities of aquatic invertebrates and indigenous fish species by depleting available food, causing increased mortality or inhibiting their spawning (Witkowski 2011). Stone moroko is considered as one of the most successful invasive species in Europe mainly because of its wide tolerance of environmental conditions, reaching sexual maturity in the first year of life and batch spawning (e.g. Pollux and Korosi 2006).

In the Czech Republic, stone moroko is an invasive species that was introduced between 1981–1982 with shipping freshwater fingerlings and spread quickly in Czech waters (Hanel and Lusk 2005).

3. 1. 2. Trematode species (cercariae)

Cercariae of three trematodes were used in this study. They were selected based on their specific-species traits such as body size, morphotype, behaviour and swimming in water column, transmission mode and patterns in emergence from their first intermediate hosts-molluscs. The cercariae are morphologically distinct making them easy to distinguish and identify. Basic aspects of specific traits of each trematode species are briefly described below.

Plagiorchis elegans (Rudolphi, 1802) (Fig. 3A–B)

Family: Plagiorchiidae Lühe, 1901

Species of the genus *Plagiorchis* have a three-host life-cycle using lymnaeid snails as first intermediate hosts, aquatic insects or amphipods as second intermediate hosts, and various birds and mammals, accidentally amphibians and reptiles, as definitive hosts (Galaktionov and Dobrovolskij 2003). The xiphidiocercariae consist of the body with a stylet and simple monocercous tail (i.e. without furcae) without finfolds (Fig. 3E). The typical characteristic is the stylet possessing anterior thickening (Fig. 3F), the ventral sucker distinctly smaller than the oral sucker and penetration gland-cells in two groups of 7–8 on each side of the body. *Plagiorchis elegans* is ca. 300–420 µm large and has typically 40–50 refractile spherical granules scattered throughout the body (Zikmundová et al. 2014). Cercariae of *P. elegans* are both negatively phototropic and negatively geotropic. Upon emergence from the snail, cercariae disperse passively and swim with occasional short resting on the bottom, which is resumed quickly by rapid movement. After several hours, swimming is ceased and cercariae crawl on the substrate using suckers (Gorman 1980, Lowenberger and Rau 1994). They exhibit rather slow swimming that maintains their position in the water column (Lowenberger and Rau 1994). Several thousands of cercariae (ca. 1,500–3,600) can be released every day and the emergence is nocturnal (i.e. higher numbers of cercariae emitted during nighttime) (Gorman 1980).

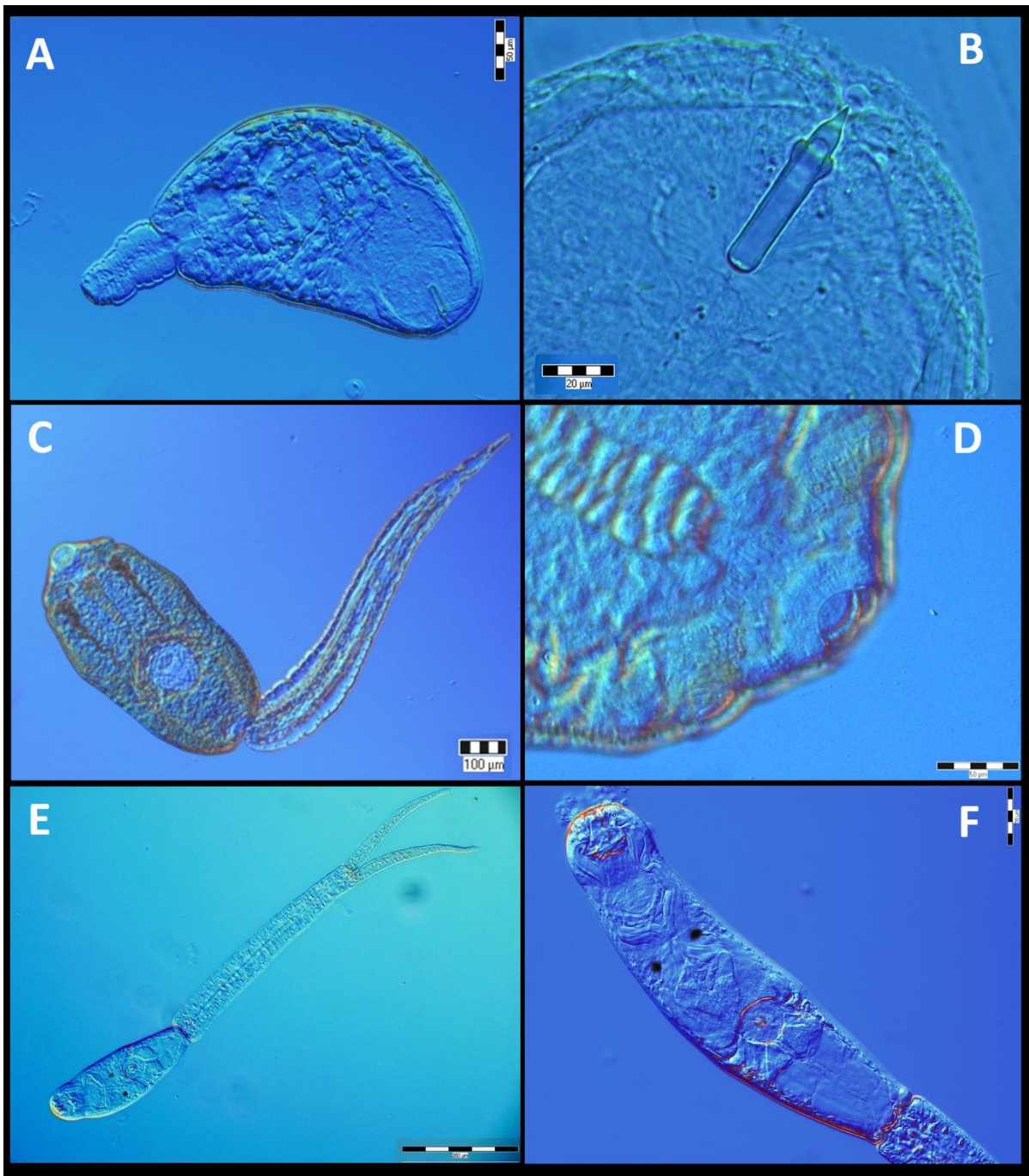


Fig. 3. Photomicrographs of live cercariae of experimental trematode species.

A–*Plagiorchis elegans*, xiphidiocercaria showing body (ventral view) (300–420 μm).

B–stylet. **C**–*Echinoparyphium aconiatum*, echinostome cercaria showing body (ventral view) (size 500–600 μm).

D–Head collar, dorsal collar spines (dorsal view). **E**–*Trichobilharzia szidati*, ocellate furcocercaria showing body (ventral view) (size 900 μm).

F–Head showing apical organ, pigmented eye-spots and penetration glands.

Scale bars: A, D, F, 50 μm ; B, 20 μm ; C, 100 μm ; E, 200 μm .

Source: Pavla Heinclová and Miroslava Soldánová.

Echinoparyphium aconiatum Dietz, 1909 (Fig. 3C–D)

Family: Echinostomatidae Looss, 1899

Echinoparyphium aconiatum has a three-host life-cycle using snails as first and second intermediate hosts, and snail-eating birds as definitive hosts (Kanev 1982). The echinostome cercariae are large *ca.* 500–600 µm in length, consisting of the body with a simple monocercous tail without finfolds (Fig. 3A) and 37 collar spines in the anterior part of the body (Fig. 3B) (Faltýnková et al. 2007). As other echinostome cercariae, those of *E. aconiatum* are typical by continuous swimming and the speed of swimming depends predominately on the size of the body and temperature. Larger echinostome cercariae swim much faster than smaller species (see review of Haas 2000). Cercariae of *E. aconiatum* show positive photo-taxis and negative geo-taxis in an initial period after being released from an infected snail (Haas 1994). The swimming and geo-orientation of *E. aconiatum* cercariae in water column are affected mainly by light intensity (low vs high) making them to swim in upward or downward direction. Such change in orientation is due to selection of habitats of snails at the bottom of waters when larval stages actively increase the chance to encounter their hosts (Haas 2003). In general, cercariae of *E. aconiatum* occur close to the surface in early stage after emergence, but later predominately near the bottom, especially when exposing to the bright light. Most of the species of the family Echinostomatidae show diurnal patterns in cercarial emergence (i.e. higher numbers of cercariae emitted during daytime) (Haas 2000). Several hundred to thousands of *E. aconiatum* cercariae can be released every day from a single infected snail (*ca.* 400–4,800 cercariae; Šarounová 2011).

Trichobilharzia szidati Neuhaus, 1952 (Fig. 3E–F)

Family: Schistosomatidae Stiles & Hassall, 1898

Species of the genus *Trichobilharzia* utilise a two-host life cycle including snails and birds with direct penetration of the definitive host. Cercariae of *Trichobilharzia* spp. are the main causative agents of swimmer's itch in Europe (Soldánová et al. 2013). The furcocercous cercariae (i.e. bifurcated tail) are large *ca.* 900 µm on average (Holická 2009) (Fig. 3C). The typical characteristics are two pigmented eye-spots in the anterior part of the body, two pairs of circumacetabular penetration glands surrounding the acetabulum and three pairs of postacetabular penetration glands in the posterior part of the body (Fig. 3D). The furcocercariae exhibit negative geotaxy and positive phototaxy with concentration of cercariae beneath the water surface, where the definitive hosts may occur (Horák et al. 2015). The behaviour of cercariae in water column is complex involving repeating movements in cycles. Their swimming is intermittent with alternation of resting phase and active swimming.

They have a tendency of attaching to substrates and their swimming behaviour is strongly regulated by light. Moving shadows trigger swimming away from the source of light to deeper water levels where cercariae can encounter and penetrate legs of definitive hosts, the ducks. The cercariae are released in several thousand every day from a single infected snail (ca. 2,500–29,500) and the emergence is diurnal (Soldánová et al. 2016).

3. 2. Animal collection and maintenance

Juvenile fish of two species, roach (*Rutilus rutilus*; mean size 82 mm) and white bream (*Blicca bjoerkna*; 60 mm) were provided from the Institute of Hydrobiology (Department of Fish and Zooplankton Ecology), Biology Centre CAS, České Budějovice, Czech Republic. Fish were sampled from Římov Reservoir (48°51'00"N, 14°29'29"E) in South Bohemia in April and May 2017. Commercially raised rainbow trout (*Oncorhynchus mykiss*; 81 mm) were obtained from fishery in Kaplice, South Bohemia (<http://www.pstruharstvics.cz>) in April 2017. Stone moroko (*Pseudorasbora parva*; 35 mm) was sampled in a small pond near the Biological Centre, BC CAS (48°58'50" N, 14°25'10") in May and July 2017. Immediately after transportation to the laboratory, fish were placed separately by species into the large aquaria (36 × 38 × 48 cm) containing aerated water and artificial vegetation providing shelter. All experiments on the predation of fish on trematode cercariae were performed in the shortest possible period after the acclimatization of fish lasting about 10 days. Fish were provided with commercially available food of *Tubifex tubifex* during acclimatization and experiments.

Snails were collected in two fishponds, Vlkovský pond (49°08'56"N, 14°43'51"E) and Hluboký u Hamru (49°9'39.399"N, 14°46'15"E). Both ponds are located in the Třeboň Basin Biosphere Reserve and Protected Landscape Area in South Bohemia in the Czech Republic. Two species of pulmonate gastropods were sampled, *Lymnaea stagnalis* (Linné, 1758) (Fig. 4A) and *Stagnicola* sp. (Lymnaeidae) (Fig. 4B). Ponds were selected based on previous parasitological research (e.g. Soldánová et al. 2011). These ponds are known to support viable persistent populations of both snail species harbouring a broad spectrum of trematodes, thus providing a sufficient supply of material (trematode cercariae) for laboratory experiments. In laboratory, snails were placed individually into beakers with dechlorinated tap water and under a light source for 24 h to stimulate the cercarial emergence and detect desired trematode species. Live cercariae were identified to species level using the keys of Faltýnková et al. (2007) and Zikmundová et al. (2014). Snails with no patent infections or with trematode species other than required were returned to their natural habitats. Collections of snails were

conducted whenever necessary, usually after death of specimens, throughout the warm seasons from May to August 2017. Snails were regularly fed with lettuce.

All animals were maintained in an experimental room under standard conditions with air temperature set to 18°C, water temperature 16.5°C and 12:12 light:dark cycle prior and during experiments.



Fig. 4. Freshwater pulmonate gastropods (Lymnaeidae). A–*Lymnaea stagnalis* (size 55 mm). B–*Stagnicola* sp. (size 38 mm). Source: Pavla Heinclová.

3. 3. Experimental set-up

Two types of experiments were designed, Experimental design I and Experimental design II (FR), in which the net effect of both trematode and predator specific traits on the consumption of cercariae was examined.

In Experimental design I, the main goal was to test the ability of different fish species to consume free-swimming cercariae of selected trematode species, and their potential effect to reduce cercarial population. The following questions were addressed:

- (1) Are fish able to consume cercariae? In case of successful predation;
- (2) Are there any interspecific and intraspecific differences in consumption of different trematode species?

- (3) What is the impact of direct predation on cercarial population and transmission success of trematodes?

Based on evidence from studies using cercariae as prey, the hypotheses tested for the Experimental design I are that fish are efficient consumers of cercariae of all trematode species significantly reducing cercarial numbers, and that the predation rates vary depending on specific traits of both fish and trematode species.

In the Experimental design II, the main goal was to investigate the effect of parasite density on the consumption of cercariae in a functional response experiment that is commonly used to describe the relationship between resource consumption rate and resource density. The following questions were addressed:

- (1) Is the number of consumed cercariae affected by parasite density?
- (2) What is the predator's functional response to changing density of trematode cercariae?

Based on evidence from studies using cercariae and other free-living organisms as prey, the hypotheses tested for the Experimental design II are that the number of consumed cercariae increase with increasing cercarial density, and that fish exhibit functional response Type III as it is typically displayed by vertebrate species.

To obtain freshly released cercariae that were required for experiments, infected snails were incubated at room temperature under light source for max 3 h to stimulate cercarial release (Fig. 5A). A common pool of cercariae was used by placing several infected snails with the same trematode species into one beaker with dechlorinated water, thus ensuring the variability of cercariae from different hosts. The necessary numbers of cercariae for each experimental design and replicate were individually pipetted under the stereomicroscope into smaller beakers with small amount of water (50 ml) (the fixed density of 100 cercariae in Experimental design I and ten different densities in Experimental design II; Table 1). Fish were individually separated into experimental plastic containers (20 cm in diameter and 12 cm height) with 500 ml of dechlorinated water (Fig. 5B). A green paper was used to cover each container to imitate natural water colour conditions and preventing fish to disturb each other while consuming cercariae. Containers were randomly placed on a bench under extra light source in the experimental room (Fig. 5C). The fish were regularly filmed (one fish per experiment) (Fig. 5D). Prior to each experiment, all fish were fasted for 24 h and kept in plastic containers for 30 min for general calming and relaxation. At the start of each experiment, 50 ml of water with counted densities of cercariae were gently stirred to homogenise the water with cercariae ensuring that no cercariae were attached to the beaker's wall or bottom. Immediately after, cercariae were added to each container with a single fish.

Each experiment lasted 30 min. After this time, fish were carefully removed from containers, and water with remaining cercariae was gently stirred and rinsed with water several times to filter it using a filtration equipment (Fig. 5E) and 12 μm -pore membrane (Fig. 5F). Filters were removed to a small Petri dish (diameter 5.5 cm). Remaining cercariae were fixed using 70% ethanol and stained with few drops of the carmine solution (Fig. 5G, H). Cercariae fixed in the filter were then counted in Petri dish under a stereomicroscope. Fixation and staining proved as appropriate methods to facilitate counting as most of the cercariae were undamaged and better visible (Fig. 6A–F). The same procedure was applied in corresponding controls, i.e. in the absence of fish predator of each experimental design, fish-trematode combinations and replicates, with the aim to the possible loss of cercariae due to human factor or during filtration procedure. The mean number of lost cercariae (calculated across replicates) was subtracted from the observed number of consumed cercariae by fish, and included in all statistical analyses as the number of predated cercariae.

Only fish of similar sizes were used for experiments to avoid the effect of fish size on the consumption of cercariae. All fish were measured (standard length – SL, in mm) after each replicate trial of each fish-trematode combination. In both experimental designs, replicates for a single fish-trematode combination were conducted over a maximum period of 14 days, thus ensuring only a small change in the growth of fish individuals. As the size of collected stone moroko ranged from 22 to 46 cm, indicating presence of both juvenile and adult fish, two size groups were distinguished in experiments of both experimental designs (Table 1).

Despite the standard operating procedures that ensure humane treatment for fish maintenance and survival in capture, the high mortality of rainbow trout, roach and white bream made running of experiments with all fish-trematode combinations impossible in both experimental designs. Hence, only stone moroko (both size groups) were used in all experiments involving all possible fish-trematode combinations in both experimental designs (Table 1). Unfortunately, juvenile fish of the other three species were not available to obtain later during the year due to their specific life-history traits, i.e. their reproduction takes place only once a year, usually in spring months. Therefore, the same fish individuals were used repeatedly in some experiments. However, marking fish by a small cut in caudal fin ensured that different individuals were recognisable and easily randomised between replicates. This is especially important in Experimental design II, because fish were not exposed to the same density of cercariae in consecutive replicates, which is known to affect predation rates (Real 1977). Fish that died during or after experiments were replaced by new individuals whenever possible.

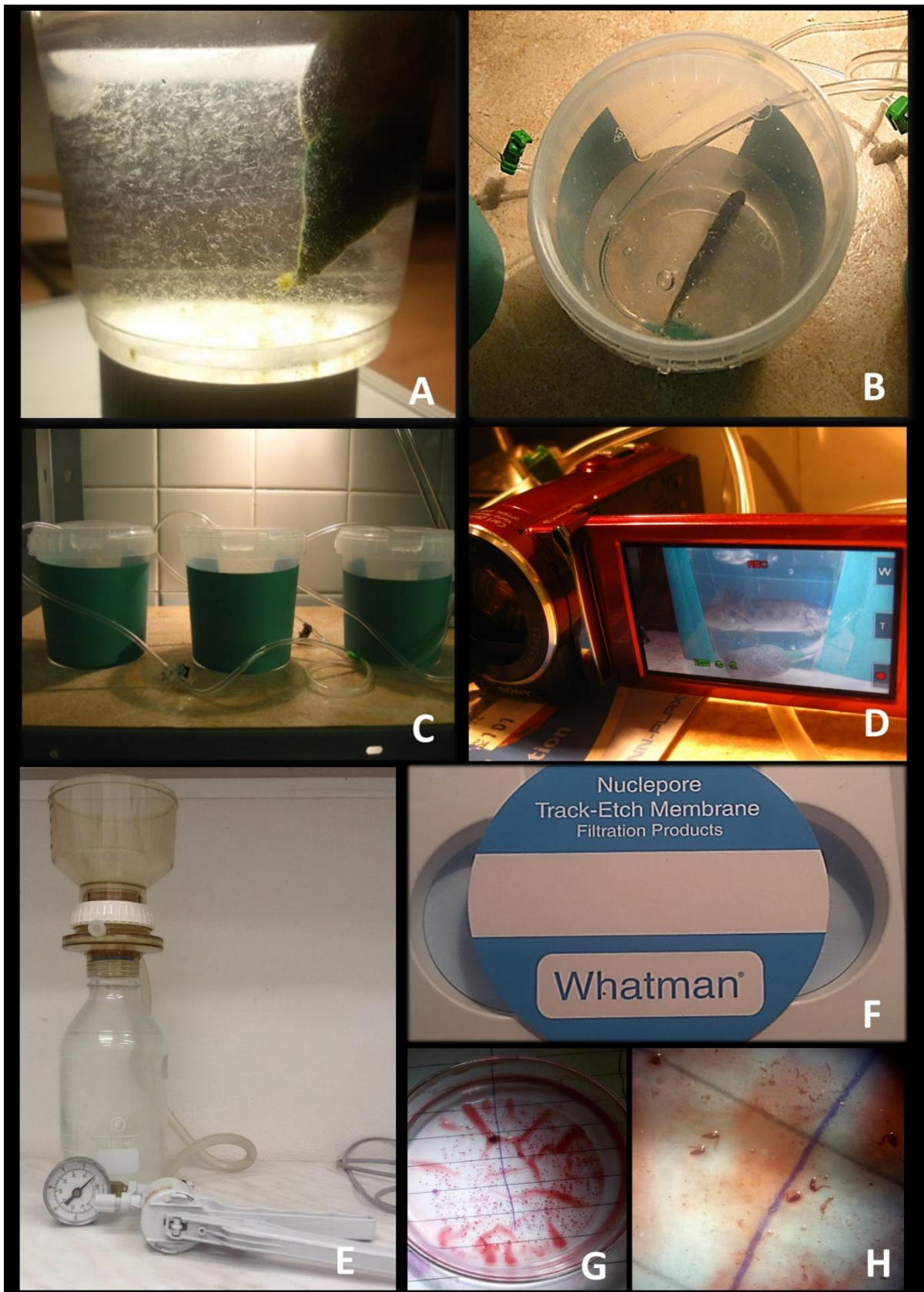


Fig. 5. Experimental set-up. **A**–emission of cercariae. **B**–fish in experimental plastic containers. **C**–containers placed on a bench under extra light source in the experimental room. **D**–videotaping of fish during experiments. **E**–filtration equipment. **F**–12µm-pore filter. **G–H**–stained cercariae by Carmine solution that remained on the filter after 30 minutes of fish consumption. Source: Pavla Heinclová.

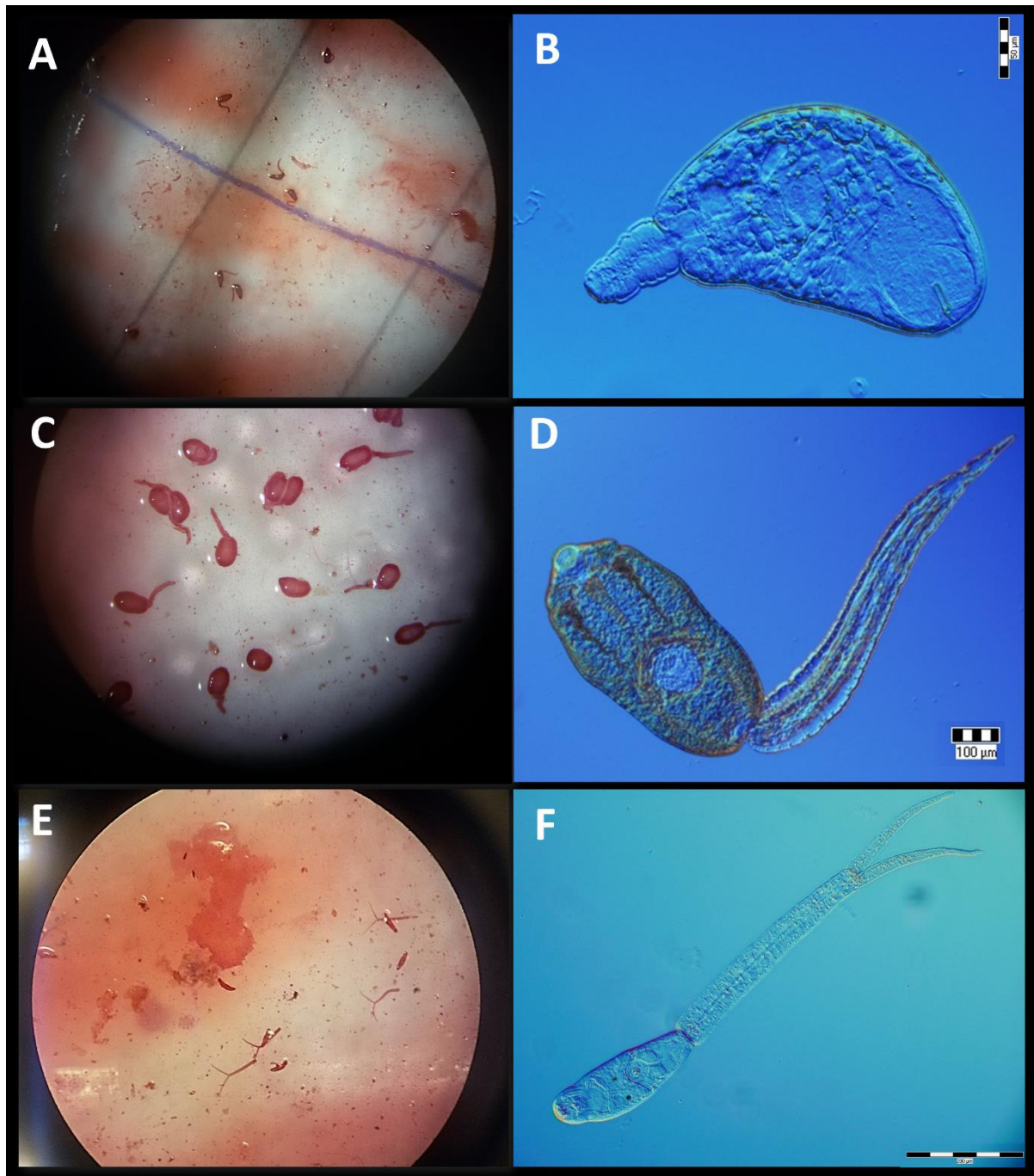


Fig. 6. Stained cercariae by carmine solution that remained on the filter after 30 minutes of fish consumption. A-B–*Plagiorchis elegans*, xiphidiocercaria (300–420 μm). C-D–*Echinoparyphium aconiatum*, echinostome cercaria (size 500–600 μm). E-F–*Trichobilharzia szidati*, ocellate furcocercaria (size 900 μm). Note that the size of stained cercariae is not corresponding to the real size because photographs were taken using stereomicroscope under different magnification. Source: Pavla Heinclová and Miroslava Soldánová

Table 1. Overview of experimental set-up for Experimental designs I and II.

	Experimental design I	Experimental design II (FR)
Density of cercariae	One density: 100	Ten densities: 10, 20, 30, 40, 50, 100, 200, 400, 600, 800
Trematode species	<i>Plagiorchis elegans</i> <i>Echinoparyphium aconiatum</i> <i>Trichobilharzia szidati</i>	<i>Plagiorchis elegans</i> <i>Echinoparyphium aconiatum</i> <i>Trichobilharzia szidati</i>
Fish species	Rainbow trout (RT) Roach (R) White bream (WB) Stone moroko: large-sized group (SM-L) small-sized group (SM-S)	Stone moroko: large-sized group (SM-L) small-sized group (SM-S)
Fish-trematode combination	RT <i>P. elegans</i> R <i>P. elegans</i> WB <i>P. elegans</i> WB <i>E. aconiatum</i> WB <i>T. szidati</i> SM-L <i>P. elegans</i> SM-L <i>E. aconiatum</i> SM-L <i>T. szidati</i> SM-S <i>P. elegans</i> SM-S <i>E. aconiatum</i> SM-S <i>T. szidati</i>	SM-L <i>P. elegans</i> SM-L <i>E. aconiatum</i> SM-L <i>T. szidati</i> SM-S <i>P. elegans</i> SM-S <i>E. aconiatum</i> SM-S <i>T. szidati</i>
No. of experiments	38	40
No. of experiments used for statistical analyses	35	28

Despite the standard operating procedures that ensure humane treatment for fish maintenance and survival in capture, the high mortality of rainbow trout, roach and white bream made running of experiments with all fish-trematode combinations impossible in both experimental designs. Hence, only stone moroko (both size groups) were used in all experiments involving all possible fish-trematode combinations in both experimental designs (Table 1). Unfortunately, juvenile fish of the other three species were not available to obtain later during the year due to their specific life-history traits, i.e. their reproduction takes place only once a year, usually in spring months. Therefore, the same fish individuals were used repeatedly in some experiments. However, marking fish by a small cut in caudal fin ensured that different individuals were recognisable and easily randomised between replicates. This is especially important in Experimental design II, because fish were not exposed to the same density of cercariae in consecutive replicates, which is known to affect predation rates (Real 1977). Fish that died during or after experiments were replaced by new individuals whenever possible.

3. 3. 1. Experimental design I and statistical analyses

In this type of experiment, the fixed density of 100 cercariae, three trematode species and four fish species were used in a total of 11 fish-trematode combinations. In initial experiments, each combination consisted of three replicate trials resulting in a total of 38 experiments (Table 1). However, several steps have been taken to correct data for further statistical analyses. This resulted in 'selected' data set consisting of 3 or 4 replicates (Table 2) and a total of 35 experiments (Table 1).

Analyses were carried out using program R (R Core Team, version 3.0.1). All data tested were not normally distributed (Shapiro-Wilk normality test; $p < 0.05$ in all cases). Therefore, the Krukall-Wallis test (K-W), a nonparametric method used to compare two or more independent samples of equal or different sample sizes, followed by Dunn *post-hoc* test with Bonferroni correction (package FSA, 0.8.21, Ogle 2018), was used in further analyses. The significance level was set up to 0.05.

First, the ideal time of consumption of cercariae by fish was tested in order to minimize stress during the time that fish were supposed to spend in experimental containers. Preliminary experiments were performed to check differences in the number of predated cercariae between 30 and 60 min. Tests were performed for 7 of 11 fish-trematode combinations including three trematode species. Since there were no significant differences in 6 treatment combinations (K-W; all $p > 0.05$), further experiments were run for 30 min. Hence, data on numbers of cercariae consumed during 30 and 60 minutes were merged together and used as an extra replicate in following analyses (i.e. the replicate 4 in some combinations; Table 2). Only white bream exhibited higher consumption levels of cercariae of *Echinoparyhium aconiatum* during 60 min (K-W, $H_{1,10} = 5.77$, $p = 0.016$), and therefore the extra fourth replicate was excluded.

Second, to control for the possible effect of fish size on the consumption of cercariae, the sizes/length of fish that were selected visually prior performing experiments were tested for significant differences among (K-W) and within (one-sample t-test) replicates for each fish species. Also, the differences in fish length between large- and small-sized stone moroko were analysed (K-W).

Third, differences in consumed cercariae among both fish and trematode species were analysed in two separate models. Model 1 (so-called Trematode interspecific variation) included analyses testing differences in the number of predated cercariae of different trematodes consumed by a single fish species, whereas Model 2 (so-called Trematode intraspecific variation) included analyses testing the variation in the number of predated cercariae of a single trematode species among different species of fish. To distinguish

between inter- and intraspecific variability and possible effect of replicates, the differences in numbers of predated cercariae were tested between replicates within each fish-trematode combination using the initial data set (Table 1). Only replicates showing no significant differences (K-W, $p > 0.05$) or requiring data correction were entered into inter- and intraspecific multiple comparison (Table 2).

Table 2. Summary data for the ‘selected’ data set in Experimental design I entered in statistical analyses to test the intra- and interspecific variation in consumption of cercariae. The number of fish individuals, mean number of predated cercariae and mean size of fish is provided for each replicate separately. Control represents the mean number of cercariae subtracted from the initial numbers counted in the absence of fish.

Fish species	Trematode species	No. of fish				Mean No. of predated cercariae				Control	Mean fish size			
		R1	R2	R3	R4	R1	R2	R3	R4		R1–R4	R1	R2	R3
Rainbow trout	<i>P. elegans</i> ¹	5	5	5	-	64	5	23	-	3	81	81	81	-
Roach	<i>P. elegans</i>	5	8	10	-	6	14	20	-	3	84	82	82	-
White bream	<i>P. elegans</i>	10	10	10	-	15	15	26	-	3	60	54	61	-
	<i>E. aconiatum</i> ²	10	10	10	-	57	59	63	-	2	62	62	62	-
	<i>T. szidati</i>	9	-	-	10	44	-	-	54	6	63	-	-	62
Stone moroko – large	<i>P. elegans</i>	10	10	10	-	17	16	15	-	3	45	45	45	-
	<i>E. aconiatum</i>	10	10	10	6	29	22	21	26	2	45	45	45	45
	<i>T. szidati</i> ^{3,*} (A1)	10	-	-	6	53	-	-	57	6	45	-	-	45
	<i>T. szidati</i> * (A2)	-	10	10	-	-	37	43	-	6	-	45	45	-
Stone moroko – small	<i>P. elegans</i>	10	10	10	-	14	15	10	-	3	32	32	32	-
	<i>E. aconiatum</i>	10	10	6	6	30	23	37	24	2	24	24	25	25
	<i>T. szidati</i>	10	10	10	-	50	42	41	-	6	24	24	24	-

¹*Plagiorchis elegans*; ²*Echinoparyphium aconiatum*; ³*Trichobilharzia szidati*

* Four replicates divided into two separate analyses as A1 and A2 (see Chapter 3.3.1. for details)

The number of predated cercariae varied between replicates of the following fish-trematode combinations: white bream – *T. szidati* showed significantly different numbers between initial 4 replicates consisting of 9, 4, 3 and 10 fish individuals (K-W, $H_{3,26} = 9.742$; $p = 0.021$). Therefore, two replicates were excluded due to small number of fish most likely affecting robustness of the analysis. The combination of stone moroko – large and *T. szidati* also showed different numbers of predated cercariae between the initial 4 replicates (10, 10, 10 and 6) (K-W, $H_{3,36} = 22.369$; $p = 0.0001$), thus dividing the consumption of cercariae into two replicate groups. Group A1 showing pattern of high cercarial consumption (mean of 54

cercariae) and group A2 with low consumption pattern (40 cercariae) After division, replicates within each group did not differ in numbers of consumed cercariae (K-W; both $p > 0.05$) and were entered in further statistical analyses separately as group A1 and A2. In the fourth replicate of combination stone moroko – small and *T. szidati* fish consumed distinctly higher number of cercariae (mean 57 cercariae) than in other 3 replicates (initial 4 replicates consisted of 10, 10, 10 and 6 fish; K-W, $H_{3, 36} = 9.192$; $p = 0.0268$), and therefore the fourth replicate was excluded. Last combination comprised rainbow trout and *P. elegans*. All 3 replicates considerably differed in numbers of consumed cercariae (K-W, all $p > 0.05$ in pairwise *post-hoc* test). However, as the only exception, the 3 replicates were included into further analyses on the trematode interspecific variation (Model 1) because of the possible effect of such a high variation among replicates (5 to 64 cercariae, respectively) compared to other fish species preying on *P. elegans* (Table 2). Therefore, analyses of two data sets are distinguished in Model 1, i.e. data excluding and including rainbow trout. Table 2 summarises the ‘selected’ data set that was entered into following statistical analyses evaluating intra- and interspecific variation in consumption of different trematode species.

3.3.2. Experimental design II and statistical analyses

In this second type of experiment, different densities of cercariae were used (10 increasing densities in total: 10, 20, 30, 40, 50, 100, 200, 400, 600 and 800; Table 1) to examine whether the number of consumed cercariae was influenced by parasite density, and to identify the functional response that fishes exhibit while consuming cercariae. Three trematode species and one fish species, stone moroko divided into two size groups, were used in total of 6 fish/groups-trematode combinations (Table 1). In the initial data set, each trematode combination consisted of eight replicate trials, except for *E. aconiatum* comprising four replicate trials in both size groups of stone moroko (Table 1 and 3). In total, 40 experiments were performed. However, in some replicates non-randomized fish were accidentally used (Table 3). Because the non-randomized treatment may affect predation ('learning' behaviour of fish during predation; Real 1977), only randomized data were used resulting in a ‘selected’ data set that was entered into statistical analyses (Table 3). Selected data set comprised of 2 to 6 replicates and total of 28 experiments (Table 1 and 3).

First, fish sizes/length were tested for differences between the large- and small-sized groups for each fish-trematode combination.

Table 3. Summary data for the initial data set and ‘selected’ data set in Experimental design II entered into statistical analyses. Selected data set comprises replicates with randomized fish only. Each replicate consists of 10 fish.

Fish species	Trematode species	No. of replicates	Initial data set (NR+R ¹ replicates)	Selected data set	Mean fish size (max–min) [mm]
Stone moroko – large	<i>P. elegans</i> ²	8	2 + 6	6	45 (43–45)
	<i>E. aconiatum</i> ³	4	2 + 2	2	45 (43–46)
	<i>T. szidati</i> ⁴	8	2 + 6	6	45 (43–46)
Stone moroko – small	<i>P. elegans</i>	8	2 + 6	6	24 (23–28)
	<i>E. aconiatum</i>	4	2 + 2	2	24 (22–28)
	<i>T. szidati</i>	8	2 + 6	6	24 (22–28)

¹NR, replicate trials with non-randomized fish; R, replicate trials with randomized fish;

²*Plagiorchis elegans*; ³*Echinoparyphium aconiatum*; ⁴*Trichobilharzia szidati*

However, since the main goal was to determine the type of response that would best describe the consumption rates by stone moroko, a similar pattern in consumption was assumed in both large and small fish. Therefore data were merged together and three functional response analyses were performed for each trematode species separately. Furthermore, to account for the effect of fish size on the consumption of cercariae, fish length in each size group was tested for differences among (K-W) and within (one-sample *t*-test) replicates. Second, the differences in the number of consumed cercariae between randomized replicates were tested within each fish-trematode combination (K-W). The significance level in all tests was set up to 0.05.

Third, functional responses were analysed by performing logistic regressions on the proportion of consumed cercariae as function of the prey density, using the *frair_test* function which is provided in the package R package *frair* (version 0.5.100, Pritchard et al. 2017) and enables the discrimination of Type II or Type III functional responses. These models operate with essential components of predator’s consumption such as search, capture and handling of resources that are accounted for in analyses. Type II responses are described by a significantly negative first-order term (declining proportional consumption with increasing prey density) whereas Type III is indicated by a significantly positive first-order term followed by a significant negative second-order term (initial increase and subsequent decrease in proportional consumption) (Juliano 2001). Type I was not considered since it assumes linear relationship and thus not responding to the consumption patterns of vertebrate predators such as fish.

After visual inspection of the data, the consumption of cercariae at the density of 800 was observed to decrease in most of the fish-trematode combinations suggesting that saturation in consumption occurred. Since there could be some unknown noise interaction at the highest density showing that the pattern in the rest of data is not followed as the predator stops consuming its prey, densities of 800 cercariae of all trematodes were eliminated from functional response analyses. Due to ten different increasing densities, the mean and percentage of trematodes removed by fish was calculated across low (10–50), medium (100–200), high (400–600) densities, and the density of 800 cercariae.

4. RESULTS

4.1. Experimental design I

Experiments of some fish-trematode combinations could not be performed due to rapid and frequent death of fish during the experiments, namely rainbow trout and roach, being the two species combined with *Plagiorchis elegans* only (Table 1). Therefore, the number of fish used in replicate trials varied from 5 to 10 per a replicate (Table 2). An average of 3 cercariae were recovered in the control treatments, i.e. in the absence of fish (range from 2 to 6 cercariae across all trematode species) (Table 2), illustrating the effectiveness of the filtration method.

The overall mean size across fish species ranged from 24 to 82 mm, stone moroko (small-sized group) being the smallest fish and rainbow trout the largest (Table 4). As expected, fishes of stone moroko differed significantly in length between the two size groups in general (K-W, $H_{1, 194} = 148.07$, $p < 0.001$) as well as in each fish-trematode treatment (K-W, all $p < 0.001$), proving the presence of adult fish in large-sized group and juvenile fish in small-sized group. Fish sizes varied also within rainbow trout, roach and white bream (Table 4). However, there were no significant differences among (K-W; all $p > 0.05$) and within replicates (one-sample *t*-test, all $p > 0.05$) demonstrating that selected individuals of each fish species were of similar sizes and therefore with no apparent possible effect on the consumption rates of cercariae.

The data correction for possible effects of different consumption of cercariae among replicates using the initial data set with 38 experiments (Table 1) resulted in the elimination of dissimilar replicates, except for rainbow trout and *P. elegans* (Table 2; see chapter 3.3.1. in Materials and Methods for details). Hence, altogether four species were used in 35 experiments/replicates of 11 fish-trematode combinations (Table 4). To answer the specific questions in Experimental design I, the analyses performed using this 'selected' data set (see Table 2) demonstrated that (1) all species of fish frequently prey upon trematode cercariae, but with different consumption rates. Furthermore, (2) interspecific and intraspecific differences in consumption of different trematode species were detected, as well as (3) the significant reduction of cercarial population (here represented by 100 density) by action of fish predators. Statistical results are shown in Table 5 (Kruskal-Wallis test), Table 6 (multiple comparison by *post-hoc* Dunn test) as well as in Figs. 8A–D and 9A–E.

Table 4. Summary data for ‘selected’ data set in Experimental design I entered into statistical analyses to test the intra- and interspecific variation in consumption of cercariae. Pooled data across replicates are provided.

Fish species	Trematode species	No. of replicates	Total No. of fish	Mean No. of predated cercariae	Min.–max. No. of predated cercariae	±SD ¹	Percentage of predated cercariae [%]	Mean fish size (min–max) [mm]
Rainbow trout	<i>P. elegans</i> ²	3	15	30	1–75	26	30	81 (76–85)
Roach	<i>P. elegans</i>	3	23	15	0–41	12	15	82 (75–89)
White bream	<i>P. elegans</i>	3	30	18	0–48	12	18	58 (45–75)
	<i>E. aconiatum</i> ³	3	30	60	33–78	13	60	62 (45–75)
	<i>T. szidati</i> ⁴	2	19	49	16–65	11	49	62 (45–75)
Stone moroko – large	<i>P. elegans</i>	3	30	16	0–45	12	16	45 (43–46)
	<i>E. aconiatum</i>	4	36	24	0–55	14	24	45 (43–46)
	<i>T. szidati</i> *	2 (A1)	16	54	45–62	5	54	45 (43–46)
		2 (A2)	20	40	12–54	10	40	45 (43–46)
Stone moroko – small	<i>P. elegans</i>	3	30	13	0–33	9	13	31 (25–40)
	<i>E. aconiatum</i>	4	32	28	0–59	17	28	25 (22–28)
	<i>T. szidati</i>	3	30	44	13–63	10	44	24 (22–28)

¹ Standard deviation; ²*Plagiorchis elegans*; ³*Echinoparyphium aconiatum*; ⁴*Trichobilharzia szidati*

* Four replicates divided into two separate analyses as A1 and A2 (see Chapter 3.3.1. for details)

All fish species were able to consume trematode cercariae. White bream was particularly efficient predator resulting in the removal of 41% of the three trematode species (18–60%), followed by stone maroko – large consuming on average 30% (16–54%), and stone maroko – small 28% of cercariae (13–44%) (Table 4; Fig. 7A). The two of fish species, white bream and stone moroko (both size groups) removed altogether 36% of *E. aconiatum* (24–60%), 16% of *P. elegans* (13–18%), being the least predated trematode, and 46% of *T. szidati* (40–54%), being the most consumed trematode in the two fish species (Table 4; Fig. 7B). All four fish species, rainbow trout, roach, white bream and stone moroko (both size groups) removed altogether 18% of *P. elegans* cercariae, with the greatest removal by rainbow trout (Table 4).

The significant differences were detected in analyses of both models testing the intra- and interspecific variability in consumption of cercariae. Model 1 revealed a significantly different consumption rates of the three trematode species by a single species of fish (all $p < 0.001$; Table 5). Overall, white bream consumed the most *E. aconiatum* and *T. szidati* (Table 4, Fig. 8A), while stone moroko more on *T. szidati*, independently of the fish size groups (Table 4, Fig. 8B–D).

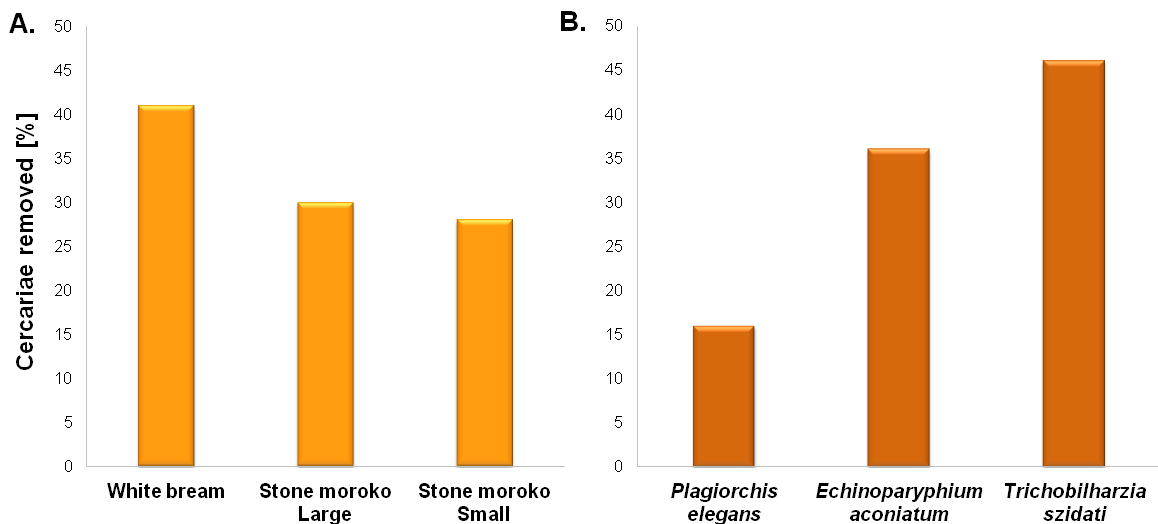


Fig. 7. Mean percentage of cercariae removed in the presence of predators in Experimental design I. A) Reduction of the three trematode species (*Echinoparyphium aconiatum*, *Plagiorchis elegans* and *Trichobilharzia szidati*) by a single fish species. B) Reduction of a single trematode species by the two species of fish (white bream and stone moroko divided into two size groups).

Table 5. Results of statistical analyses for Experimental design I assessing the variation in the number of predated cercariae of different trematodes consumed by a single fish species (Model 1) and differences in predated cercariae of a single trematode species consumed by different fish predators (Model 2). Analyses on consumption of cercariae by fish in Model 1 comprised both data for *Plagiorchis elegans*, excluding and including rainbow trout. See abbreviations for fish species in Table 1. A1, A2, separate analyses for combination stone moroko – large and *Trichobilharzia szidati* (see Chapter 3.3.1. for details).

Model	Treatment	H ¹	df ²	P ³
Model 1				
Trematode interspecific variation				
	WB – <i>P. elegans</i> ⁴ , <i>E. aconiatum</i> ⁵ , <i>T. szidati</i> ⁶	52.727	2	< 0.001***
	SM-L – <i>P. elegans</i> , <i>E. aconiatum</i> , <i>T. szidati</i> (A1)	40.428	2	< 0.001***
	SM-L – <i>P. elegans</i> , <i>E. aconiatum</i> , <i>T. szidati</i> (A2)	29.874	2	< 0.001***
	SM-S – <i>P. elegans</i> , <i>E. aconiatum</i> , <i>T. szidati</i>	45.77	2	< 0.001***
Model 2				
Trematode intraspecific variation				
	<i>P. elegans</i> ^a – R, WB, SM-L, SM-S	2.481	3	0.479
	<i>P. elegans</i> ^b – RT, R, WB, SM-L, SM-S	5.631	4	0.228
	<i>E. aconiatum</i> – WB, SM-L, SM-S	48.678	2	< 0.001***
	<i>T. szidati</i> – WB, SM-L (A1), SM-S	12.976	2	0.0015**
	<i>T. szidati</i> – WB, SM-L (A2), SM-S	9.287	2	0.0096**

¹H-test values (Kruskal-Wallis); ²df, degrees of freedom; ³P, significance level of H-test; ⁴*Plagiorchis elegans*; ⁵*Echinoparyphium aconiatum*;

⁶*Trichobilharzia szidati*

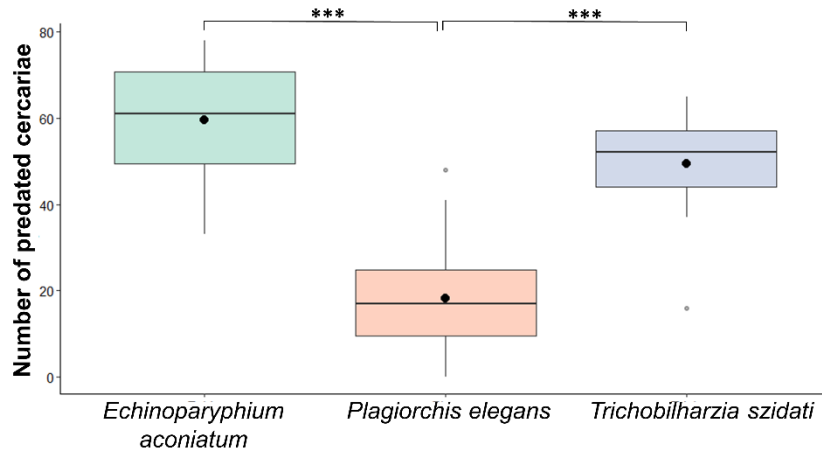
^a data excluding rainbow trout

^b data including rainbow trout

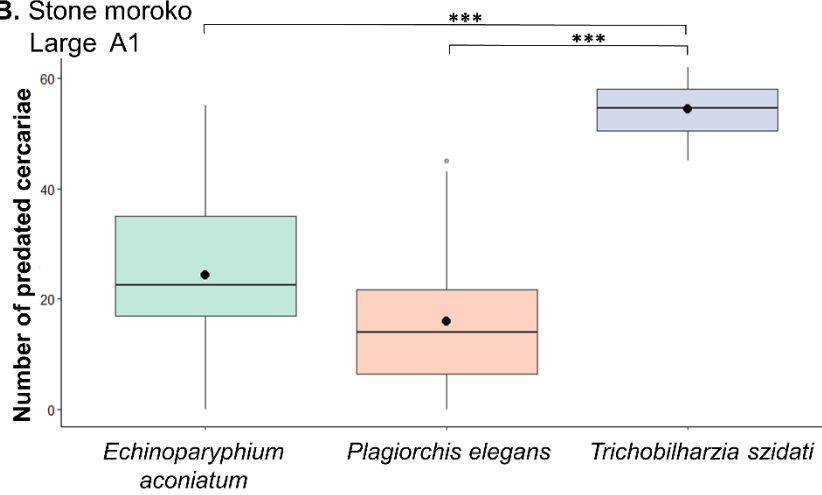
** significant at P < 0.01

*** significant at P < 10⁻⁴

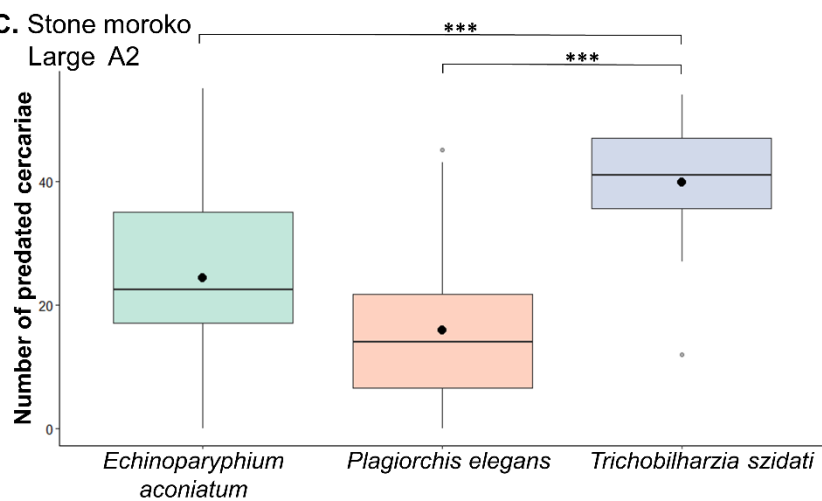
A. White bream



**B. Stone moroko
Large A1**



**C. Stone moroko
Large A2**



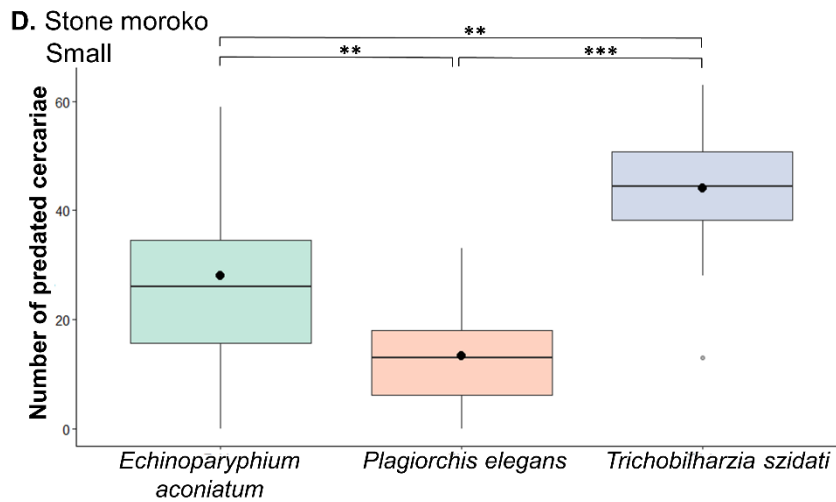


Fig. 8. Trematode interspecific variation in consumption of cercariae in Experimental design I (Model 1). Number of predated cercariae of three different trematode species, *Echinoparyphium aconiatum*, *Plagiorchis elegans* and *Trichobilharzia szidati* are shown for each fish: A) white bream, B) stone moroko – large (A1), C) stone moroko – large (A2), and D) stone moroko – small. Middle line in the box represents median, black dot the mean value, box edges interquartile range, first and third quartile (the 25th and 75th percentiles) and whiskers show standard error. Statistically significant results are marked with an asterisk (**P=0.01; ***P<10⁻⁴) representing the results of multiple comparison using *post-hoc* Dunn test with Bonferroni correction (see also Table 6). A1, A2 are separate analyses for the combination stone moroko – large and *Trichobilharzia szidati* (see Chapter 3.3.1. for details). Note the different y-axis.

Table 6. Results of statistical pairwise comparison assessing the interspecific (Model 1) and intraspecific (Model 2) variation in consumption of cercariae in Experimental design I. Results from pairwise comparisons and corresponding P-values using *post-hoc* Dunn test with applied Bonferroni correction are shown for Model 1 in left part and for Model 2 in right part of the Table. A1, A2 are separate analyses for combination stone moroko – large and *Trichobilharzia szidati* (see Chapter 3.3.1. for details).

Model 1 (Trematode interspecific variation)				Model 2 (Trematode intraspecific variation)		
White bream (WB)	Stone moroko – large (SM-L) [A1]	Stone moroko – large (SM-L) [A2]	Stone moroko – small (SM-S)	<i>Trichobilharzia szidati</i> (TS)	<i>Echinoparyphium aconiatum</i> (EA)	<i>Plagiorchis elegans</i> (P)
EA > P (p < 0.001)***	EA = P (p = 0.110)	EA = P (p = 0.069)	EA > P (p = 0.003)**	WB = SM-L [A1] (p = 0.456) WB > SM-L [A2] (p = 0.007)**	WB > SM-L (p < 0.001)***	^a R = WB = SM-L = SM-S (all p = 1, except SM-S/WB, p = 0.773)
EA = TS (p = 0.265)	EA < TS (p < 0.001)***	EA < TS (p < 0.001)***	EA < TS (p = 0.001)**	WB = SM-S [A1] (p = 0.122) WB = SM-S [A2] (p = 0.149)	WB > SM-S (p < 0.001)***	^b R = WB = SM-L = SM-S = RT (all p = 1, except SM-S/RT, p = 0.347)
P < TS (p < 0.001)***	P < TS (p < 0.001)***	P < TS (p < 0.001)***	P < TS (p < 0.001)***	SM-L > SM-S [A1] (p = 0.001)** SM-L = SM-S [A2] (p = 0.514)	SM-L = SM-S (p = 1)	

^a data excluding rainbow trout

^b data including rainbow trout

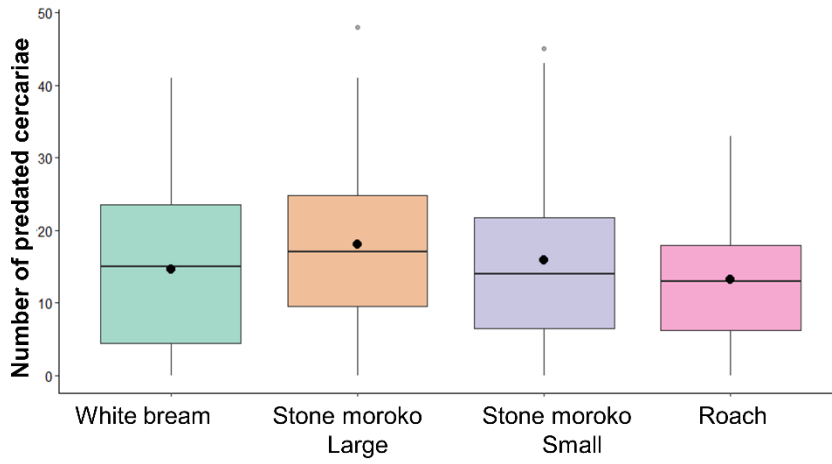
** significant at P < 0.01

*** significant at P < 10⁻⁴

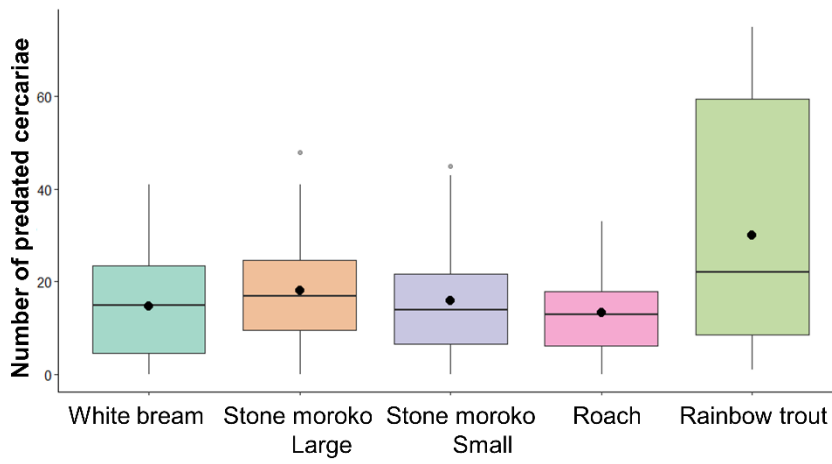
In contrast, both fish species consumed cercariae of *P. elegans* at a constantly low rate (Table 4, Fig. 8 A–D). Pairwise *post hoc* comparisons showed significant differences in consumption of cercariae by white bream and the large-sized stone moroko for some trematode species combinations only (Table 6, Fig. 8A–C), whereas the consumption by small-sized stone moroko differed among all three trematodes (Table 6, Fig. 8D), because distinctly different mean numbers were recorded for *E. aconiatum*, *P. elegans* and *T. szidati* (Table 4). The same predation patterns by large-sized stone moroko were observed for all trematode species in the two groups A1 and A2, despite the different numbers of consumed cercariae of *T. szidati* between replicates (Table 2, 4 and 6, Fig. 8B and C). Overall, large- and small-sized stone moroko showed similar consumption pattern (Table 6, Fig. 8B–D).

Results of Model 2 revealed trematode intraspecific variation in consumption, i.e. number of predated cercariae of a single trematode species consumed by different fish predators (all $p < 0.01$), except for *P. elegans* ($P > 0.05$) (Table 5). However, only some combinations are responsible for these differences (Table 6, Fig. 9). *Plagiorchis elegans* is consumed considerably less with different pattern than other two trematode species (Fig. 7 and Fig. 9A–B). Comparison of *P. elegans* cercariae among the three fish species, roach, white bream and stone moroko, revealed similar consumption rates (Table 6, Fig. 9A). The same result was observed for data set including rainbow trout (Table 3, Fig. 9B), despite the higher mean number of consumed cercariae and significant differences among replicates compared to other species of fish (Table 2 and 4). The consumption of *E. aconiatum* by stone moroko was independent of fish size, whereas distinctly more cercariae were consumed by white bream (Table 4, and 6, Fig. 9C). *Trichobilharzia szidati* is the most consumed trematode by white bream and both size groups of stone moroko (46%, Fig. 7B). The high and low predation rates observed by the large-sized stone moroko (group A1 and A2, respectively; Table 4) are responsible for the differences rather than the effect. At high consumption, there are no significant differences among the two fish species, white bream and stone moroko. Large fish of stone moroko consumed significantly more cercariae than small fish (Table 6, Fig. 9D). In contrast, at low consumption levels, the removal of cercariae was comparable between large and small fish of stone moroko (Table 4), but showing differences between white bream and large-sized stone moroko (Table 6, Fig. 9E).

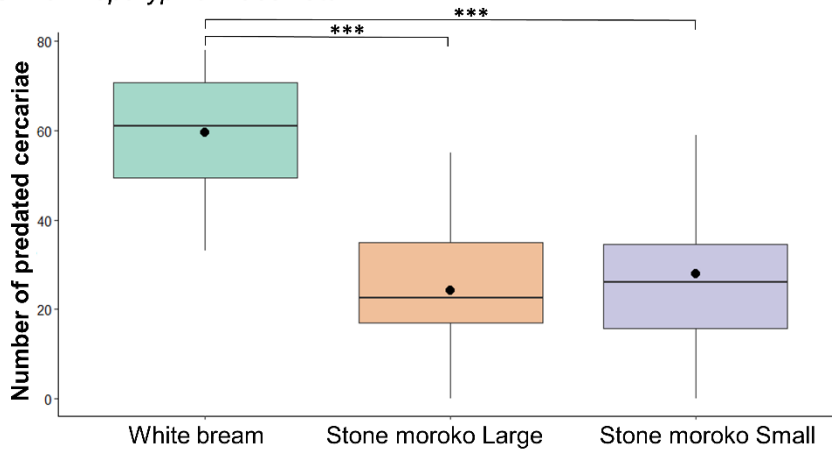
A. *Plagiorchis elegans* A



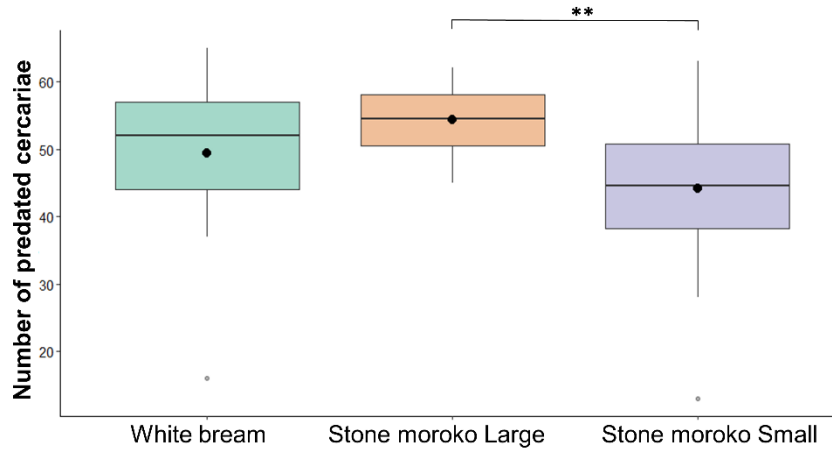
B. *Plagiorchis elegans* B



C. *Echinoparyphium aconiatum*



D. *Trichobilharzia szidati* A1



E. *Trichobilharzia szidati* A2

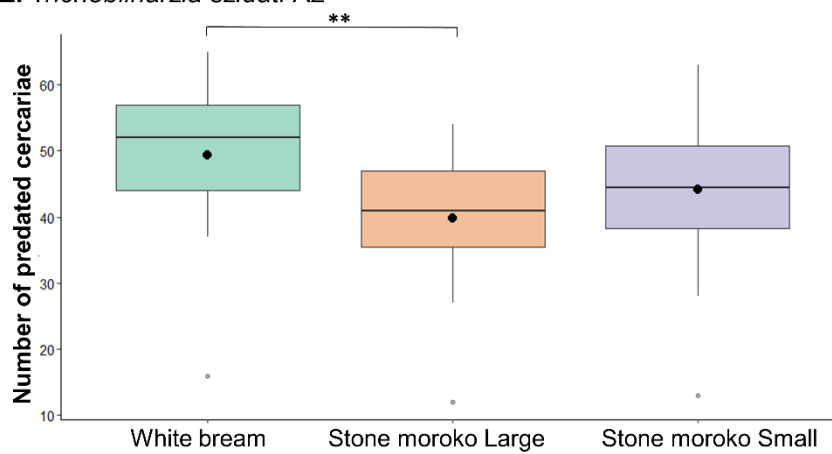


Fig. 9. Trematode intraspecific variation in consumption of cercariae in Experimental design I (Model 2). Number of predated cercariae by four different fish, rainbow trout, roach, white bream, stone moroko (divided into two size groups; large and small) are shown for: A) *Plagiorchis elegans* A (excluding rainbow trout), and B) *Plagiorchis elegans* B (including rainbow trout). Number of predated cercariae by two different fish, white bream and stone moroko (divided into two size groups; large and small) are shown for each trematode species: C) *Echinoparyphium aconiatum* and D) *Trichobilharzia szidati* (A1), E) *Trichobilharzia szidati* (A2). Middle line in the box represents median, black dot the mean value, box edges interquartile range, first and third quartile (the 25th and 75th percentile) and whiskers show standard error. Statistically significant results are marked with an asterisk (**P<0.01; ***P<10⁻⁴) representing the results of multiple comparison using *post-hoc* Dunn test with Bonferroni correction (see also Table 6). A1, A2 are separate analyses for the combination stone moroko – large and *T. szidati* (see Chapter 3 3.1. for details). Note the different y-axis.

4. 2. Experimental design II

Altogether, stone moroko fish of two sizes (large fish: mean length of 45 mm; small fish: 24 mm) were used in selected data set comprising 28 experiments/replicates of 6 fish/size group-trematode combinations (Tables 3 and 7–9). The mean loss of cercariae in the control treatments, i.e. in the absence of fish, ranged from 0–1 in low densities, 2–8 in medium (maximum of 13 cercariae), 18–56 in high (maximum of 60) and 44–76 in a density of 800 cercariae in all trematode species (Tables 7–9).

Individuals of stone moroko differed significantly in length between the two size groups in general (K-W, $H_{1, 280} = 213.62$, $p < 0.001$) as well as in each fish-trematode treatment (K-W, all $p < 0.001$), and no differences were found within replicates of each fish size group (one sample *t*-test, all $p > 0.05$). Both results demonstrate the presence of adult and juvenile fish in the separated size groups, and similar sizes of fish within large- and small-sized group. No differences in numbers of predated cercariae were found between non-randomized replicates in any of the fish-trematode combination (*P. elegans*, K-W, $H_{5, 120} = 3.462$, $p = 0.629$; *E. aconiatum*, K-W, $H_{1, 40} = 0.115$, $p = 0.734$; *T. szidati*, K-W, $H_{5, 120} = 2.288$, $p = 0.808$).

The number of consumed cercariae was influenced by prey density (Tables 7–9, Figs. 10, 11). Overall, cercariae of all three trematodes were the most consumed at high densities of 400 and 600 cercariae while none or hardly any cercariae were recovered from low densities (Table 7–9, Fig. 10), except for *T. szidati* (mean range of 8–13; Table 9). At the highest density (800 cercariae), a pronounced decrease in cercarial consumption was observed for most trematodes in all fish groups, except for stone moroko – small and *T. szidati* (Table 9). *Plagiorchis elegans* was the least consumed trematode in all density groups, i.e. low, medium, high and the density of 800 cercariae (Table 7, Fig. 10). The highest average consumption of *E. aconiatum* was observed at high cercarial densities in both large- and small-sized fish groups whereas at medium densities was quite low (Table 8, Fig. 10). *Trichobilharzia szidati* was the most frequently predated species by both size groups of fish at both low and medium cercarial densities compared to other two trematodes (Table 9, Fig. 10). The large and small-sized groups of stone moroko removed across all densities altogether 7% of *P. elegans* cercariae (maximum 8–18% at low densities, but also up to 10–16% at high densities; Table 7), 23% of *E. aconiatum* (maximum 37–38% at high densities; Table 8), and 25 % of *T. szidati* (maximum of 35–36% at high densities, but also up to 31–33% at medium and 27–33% at low densities; Table 9).

Table 7. Summary data for ‘selected’ data set for stone moroko – *Plagiorchis elegans* combination in Experimental design II entered into statistical analyses to test the effect of parasite density on the consumption of cercariae. The mean number of trematodes removed by fish is presented for each prey density separately and across low (10–50), medium (100–200), high (400–600) densities, and the density of 800 cercariae. Pooled data across replicates are provided. See Table 3 for number of replicates used in each fish-trematode combination. Each replicate consists of 10 fish. Control represents the mean number of cercariae subtracted from the initial numbers counted in the absence of fish. SD, standard deviation.

Fish species	Trematode species	Density of cercariae	Mean No. of predated cercariae	Min.–max. No. of predated cercariae	±SD	Mean control	Percentage of predated cercariae [%]	Density group	Mean No. of predated cercariae
Stone moroko – large	<i>Plagiorchis elegans</i>	10	0	0	0	0	0	Low	1
		20	0	0–2	1	0	2		
		30	1	0–3	2	0	4		
		40	3	0–7	3	0	8	Medium	10
		50	2	0–11	4	0	4		
		100	2	0–8	3	3	2		
		200	19	0–82	31	4	10	High	40
		400	18	0–74	28	6	5		
		600	63	0–264	103	30	10		
		800	24	0–73	33	44	3	800	24
Stone moroko – small	<i>Plagiorchis elegans</i>	10	0	0	0	0	0	Low	3
		20	0	0	0	0	0		
		30	1	0–4	2	0	2		
		40	3	0–7	3	0	8	Medium	12
		50	9	0–35	14	0	18		
		100	7	0–25	10	3	7		
		200	17	0–58	22	4	9	High	53
		400	64	34–84	18	6	16		
		600	43	0–131	48	30	7		
		800	28	0–111	43	44	4	800	28

Table 8. Summary data for ‘selected’ data set for stone moroko – *Echinoparyphium aconiatum* combination in Experimental design II entered into statistical analyses to test the effect of parasite density on the consumption of cercariae. The mean number of trematodes removed by fish is presented for each prey density separately and across low (10–50), medium (100–200), high (400–600) densities, and the density of 800 cercariae. Pooled data across replicates are provided. See Table 3 for number of replicates used in each fish-trematode combination. Each replicate consists of 10 fish. Control represents the mean number of cercariae subtracted from the initial numbers counted in the absence of fish. SD, standard deviation.

Fish species	Trematode species	Density of cercariae	Mean No. of predated cercariae	Min.–max. No. of predated cercariae	±SD	Mean control	Percentage of predated cercariae [%]	Density group	Mean No. of predated cercariae
Stone moroko – large	<i>Echinoparyphium aconiatum</i>	10	0	0	0	0	0	Low	2
		20	1	0–2	1	0	5		
		30	2	0–3	2	0	5		
		40	3	1–5	3	0	8		
		50	4	3–5	1	0	8	Medium	14
		100	8	3–12	6	2	8		
		200	20	8–32	17	2	10		
		400	93	67–119	37	13	23	High	157
		600	222	137–306	120	24	37	800	126
800	126	126–126	0	54	16				
Stone moroko – small	<i>Echinoparyphium aconiatum</i>	10	0	0	0	0	0	Low	2
		20	2	0–4	3	0	10		
		30	3	2–3	1	0	8		
		40	4	1–7	4	0	10	Medium	6
		50	3	2–4	1	0	6		
		100	9	6–11	4	2	9		
		200	4	0–7	5	2	2	High	186
		400	147	122–171	35	13	37		
		600	226	60–392	235	24	38		
800	182	14–350	238	54	23	800	182		

Table 9. Summary data for ‘selected’ data set for stone moroko – *Trichobilharzia szidati* combination in Experimental design II entered into statistical analyses to test the effect of parasite density on the consumption of cercariae. The mean number of trematodes removed by fish is presented for each prey density separately and across low (10–50), medium (100–200), high (400–600) densities, and the density of 800 cercariae. Pooled data across replicates are provided. See Table 3 for number of replicates used in each fish-trematode combination. Each replicate consists of 10 fish. Control represents the mean number of cercariae subtracted from the initial numbers counted in the absence of fish. SD, standard deviation.

Fish species	Trematode species	Density of cercariae	Mean No. of predated cercariae	Min.–max. No. of predated cercariae	±SD	Mean control	Percentage of predated cercariae [%]	Density group	Mean No. of predated cercariae
Stone moroko – large	<i>Trichobilharzia szidati</i>	10	0	0	0	0	0	Low	4
		20	0	0	0	0	0		
		30	0	0–1	0	0	1		
		40	11	0–19	7	1	27	Medium	40
		50	8	0–15	6	1	15		
		100	33	18–51	11	2	33		
		200	48	7–122	47	13	24	High	153
		400	145	120–199	28	51	36		
		600	160	0–319	133	60	27		
800	77	0–282	111	76	10	800	77		
Stone moroko – small	<i>Trichobilharzia szidati</i>	10	0	0	0	0	0	Low	5
		20	0	0	0	0	0		
		30	1	0–3	1	0	2		
		40	13	5–22	7	1	33	Medium	40
		50	12	0–22	10	1	24		
		100	18	4–39	13	2	18		
		200	63	26–117	35	13	31	High	160
		400	140	54–222	54	51	35		
		600	181	114–250	45	60	30		
800	206	36–504	157	76	26	800	206		

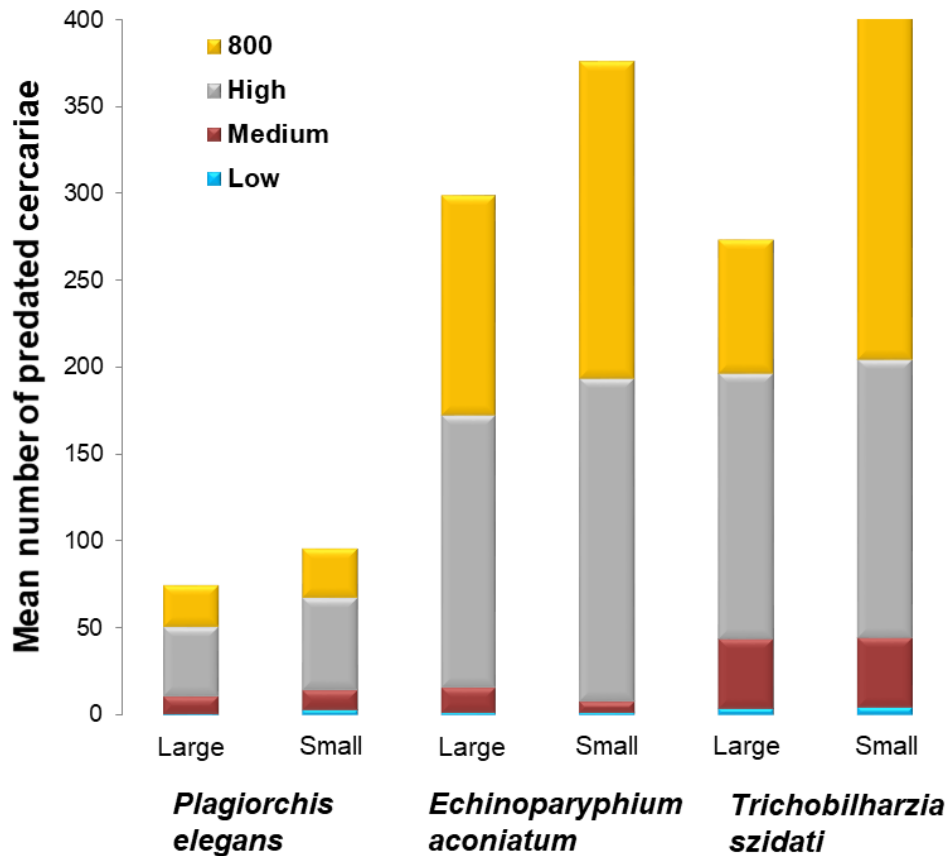
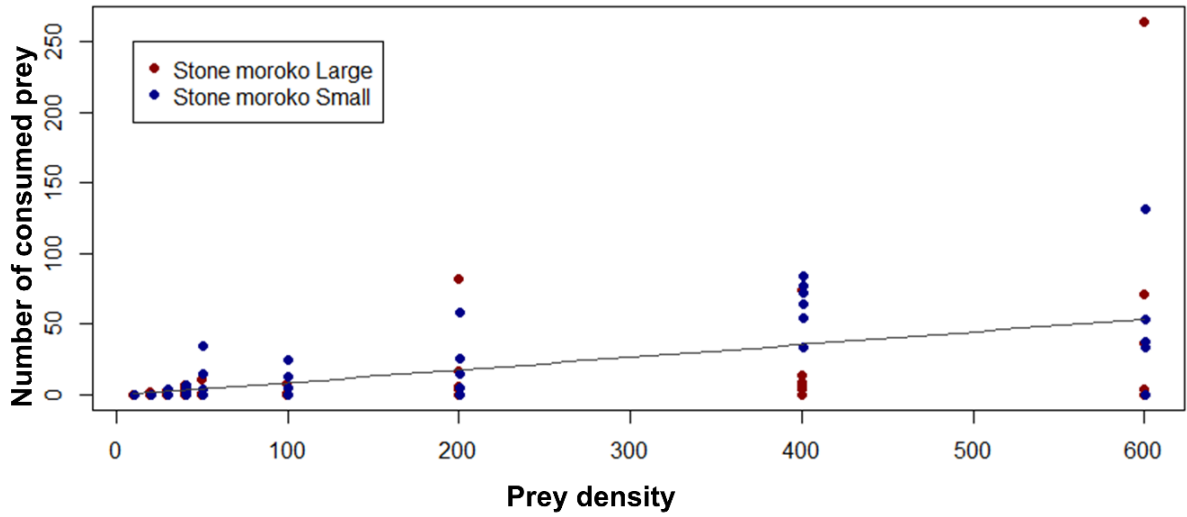


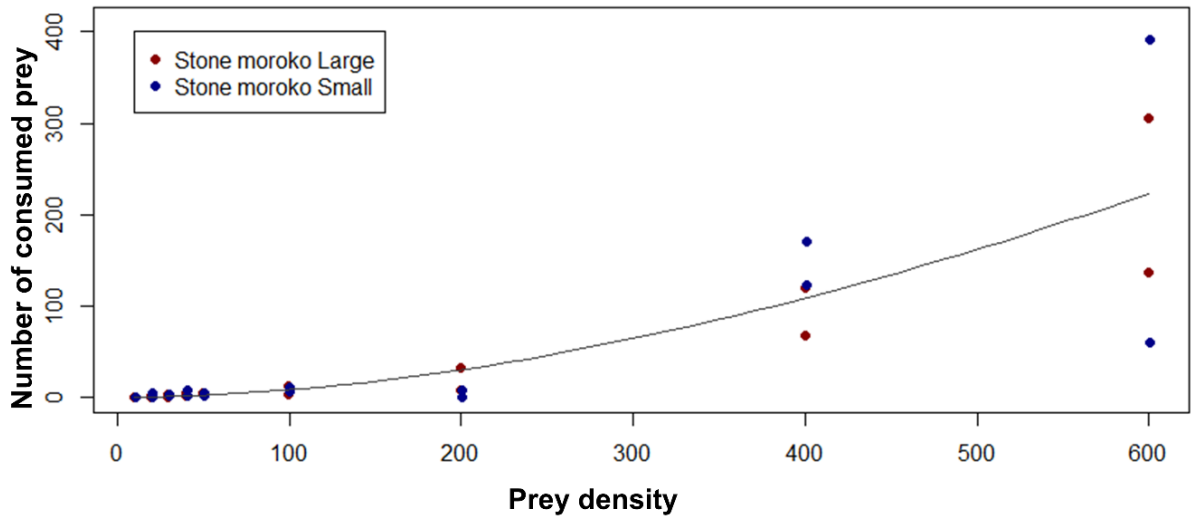
Fig. 10. Mean number of predated cercariae of three trematode species across low (10–50), medium (100–200) and high (400–600) densities, and for a density of 800 cercariae between two size groups of stone moroko (large-sized group: mean 45 mm; small-sized group: mean 24 mm) recovered in Experimental design II. Pooled data across replicates are provided. Each replicate consists of 10 fish.

The functional response analyses performed for each trematode separately suggested that changes in the feeding rates of stone moroko with increasing densities of *P. elegans*, *E. aconiatum* and *T. szidati* displayed a distinctly non-linear consumption pattern, but with somewhat weak pattern for *P. elegans* (Fig. 11). Logistic regressions indicated the functional response of Type III since the first-order terms were all significantly positive followed by a significantly negative second-order terms (all p-values < 0.001; Fig. 11). The consumption of the three trematode species most likely does not differ between large and small fish as indicated by overlapping data points (Fig. 11), but further analyses are needed to confirm this in the future.

A. *Plagiorchis elegans*



B. *Echinoparyphium aconiatum*



C. *Trichobilharzia szidati*

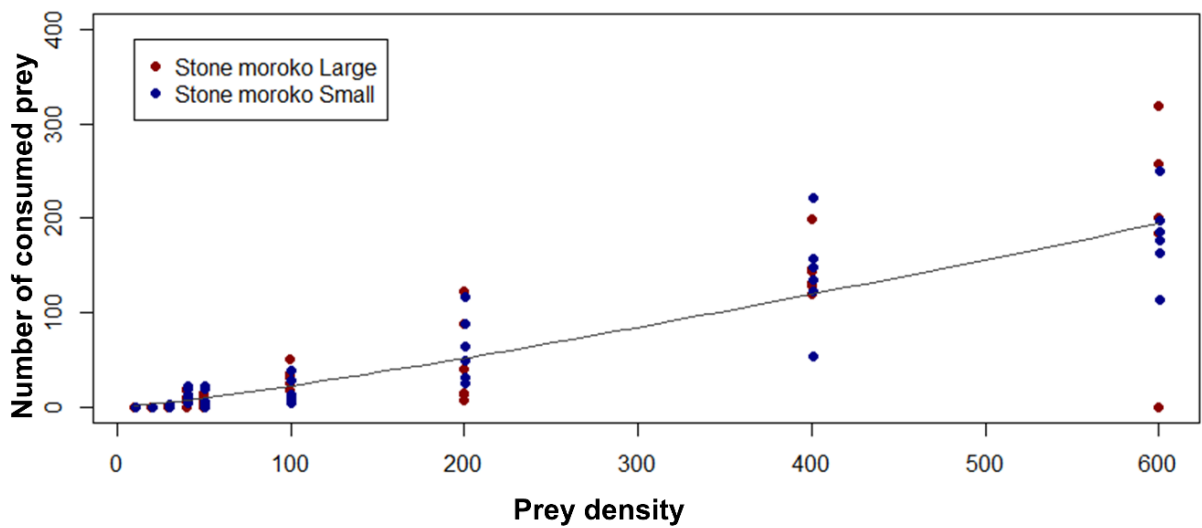


Fig. 11. Type III functional response curves, where feeding rate increases with prey density until it reaches saturation, for stone moroko preying on different densities (10, 20, 30, 40, 50, 100, 200, 400, 600) of trematode cercariae: A) *Plagiorchis elegans*, B) *Echinoparyphium aconiatum*, and C) *Trichobilharzia szidati*. The curve represents the fitted line for Type III response and the dots show the mean values for each replicate for both stone moroko – large and stone moroko – small. Note the different y-axis.

5. DISCUSSION

The transmission of trematode cercariae from first intermediate molluscan hosts to next hosts in their life-cycle is a challenging process due to non-host organisms that may interfere with free-living stages in many possible ways (Thieltges et al. 2008a, Johnson and Thieltges 2010, Johnson et al. 2010, Goedknecht et al. 2012). Trematode cercariae appear to be regular diet components of many organisms resulting in a reduced probability that trematodes reach their target hosts. However, there is a scarce evidence for the role of fish acting as effective predators of cercariae (Kaplan et al. 2009, Orlofske et al. 2012, 2015).

In this study, the predation effects of four fish species on the consumption of free-living cercariae of three trematode species (*Plagiorchis elegans*, *Echinoparyphium aconiatum* and *Trichobilharzia szidati*) were experimentally investigated with regard to specific life-history traits of both predator and parasite. However, due to the decreased ability of rainbow trout, roach and white bream to survive in laboratory conditions, only stone moroko could be employed in both experimental designs with all trematode species.

All fish species recognised trematode free-living cercariae as a food item, thus being capable of consumption. The efficiency of fish to reduce numbers of cercariae is evidenced by relatively high predation rates within 30 minutes of laboratory settings (single density: 18–41% reaching up to 60%; increasing densities: 7–25% reaching up to 40%). The same trend in consumption was found in both experimental designs using a single and different, increasing densities of cercariae, *E. aconiatum* and *T. szidati* being always the most and *P. elegans* the least consumed trematode. The high predation rates are partly consistent with results from earlier studies using marine and freshwater fish-trematode systems, just showing somewhat higher predation rates. Kaplan et al. (2009) demonstrated that up to 100% of cercariae can be removed by different species of California killifish. In a freshwater system, high consumption rates ranging from 38 to 75% and reaching up to 93% were recorded for a mosquitofish (*Gambusia affinis*) depending mainly on the biological and ecological traits of both predator and prey (Orlofske et al. 2012, 2015). Since experiments in the present and previous studies lasted for 30 minutes, it is possible that the use of different water volumes in experimental containers resulted in higher consumption rates (Kaplan et al. 2009, Orlofske et al. 2013, 2015). Smaller space could therefore constrain predators leading to an enhanced contact and visual detection of cercariae.

Direct predation and thus parasite removal can be affected by size of both hosts and trematodes in predator-prey systems. Small-bodied juvenile fish consumed the most cercarial stages, especially those of a large size (Kaplan et al. 2009, Orlofske et al. 2015).

Similar pattern was confirmed for larval insects (Catania et al. 2016, Welsh et al. 2018). Contrary to previous studies, results of this study indicate contrasting effects of fish size. White bream (mean standard length 60 mm) was the most effective fish in reducing cercarial numbers compared to stone moroko (35 mm), showing that larger fish species consume more cercariae. However, there were no significant differences in consumption between juvenile and adult stone moroko (SL 24 and 45 mm, respectively). Lower consumption rates could be expected for adults as they are larger and their feeding on zoobenthos would coincide with specific traits of trematode cercariae (e.g. Boltachev et al. 2006). Likely, the ability of stone moroko to feed on diverse food items may explain the similar predation rates (Hliwa et al. 2002). Furthermore, all four fish species consumed cercariae of *P. elegans* with equally lowest consumption rates, rainbow trout being the largest and stone moroko the smallest fish (81 and 35 mm, respectively). However, this is rather related to the size of cercariae than to the size of fish. The size of cercariae is similar to that of zooplankton which dominates in juvenile fish diet (Blanco et al. 2004), but the cercariae of *P. elegans* may be too small to be detected or represent an energetically unprofitable food item for fish of any size. These findings lead to the assumption that the predator's size may not be a determining factor reducing cercarial numbers, suggesting that size of cercariae and specific traits of both predators and trematodes are involved in the complexity of consumption processes.

Predation rates of cercariae varied among fish species and trematode species. The detected diverse intra- and interspecific consumption patterns are congruent with the hypothesis established in this study that fish would consume cercariae differently depending on species-specific traits. For example, cercariae that swim more frequently close to the bottom would be more frequently predated by a benthic fish. It was also observed previously that larger cercariae are most vulnerable to predation by fish (Kaplan et al. 2009, Orlofske et al. 2015). This is consistent with results of this study because the highest consumption was observed for the two large-sized cercariae of *E. aconiatum* and *T. szidati* and the lowest for the smallest cercariae of *P. elegans*. The size of *T. szidati* and *P. elegans* cercariae may be the main factor affecting their consumption. However, the feeding preference of white bream for smaller cercariae *E. aconiatum* compared to the lower consumption of the largest cercariae of *T. szidati* suggests that other mechanisms are involved. The combination of fish distribution in the water column, distinct morphotypes of cercariae, transmission modes, patterns in emergence and swimming behaviours in water column (e.g Haas 2000, Horák et al. 2015, Soldánová et al. 2016) may contribute to these contrasting consumption relationships.

Echinoparyphium aconiatum has benthic cercariae that actively select snail habitats to increase the chance of their contact. Cercariae occur near the bottom, but swim to the surface in early stage after emergence from a snail (Haas 2003). Since white bream prefers bottom habitats but can also occur in open water column (Kottelat and Freyhof 2007), the overlapping distribution of both predator and parasites in the whole water range reflects the observed high predation rates. Stone moroko consumed mostly cercariae of *T. szidati*. However, being a benthopelagic fish with rare swimming to open waters (Kapusta et al. 2008), stone moroko exhibits an inconsistent behaviour with the behaviour of *T. szidati*. Active swimming and aggregation of cercariae may increase the predation by a predator (Orlofske et al. 2015). This is particularly true for pelagic cercariae of *T. szidati* which are distributed within the water column and swim intermittently alternating resting and active periods (e.g. Horák et al. 2015). Fish use visual cues to detect a prey (Hall 1982), making cercariae of *T. szidati* potentially more attractive as the fish feeding may be triggered by burst of active swimming after the resting period. In support to this assumption, *T. szidati* was the most frequently predated species by stone moroko at all cercarial densities. This suggests that cercariae are a frequent subject to predation, being visible to predators even in low numbers. Yet, in nature context, the successful predation would require parasite traits matching to specific traits of predators in space and time.

Cercarial release from their first molluscan hosts is often synchronised with periods of host activity and behaviour, making the emergence rhythms adaptive to enhance contact with target hosts (Combes et al. 1994). The variety of patterns in cercarial release depends on the host life-history, which affects the distribution of cercariae within the water column. It is speculative how the diverse, and often species-specific, trematode emission strategies would influence the trophic relationships between the predator and prey in natural conditions. The overlapping circadian rhythms in feeding behaviour and cercarial emergence of *E. aconiatum* and *T. szidati* may facilitate predator-prey interactions in aquatic habitats (e.g. Sunardi et al. 2015, Říha et al. 2015). Both trematode species exhibit diurnal emergence, the former peaking in the middle of day (Haas 2000), and the latter early in the morning (Soldánová et al. 2016). In littoral zones of aquatic habitats, cercariae of *T. szidati* may form dense clouds, especially during sunny days, while awaiting ducks serving as definitive hosts (Soldánová et al. 2013, Horák et al. 2015). The co-occurrence of fish, snails, abundant populations of cercariae and the highest activity of stone moroko in the morning may result in a substantial impact on the cercarial population and transmission to definitive hosts. Similar mechanisms are involved in *E. aconiatum*, but the emergence patterns of *P. elegans* would make the likelihood for interactions unlikely due to the nocturnal emergence

and predominant feeding of fish during the daytime (e.g. Gorman 1980, Říha et al. 2015). Overall, there is no evidence to support these assumptions as it would require thorough studies on the circadian rhythms of both predator's feeding behaviour and cercarial emergence determining the day period at which are cercariae the most vulnerable to predators.

Functional responses describe the relationship between consumption patterns of a predator and prey density to understand the dynamics of predator-prey systems (Holling 1959). Functional responses have generally been divided into three types in which factors such as searching for a prey and handling of prey are included. Type I shows the linear increase of consumption rate with increasing prey density, until it reaches saturation point. It has been reported only in filter-feeding species (Jeschke et al. 2004). In Type II (curve shape of decelerating hyperbola) the capture increases with increasing prey density gradually approaching saturation when predators spend most of their time hunting for food at low densities. Type III produces a typical sigmoidal curve describing negligible consumption at low densities, increasing with increasing prey densities and reaching saturation at high levels of prey. This type is typical for vertebrate predators capable of learning from experience.

Consistent with predictions in this study, the numbers of consumed cercariae were affected by cercarial density, showing the Type III functional response of stone moroko towards the three trematode species. The same pattern was observed in relationship between stone moroko and zooplankton (Jackson et al. 2015). Only few studies have been concerned about the effect of varying cercarial densities on the consumption of cercarial stages using marine crustaceans and bivalves (Welsh et al. 2017) and freshwater fish and larval insects (Orlofske et al. 2015) but they did not determine the predator's functional response. Congruent with this and previous studies, cercariae were consumed most at high densities while few were recovered from low densities. This indicates the relatively high effectiveness of stone moroko in removing cercariae over a range of densities that may be present in natural aquatic conditions due to cercarial emission strategies.

However, only three or four different densities were used in the previous studies with a maximum density of 300 cercariae (Orlofske et al. 2015, Welsh et al. 2017). In the present study a range of densities from 10–800 cercariae was offered to fish. This was especially important to distinguish between functional response Type II and Type III to increase accuracy and robustness of analyses (e.g. Uzsko et al. 2015). This may probably be the reason why predators continued consuming cercariae at high densities without reaching saturation (Orlofske et al. 2015, Welsh et al. 2017). In this study, the use of a wider range of

cercarial densities allowed to detect the saturation point. This suggests that fish would need a minimum of 800 cercariae to lower feeding, supporting the notion that stone moroko represents potential threat to aquatic biodiversity by rapid depletion of food resources (Witkowski 2001). However, although the analyses clearly supported the Type III response for all trematodes, the trend is somewhat weak for *P. elegans*, probably because of the general lower consumption of cercariae.

The functional response analyses may allow predictions on the density-dependent ecological impact of invasive predators on the native prey (e.g. Dick et al. 2014). Stone moroko showed the highest ability to decrease *T. szidati* cercarial populations at all densities, thus representing a promising means of biological control. Cercariae of *T. szidati* are important causative agents of cercarial dermatitis in Europe, an itchy inflammatory skin reaction to penetrating cercariae entrapped in human skin (Soldánová et al. 2013, Horák et al. 2015). Fish predation may act as barrier significantly reducing the transmission to definitive host and infection in humans. Unfortunately, stone moroko is an invasive pest species able to modify the structure of aquatic invertebrates and indigenous fish communities (Witkowski 2011, Jackson et al. 2015). Therefore, using stone moroko to reduce risk of cercarial dermatitis would result in cascading impacts on lower trophic levels in food webs and severe consequences for aquatic ecosystems.

A significant reduction in cercarial numbers by fish supports a contention that fish may serve as important source of cercarial mortality (Lafferty 2008). The ability of fish to consume more than a half cercariae of *E. aconiatum* and *T. szidati* within a short period of 30 minutes would translate to a considerable decrease in infection levels in next hosts and trematode transmission success. For example, in natural conditions 118 cercariae (maximum of 1,303) of *T. szidati* can be released from a single snail during the two-hour morning shedding peak (Soldánová et al. 2016). This amount corresponds to the density of 100 cercariae in both experimental designs. Hence, 39 to 63 cercariae released from a single snail can be potentially consumed by a single fish under favourable conditions, i.e. being ‘at the right time at the right place’. It is worth nothing that both trematode cercariae of *T. szidati* and its snail host *Lymnaea stagnalis* are common and abundant in freshwater ecosystems, so that the cumulative annual biomass of cercariae may reach up to 4.65 tons (Soldánová et al. 2016). This suggests that the energetic benefit for predators is essential and supports the suggestion that cercariae provide energy for higher consumers, contribute to the energy flow through food webs, thereby significantly affecting the functioning, structure and dynamics of food web in natural ecosystems (e.g. Kuris et al. 2008, Thieltges et al. 2008b, Johnson et al. 2010)

Overall, the results of this study indicate that fish are potentially important predators and regulators of the trematode abundance and transmission processes in European freshwater ecosystems with negative impact for the parasite by decreasing its population sizes and excluding infective stages from the ecosystem, and positive impact for the predator by providing food and lowering infection levels and risk of disease. It also demonstrates the net-effect of parasite and trematode specific traits on the consumption of free-living stages, emphasising that biological and ecological aspects should be always considered when evaluating the role of parasites in trophic interactions. However, we should be aware that the presented data may not provide a sufficient evidence of truly cercarial consumption and digestion by fish as cercariae may be trapped on fish gills or lost by manipulation during experiments. In this study, several trials were made to visually confirm the presence of predated cercariae in necropsied stone moroko. However, the 30 minutes of experiments correspond to the time period when soft-bodied cercariae are not distinguishable (Kaplan et al. 2009). Another attempt with a specific fluorescent dye, making cercariae visible under a fluorescence microscope, also failed. Therefore, several parts of the fish body such as mouth, gills and sections of gut were preserved in molecular grade ethanol for future molecular analyses. Although the results are based on experiments in artificial conditions, this study has little reservation to believe that fish would not have consumed cercariae for three main reasons. First, the low number of cercariae retrieved from control trials demonstrates effectiveness of the applied method as well as minimal effect of human factor. Second, the proportion of consumed cercariae is high enough to believe that cercariae are not lost by any other means than predation. Third, the camera footage showed that the activity of fish actually corresponds to the consumption, as evidenced by the number of remaining cercarie after the end of experiments.

This study raised several ideas that should be addressed in the future for a better understanding of complex relationships between predators and free-living trematode cercariae and the role of parasites in the energy aspect of food webs and ecosystem functioning in general.

- 1) Further studies are needed to more closely examine the consumption of cercariae by other organisms such as larval insects or bivalves to assess their importance in trematode transmission.
- 2) It is essential to study, so far largely unexplored, the predator's functional response to a changing prey density in various predator-trematode cercariae systems in prediction of their effect on disease risks.

- 3) It would be interesting to test the extent to which fish reduce numbers of cercariae in the presence of alternative hosts or decoy organisms, which act as diluting mechanisms decreasing infection levels in next hosts. Offering an alternative prey to a predator, such as small crustaceans that are of similar size as cercariae, in the presence of trematode cercariae may yield different results than those observed in this study as fish may selectively feed on one prey. This would help to assess the energetical benefit of trematode cercariae for fish.
- 4) The most interesting would be to conduct predator-prey experiments in the presence of two different trematode species to test the validity of fish feeding preferences with respect to the specific traits of trematode cercariae. To the best knowledge of this study, this has not been tested yet.
- 5) Investigations on circadian rhythms in the feeding behaviour of fish and cercarial emission by means of short time intervals during a day would help to determine the highest risk for the transmission of trematodes, and possibly serve as a tool for controlling risk disease of medical and veterinary important parasites.
- 6) Performing experiments under natural conditions would provide further support to the findings in the present and previous studies that were conducted under simplified laboratory environment.

6. CONCLUSIONS

The following conclusions can be drawn as results of the present study:

- 1) Fish are able to consume free-living cercariae of the three trematode species studied, but with different consumption patterns depending on the specific traits of both fish and trematodes.
- 2) Large-sized fish prey upon trematode cercariae as evidenced by the highest consumption by white bream, but no significant differences in cercarial consumption was found between small-sized (juveniles) and large-sized (adults) stone moroko.
- 3) Large cercariae are most vulnerable to predation compared to small ones, but other species-specific traits are likely involved such as morphotype, transmission mode, patterns in cercarial emergence and swimming behaviour in water column.
- 4) Fish consumption of cercariae is affected by cercarial density with increasing number of consumed cercariae with increasing densities.
- 5) The functional response of Type III was found for the consumption relationships between stone moroko and the three trematode species. The low consumption at low prey densities proved that fish do not consume cercariae at these densities, which is characteristic for this type of response.
- 6) Stone moroko appears to be the best model to study the complex interactions between predator and trematode cercariae, but its practical use for biological control is impossible because it is an invasive pest species.
- 7) The results of this study indicate that fish are potentially important predators and regulators of the trematode abundance and transmission processes in European freshwater ecosystems.
- 8) The results also confirm that trematode cercariae play an important ecological role in the energy flow in food-webs of aquatic ecosystems.

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