Master's thesis

Australapatemon sp. (Trematoda) infection in *Valvata macrostoma* and in two leech species, *Helobdella stagnalis* and *Erpobdella octoculata.*

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ABSTRACT

The infectivity of *Australapatemon sp*. was examined in three hosts species; Large-mouthed valve snail, *Valvata macrostoma*, the first intermediate host and two leech species, *Helobdella stagnalis* (Glossiphoniidae) and *Erpobdella octoculata* (Erpobdellidae), the second intermediate hosts. Hosts individuals were sampled monthly (May-September 2008) from Lake Konnevesi, Central Finland, from a shallow littoral site (<2 m) and a deep sublittoral site (5-6 m), located ca. 50-60 m apart. 20 individuals of both leech species were each experimentally exposed to 30 *Australapatemon sp*. cercariae shed by the snails. 1.15% snails were infected with *Austalapatemon sp*. cercariae while 22.3% with other trematode species (shallow site). *Australapatemon sp.* did not occur in deep site snail samples. Infection rate between the two leech species was significantly different i.e. higher in *E. octoculata* than *H. stagnalis*. There was significant seasonal variation in the prevalence and intensity of *Australapatemon sp.* in *E. octoculata*. Experimental results revealed that both leech species were equally susceptible and acted as suitable second intermediate hosts. 75% of the cercariae successfully penetrated and established metacercarial cyst infection. This result indicates that infection difference between two leech species is not a question of evolutionary specificity (genetical) but ecological differences in activity that exists between them.

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TIIVISTELMÄ

Australapatemon sp. loisintaa tutkittiin kolmessa väli-isäntälajissa; *Valvata macrostoma* kotilossa , joka on tämän loisen ensimmäinen väli-isäntä ja kahdessa juotikaslajissa, *Helobdella stagnalis* (Glossiphoniidae) ja *Erpobdella octoculata* (Erpobdellidae), jotka ovat toisia väli-isäntiä. Näytteitä kerättiin kuukausittain (toukokuusta syyskuuhun v. 2008) Konnevedestä Keski-Suomessa. Keräys tehtiin kahdelta paikalta, matalasta litoraalista (<2 m) ja 5-6 m syvyydestä. Näiden paikkojen etäisyys toisitaan on n. 50-60 m. Lisäksi altistin kokeellisesti molempien lajien juotikkaita siten, että kukin juotikas altistettiin 30 *Australapatemon sp*. kerkarialle, 20 juotikasta molemmista lajeista. *Australapatemon sp* loisinnan prevalenssissa sekä abundanssissa oli merkitsevää vaihtelua kuukausien välillä kaikissa kolmesssa isäntälajissa. Matalasta kerätyistä kotiloista ainoastaan 1.15% oli *Australapatemon sp* lajin loisimia, muiden imumatolajien loisinta samoissa kotiloissa oli 22.3% . Syvältä kerätyissä kotiloissa loista ei esiintynyt. Loisinta erosi merkitsevästi kahden juotikaslajin välillä (p= 0.02), *E. octoculata* juotikkaissa loisinta oli merkitsevästi korkeampi kuin *H. stagnalis* yksilöillä. Kokeellisessa altistuksessa molemmat juotikaslajit olivat yhtä alttiita loisille ja sopivia toisiksi väli-isänniksi. Jopa 75% kerkariatoukista pystyi tunkeutumaan juotikkaisiin ja kehittymään niissä metakerkaria toukiksi. Johtopäätöksenä voidaan todeta, että luonnossa havaitut erot juotikaslajien loisinnassa johtuvat ekologisita syistä (esim. eroista aktiivisuudessa) eikä eroista geneettisessä yhteensopivuudessa (spesifiteetissä).

Contents

1 INTRODUCTION

1.1 Parasitism

Parasites make up the majority of species on earth and may outnumber free-living species. Parasitism is one type of the symbiotic relationships that exist between two different organisms. The parasite lives for a reasonable period of its life in or on another organism, the host, from where it obtains food and shelter (Poulin 2007). To cope with their parasitic mode of life parasites show a high level of morphological adaptation and specialization. Parasites are extremely common. It is thus difficult for any free-living organism to escape parasite infection although the infection intensity amongst the host species varies greatly. Parasites rarely kill their host but heavily infected hosts can be more prone to die of additional stress. Some findings have revealed that this parasitic association has some negative effects on the fitness of the host organism by reducing host survival (Hudson *et al*. 2006) through impairment of feeding and assimilation, growth, fecundity, mediate host vulnerability to predators (Seppälä *et al*. 2004) and alteration of host behaviour (Holmes & Bethel 1972, Kinne 1980, 1983, Sousa 1983). The biodiversity and natural history of parasites in many systems are still uncharacterized.

1.1.1 Digenean trematodes

There are approximately 25,000 species of digenetic trematodes (flukes), the adults and larvae of which parasitize a variety of vertebrate and invertebrate animals. Trematode infections are cosmopolitan. The main character of the trematodes is the presence of suckers around the mouth and/or ventral suckers, both for attachment and locomotion. Although the life cycles of some trematode species have been thoroughly studied, much is still unknown about the life cycle/history of a great majority of them. The life cycle of a trematode is complex involving two or three different host species through which the parasite has to pass for completion of the cycle. Some few uncommon exceptions have been found not to follow this pattern. For example, Barker & Gibbs (1993) and Barger & Esch (2000) reported a one-host life cycle in a prohemistomid fluke, *Mesostepharias haliaturis* and an opecoelid trematode, *Plagiophorus sinitsini* respectively. Esch *et al.* (1997) on the other hand reported a four-host life cycle of the hemiurid fluke, *Halipegus occidualis*. However, the three-host life cycle is the basic, ancestral and probably the most common for digenetic trematodes (Brooks & McClennan 1993, Poulin 1998, Bush *et al*. 2001). Irrespective of the number of hosts needed, in almost all trematode species the first host in the life cycle is a mollusc that plays the key role (Esch *et al.* 2001).

In a typical digenean trematode life cycle, sexual reproduction usually occurs in the vertebrate definitive host in which the trematode reaches sexual maturity. They release the eggs mostly with faeces/urine into the water where the first intermediate hosts (usually a snail or a clam) get infected either passively or actively depending on the trematode species. Some eggs are passively eaten by a snail where they hatch within the snail releasing a larva called miracidium which is not free swimming (e.g. Lancelet fluke, *Dicrocoelium dendriticum*) while eggs of other species hatch in water under suitable conditions into an active first free-swimming ciliated miracidium larva. The free-swimming miracidium does not feed hence it has a brief life span generally between 24-36 hours. (Esch *et al*. 2001) It uses a variety of sense receptors to find the first intermediate mollusc host (Wrigth 1971). Trematodes are highly specific for their mollusc hosts (Basch 1976, Van deer Knaap & Loker 1990). Thus the first swimming behaviour of the miracidium is to find its specific mollusc which it penetrates, loses its ciliated epithelium and metamorphoses into the mother sporocyst. Different trematode species will undergo different patterns of larval development, the mother sporocyst of some species will undergo asexual reproduction to give daughter sporocysts which migrates towards gonads and/or digestive glands of the intermediate host. Daughter sporocysts give rise to cercariae. In others, mother sporocyst may give rise to either mother rediae that produces cercariae (*Clonorchis sinensis*, *Opisthorchis felineus*) or daughter rediae that produce cercariae (*Fasciola hepatica*, *Fasciolopsis buski*). Cercariae of different species show different strategies, some stay and encyst while other species release their cercariae. For species with emergent cercariae the daughter sporocyst and redia will undergo further asexual reproduction producing embryos that develop to hundreds, thousands or hundreds of thousands of clones of free-swimming cercariae per day (Esch & Fernández 1994, Esch *et al*. 2001).

Generally, fully mature cercariae leave the molluscan host either actively or passively and infect the second intermediate host in the life cycle. Experimental findings by Karvonen *et al*. (2006) reported that the trematodes, *Diplostomum spathaceum* and *Diplostomun gasterosteri* shed 11,966 and 4,471 cercariae respectively, per day. Some trematode species do not require a second intermediate host, but may penetrate the definitive host directly (schistosomes), or may encyst on vegetation (fasciolids), or may encyst in the same host in which they are produced (some echinostomes). Transmission to the final host occurs when an appropriate vertebrate host eats a parasitized second intermediate host. Metacercariae excyst in the gut of the definitive host in response to host digestive enzymes, pH or temperatures and migrates to a site, which is specific for the particular species of fluke, and matures sexually and cycle starts all over again.

1.2 *Valvata macrostoma* snails

Large-mouthed valve snail, *Valvata macrostoma,* a prosobranch hermaphroditic species can be commonly found in aquatic systems in Northern and Western Europe. They are in the UK Red Data Book (RDB) (Bratton 1991) and are recorded as ‘declining but widespread’ in Europe (Wells & Chatfield 1992, 1995). They can be found inhabiting a range of ecosystems like fluvial flats in rivers, marshy littoral areas of lakes, small water bodies with abundant aquatic vegetation and temporary waters (Glöer 2002, Watson & Ormerod 2004). The adults often die after reproduction (semelparous). Details of their life span and life cycle are not available except for a related species *V. piscinalis* (Glöer 2002, Ducrot *et al*. 2006). Despite the fact that *V. macrostoma* is common in some parts of Europe, little is still known about its trematodes. Previous studies have described spatial and temporal structure of the trematode component community in *V. macrostoma* and the different trematode cercariae shed by *V. macrostoma* one of which is *Australapatemon sp*. (Faltýnková *et al*. 2007, 2008) but there are no studies on their life cycle and seasonality (see also Gérard 1997, 2001, Gérard *et al*. 2008). Thus, *Valvata* species serves as hosts for a considerable range of trematode species.

Faltýnková *et al*. (2007) recorded the first findings of *V. macrostoma* for larval stages of *Australapatemon sp*. in Lake Konnevesi, Central Finland. To complete the life cycle of *Australapatemon sp.,* the definitive host, the birds (probably ducks), in which asexual reproduction takes place releases eggs into the water where the free-swimming ciliated miracidium hatches. They are infective to their first intermediate host, *Valvata macrostoma*.

Using their sense receptors, the miracidium penetrates the first intermediate host and loses its ciliated epithelium. Within the snail the miracidium undergoes asexual reproduction and develops into cercariae which are released into the water. The cercariae in turn penetrate the leeches, their second intermediate host and encyst to metacercariae. For effective transmission birds have to consume parasitized leeches. Metacercariae then excyst in the definitive hosts, the birds and the cycle recommences again.

1.3 Leeches (Hirudinea)

Leeches occur worldwide, except for the Antarctica, in many ecosystems including marine, estuarine, moist terrestrial and freshwater systems (Govedich & Bain 2006). They can be found in lentic and lotic waters where they form useful components of the benthic communities. Leeches can be found attached to the underside of rocks, stones, vegetation, logs and other submerged substrates (Govedich & Bain 2006).

Leeches are protandrous hermaphrodites (Kutschera & Wirtz 2001) and when the eggs hatche, the emerged juvenile leeches have unchanging number of segments. Clitellum is visible only during reproductive period and courtship procedures are usually not known except in some land leeches, which might be easier to observe than aquatic ones (Leslie 1951). Mating usually involves the intertwining of the two leeches where each deposits sperm in the clitellar opening of the other usually during night time when leeches are most active (Kutschera & Wirtz 2001). Eggs are deposited in a gelatinous cocoon containing nutrients, further treatment of this cocoon depends on the parent leech species. Leeches of the family Erpobdelliidae either bury or attach their cocoons to a rock, or leaf and leave them afterwards. The cocoon darkens and hardens after some few days. Leeches of the family Glossiphoniidae show a high level of parental care. They brood their eggs until the eggs hatch and carry their young under their dorsoventrally flattened bodies (Light & Siddall 1999). The offsprings stay attached until they reach a certain size. After their first meal some can survive on their own while others still stay attached for some time.

Leeches make up important components of aquatic ecosystems (Koval'chuk & Chernaya 2003). They significantly contribute to the biological diversity and take part in some ecological processes where they act as second intermediate hosts of some trematode parasites. As important link of trophic chains, ecological studies on parasites of aquatic animals including leeches is now of special importance (Koval'chuk & Chernaya 2003).

Helobdella stagnalis (Glossiphoniidae) and *Erpobdella octoculata* (Erpobdelliidae) are two common European leeches that occur regularly in the same water bodies, usually under the same stones (Young *et al*. 1995) but differ from each other. They are also common in Lake Konnevesi, Central Finland. *E. octoculata* do not take care of their offsprings, they produce cocoons which they ventilate only for about 10 minutes and abandon (Kutschera & Wirtz 2001). These unprotected fresh cocoons are in danger of being eaten by many potential predators (conspecifics and water snails) and even after hardening the dark-brown cocoons can be attacked by water snails (Pawlowsky 1955, Sawyer 1970, Elliot 1973 & Young 1988). On the contrary, *H. stagnalis* shows the most developed level of parental care to their offsprings (Kutschera & Wirtz 2001). Hatched larvae develop into juvenile leeches still attached to the ventral side of the parent leech for about three to four weeks. The young are frequently fed by the parent. With this high level of protection and consistent food, juveniles grow during the three weeks of post-embryonic parental care. They leave the parent after they have reached about one third the body length of the adult (Kutschera & Wirtz 1986a).

1.4 Objectives

In this work I studied the seasonality and a part of the life cycle of *Australapatemon sp.* in *Valvata macrostoma* (first intermediate host) and in two leech species (second intermediate hosts), collected from Lake Konnevesi. The two leech species I found to be second intermediate hosts for *Australapatemon sp.* are *Helobdella stagnalis* and *Erpobdella octoculata*. Before this study *Austalapatemon sp.* was only known from its first intermediate host *V. macrostoma* (Faltýnková *et al.* 2007).

I followed *Australapatemon sp.* cercariae emerging from *Valvata macrostoma* and its metacercariae in the two leech species (*Helobdella stagnalis* and *Erpobdella octoculata*). I studied the seasonality alongside infection parameters like prevalence, mean abundance and intensities of *Australapatemon sp.* in these hosts. I also carried out an experiment exposing both leech species to *Australapatemon sp* cercariae in order to study the possible specificity of the leeches to this trematode.

2 MATERIALS AND METHODS

2.1 Study area

The study was conducted in Lake Konnevesi (62° N, 26° E), a large oligotrophic lake in Central Finland with an area of 113 km^2 and mean depth of 13 m (maximum depth 56 m). All animals were sampled from benthic samples that were collected from two main areas of the lake: shallow littoral site (depth <2 m) and deep sublittoral site (depth 5-6 m). The shallow site had a muddy bottom substrate and benthic and floating aquatic plants (e.g. *Isoëtes sp., Potamogeton sp.*), while the deep sublittoral site had a bottom substrate of mud and small rocks but no plants. The two sites were in close proximity to each other, ca. 50–70 m. Leeches where collected from the same place where *Valvata macrostoma* occurred.

2.2 Sampling of *Valvata macrostoma* and processing of cercariae

Large-mouthed valve snails, *Valvata macrostoma* Mörch, 1864 [the nomenclature follows that of Glöer (2002)] (Figure 1) were collected monthly between June and September 2007 by grap samples from the two study sites of Lake Konnevesi, Central Finland. These samples were brought immediately to the laboratory for processing.

In the laboratory, samples were aerated and taken in bits and placed on white trays for isolation of *Valvata* snails. The snails were placed into different transparent jars depending on the area from where they were collected. After snails were isolated, they were placed into small multi-well plates filled with lake water. These dishes were then kept overnight (11 pm – 9 am) under light to stimulate shedding of cercariae and the snails were examined. After observation, each snail was placed into a transparent cup (about 5 cm in diameter) with about 300 ml of fresh lake water. This water was changed on every second day. These snails were fed with algae growing on submerged parts of *Equisetum fluviatile*. For snails shedding cercariae four to five live cercariae were pipetted and placed on a slide for observation. The cercariae were observed under a light microscope for identification which was based on the study of Faltýnková *et al*. (2007). Snails shedding *Australapatemon* sp. were kept aside for the exposure experiment.

Figure 1. Picture of large-mouthed valve snail, *Valvata macrostoma.*

2.3 Sampling of leeches and processing of metacercariae

Leeches (Annelida: Hirudinea) of two species, *Erpobdella octoculata* Linnaeus, 1758 (Erpobdellidae) and *Helobdella stagnalis* Linnaeus, 1758 (Glossiphoniidae) (Figures 2 and 3), were collected monthly between May and September 2007 by grap samples from the two study sites of Lake Konnevesi, Central Finland. These samples were brought immediately to the laboratory for processing. Some leeches were hand-picked from under the stones on the shore of the lake from May to September. In the laboratory, samples were aerated, taken in bits and placed on flat trays for isolation of leeches using forceps. Leeches were placed into different transparent containers filled with lake water depending on the area from where the benthic sample was collected. When isolation was completed, each leech was identified based on a key to leeches (Lukin 1976) (main characters important for identification were size, pigmentation, position and number of eyes). Afterwards, each leech was slightly pressed between two glass slides and observed under a stereomicroscope to assess the state of infection and the number of metacercariae was counted. After this examination, each leech was placed in a plastic container of 100-300 ml of fresh lake water. A layer of sand and a stone was added into each dish to provide shelter. Details of each leech were recorded behind (i.e., species, site and date of collection, infected/uninfected and the number of metacercariae). Containers with the bigger and active species (*E. octoculata*) were covered to prevent them from escaping. Leeches were fed every second day after water had been changed and remaining food was removed. The leeches were fed with crushed aquatic crustaceans (*Asellus aquaticus*), annelids (*Tubifex)* and insect larvae (mayflies, stoneflies, caddis flies), collected from the same place as the leeches. These animals were maintained in an aerated aquarium with a layer of sand and aquatic plants (*Calitriche palustris*) to serve as shelter.

Some infected leeches were randomly selected and dissected. Metacercariae were isolated mechanically from the tissue and examined under the stereomicroscope for identification. Ten replicates of each species from each month were fixed in hot 4% formaldehyde solution in a fume cupboard and their length (in mm) was measured. This same protocol was followed each time benthic samples were brought to the laboratory. All leeches were maintained alive for other experiments.

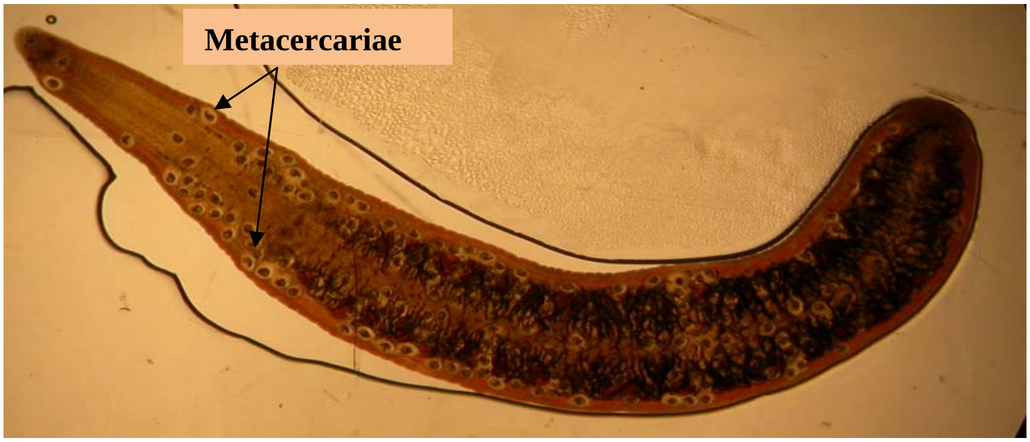

Figure 2. Picture showing the dorsal view of *Erpobdella octoculata* infected with metacercariae.

Figure 3. Picture showing the dorsal view of *Helobdella stagnalis* infected with metacercariae.

2.4 Experimental exposure of leeches to *Australapatemon sp.* cercariae

Twenty individuals of both *Erpobdella octoculata* and *Helobdella stagnalis* were selected for the experimental exposure to cercariae of *Australapatemon sp*. At the same time, it was proved that the metacercariae from natural infections belongs to *Australapatemon sp*. Ten individuals of each species served as unexposed controls. Five infected and five uninfected individuals from *E. octoculata*, and four infected and six uninfected individuals from *H. stagnalis* were used. They were kept two days without food prior to exposure to *Australapatemon sp*. Each leech was exposed to ca 10 cercariae of *Australapatemon* sp. in an amount of 3 ml of water for 3 h, after this the water was filled up to 120 ml. Because of few cercariae emerging from the snails, this was repeated three times during three consecutive days. In total, each leech thus received a dose of 30 cercariae. Exposed leeches were covered with nets and maintained for ca three months. The exposure was done in July and observation was in September (three months).

2.5 Statistical Analysis

Even with logarithm transformation the assumptions of ANOVA were not satisfied in the field data. Therefore, non-parametric Mann-Withney U Tests and Kruskal-Wallis Tests were used to analyse differences in the intensity of *Australapatemon sp.* in the two leech species. The effect of initial infection on parasite number and the degree of susceptibility between the two leech species after three months of experimental exposure were analysed using AVOVA (covariate=parasite number, fixed factor=infected/uninfected). Chi-square Tests were also used to analyse differences in the prevalence of infection and the seasonal variation of infection. Data were analyzed with SPSS software version 15.00.

3 RESULTS

3.1 Seasonality and prevalence of *Australapatemon sp.* cercariae in *Valvata macrostoma*

A total of 812 *Valvata macrostoma* were collected from June to September from the two sites in Lake Konnevesi. There was a striking difference in the total number/proportion of snails collected from the two sites, 75.1% (n=610 out of 812) (Table 1) from the shallow site and 24.9% (n=202 out of 812) from the deep site. The highest numbers/proportion of snails were collected in July (28.7%, n=233) and the lowest in June (19.6%, n=159) from the two sites.

The prevalence of infection of *Valvata macrostoma* by *Australapatemon sp*. cercariae was very low and varied between the months and the two sites. *Australapatemon sp*. cercariae were found only in samples from the shallow site. However, the monthly variation in the prevalence of *Australapatemon sp*. was statistically significant (χ^2=1.531, df=3, P=0.675). In total, only 1.2% of *V. macrostoma* individuals collected from the shallow site were infected by *Australapatemon sp*. with the highest prevalence in June (1.71%) and lowest in August (0.69%) (Table 1 and Figure 4), while 22.3% snails were infected by other trematode species. From the deep site, none of the snails were infected with *Australapatemon sp.* cercariae (Table 1) while 7.4% (n=12) snails were infected by other trematode species. The prevalence of *Australapatemon sp.* cercariae between the two sites was statistically insignificant (χ^2=2.414, df=1, P=0.120).

Table 1. *Valvata macrostoma* collected from June to September 2008 from the study sites in Lake Konnevesi and the seasonal variability in the prevalence (%) of *Australapatemon sp.* cercariae.

Sampling sites	Sampling months	No collected	Prevalence (%)	No infected by *Australapatemon sp.* cercariae
Shallow littoral site (< 5m)	June	117	1.71	2
	July	211	1.42	3
	August	144	0.69	1
	September	138	0.72	1
	Total	610	1.15	7
Deep sublittoral site (2-5 m)	June	42	0	0
	July	22	0	0
	August	49	0	0
	September	89	0	0
	Total	202	0	0

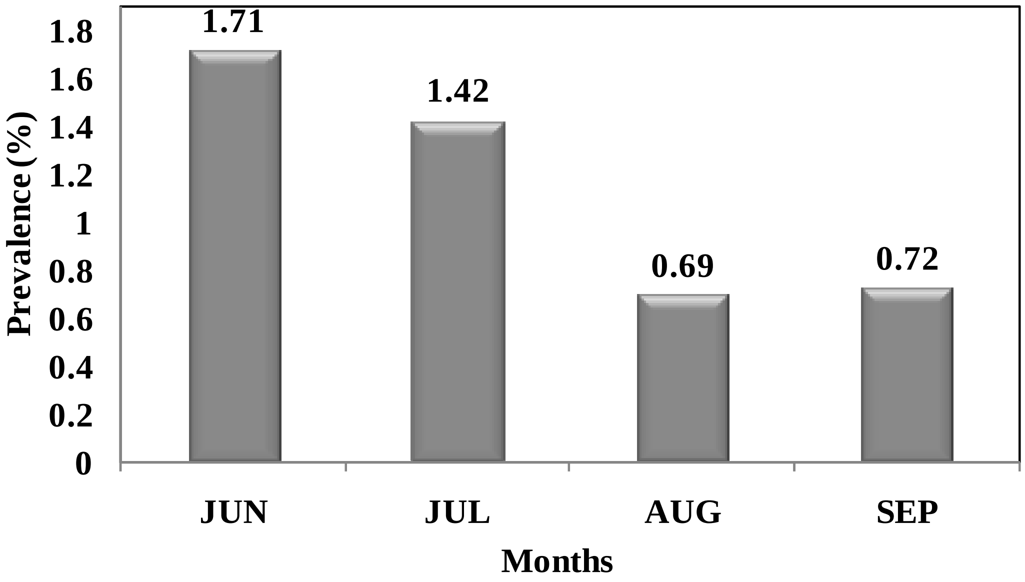

Figure 4. Seasonal variation in the prevalence (%) of *Australapatemon sp.* cercariae in *Valvata macrostoma* collected from June to September 2008 from the shallow site (<2 m) in Lake Konnevesi. Values above bars represent the monthly percentage of infected snails.

3.2 Seasonality, prevalence and abundance of *Australapatemon sp.* metacercariae in *Erpobdella octoculata* and *Helobdella stagnalis*

A total of 750 leeches were collected from May to September from the study sites in Lake Konnevesi, 437 individuals of *Helobdella stagnalis* and 313 individuals of *Erpobdella octoculata* (Table 2). The number of leeches collected varied monthly with the highest numbers/proportions of leeches collected in September 34.1% (n=152 out of 750) and lowest in May 1.7% (n= 13) for the two leech species (Table 2). There was a clear difference in the total number/proportions of both leech species collected from the two sites with a higher number/proportion from the shallow site 86.8% (n=651) compared to the deep site 13.2% (n=99).

The infection rate between the two leech species differed significantly (χ^2=6.742, df=1, P=0.009) with a higher prevalence of *Australapatemon sp*. metacercariae in *Erpobdella octoculata* (38.7%) compared to *Helobdella stagnalis* (29.6%). Also the mean intensity (± SE) of infection was higher in *E. octoculata* (40.6 ± 6.5) than in *H. stagnalis* (1.9 ± 0.1) (Mann Withney U-Test, U=3931.0, P<0.001). There was a seasonal variation in the prevalence and abundance (± SE) of *Australapatemon sp.* metacercariae in the two leech species. They were higher in June (58.5% prevalence and 1.23 ± 0.15 abundance) and lower in May where no leech was infected for *H. stagnalis* (Table 2, Figures 5 and 6). In *E. octoculata*, the highest prevalence was in July (53.6%) and highest abundance in August (36.34±9.86) and lowest prevalence and abundance in May (18.2% and 0.45±0.31) respectively, (Table 2, Figures 7 and 8). However, from the shallow site, there was a significant seasonal variation in the prevalence of *Australapatemon sp.* metacercariae in *E. octoculata* (χ^2=14.079, df=3, P=0.003) but in *H. stagnalis* it was statistically insignificant (χ^2=3.111, df=3. P=0.375). There was an aggregation of *Australapatemon sp*. metacercariae in *E. octoculata* where each leech contained more than 50 metacercariae in 9.9% (n= 31 out of 313) individuals that contained 88.6% (n= 4349 out of 4910) of the total metacercariae.

A greater number of the metacercariae found in these two leech species were encysted and a lesser number were unencysted (new infection). In *H. stagnalis* unencysted metacercariae were observed only in August while in *E. octoculata* they were observed between June to September, but varied monthly with the peak in July (Figures 10 and 11).

Table 2. Total number of *Helobdella stagnalis* and *Erpobdella octoculata* individuals collected from May to September 2008 from the study sites in Lake Konnevesi and the seasonal variability in the prevalence and average mean number (± SE) of *Australapatemon sp*. metacercariae in these two leech species.

Species	Site	Sampling months	No collected	No infected	Prevalence (%)	Abundance ± SE	Range in Mtc (Min - Max)
H. stagnalis		May	2	0	0	0	0
	Shallow littoral site (<2 m)	June	88	53	60.2	1.25 ± 0.16	0 - 6
		July	83	21	25.3	0.46 ± 0.09	0 - 3
		August	42	18	42.9	0.71 ± 0.19	0 - 7
		September	184	19	10.3	0.18 ± 0.05	0 - 6
		Total	399	111	27.8	0.53 ± 0.05	
	Deep sublittoral site (5-6 m)	May	–	–	–	–	–
		June	6	2	33.3	1.00 ± 0.68	0 - 4
		July	30	15	50	0.90 ± 0.21	0 - 4
		August	2	1	50	1.00 ± 1.00	0 - 2
		September	–	–	–	–	–
		Total	38	18	47.4	0.92 ± 0.91	
E. octoculata		May	11	2	18.2	0.45 ± 0.31	0 - 3
	Shallowlittoral site (<2 m)	June	6 1	15	24.6	0.85 ± 0.31	0 - 14
		July	49	37	75.5	27.69 ± 5.61	0 - 157
		August	71	30	42.3	36.86 ± 9.93	0 - 385
		September	60	16	26.67	12.97 ± 5.46	0 - 225
		Total	252	100	39.7	19.08 ± 3.38	
	Deep sublittoral site (5-6 m)	May	–	–	–	–	–
		June	1	0	0	0	0
		July	48	15	31.3	1.65 ± 1.25	0 - 60
		August	–	–	–	–	–
		September	12	6	50	1.83 ± 0.89	0 - 10
		Total	61	21	34.4	1.66 ± 0.99	

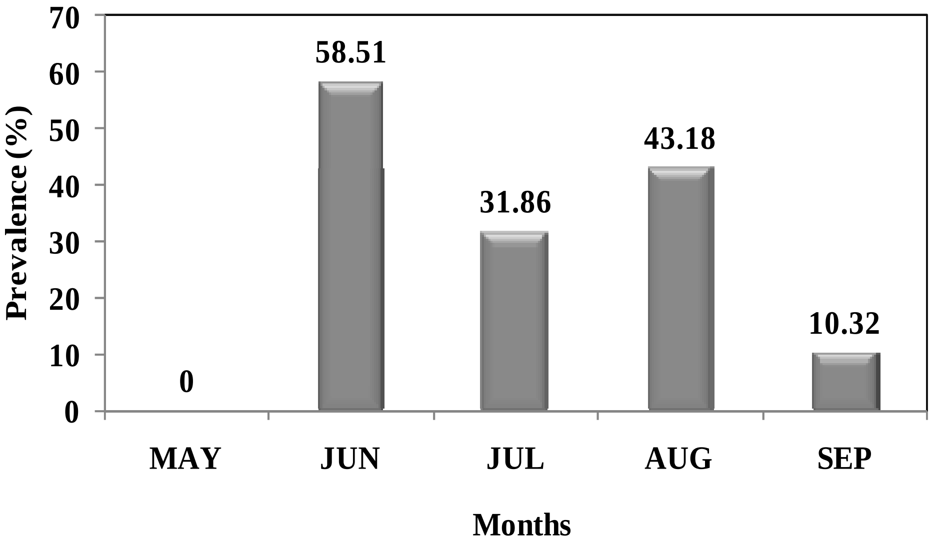

Figure 5. Seasonal variation in the prevalence (%) of *Australapatemon sp*. metacercariae in *Helobdella stagnalis* collected from May to September 2008 from the study sites in Lake Konnevesi. Values above the bars indicate the monthly percentage of infected leeches.

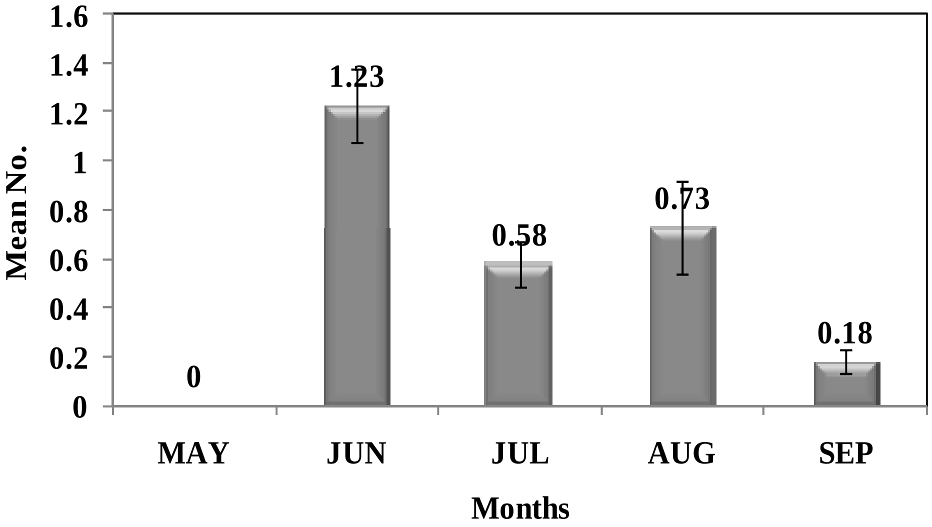

Figure 6. Seasonal variation in the average mean number (± SE) of *Australapatemon sp.* metacercariae in *Helobdella stagnalis* collected between May to September 2008 from the study sites in Lake Konnevesi. Error bars represent the monthly standard deviation of the average number of metacercariae in the leeches and the values above the bars represent the monthly average mean number of metacercariae.

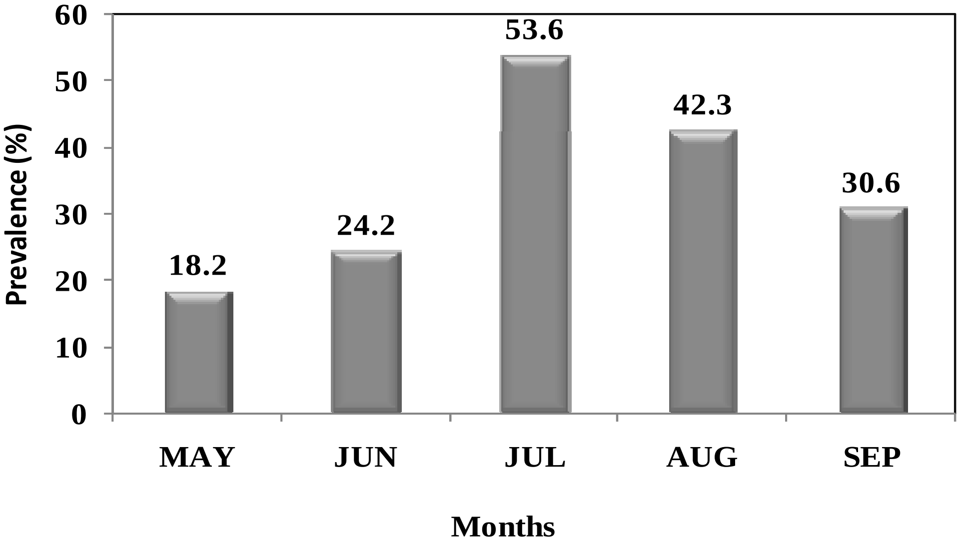

Figure 7. Seasonal variation in the prevalence (%) of *Australapatemon sp.* metacercariae in *Erpobdella octoculata* collected from May to September 2008 from the study sites in Lake Konnevesi**.** Values above the bars indicate the monthly percentage of infected leeches.

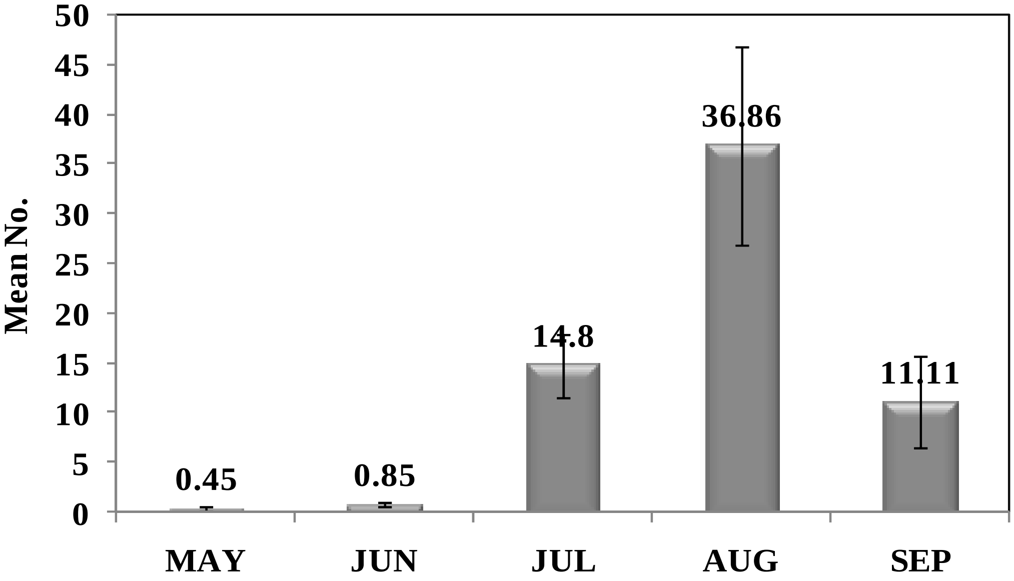

Figure 8. Monthly variation in the average mean number (± S.E) of *Australapatemon sp.* metacercariae in *Erpobdella octoculata* collected between May to September 2008 from the study sites in Lake Konnevesi. Error bars represent the monthly standard deviation of the average number of metacercariae in the leeches and the values above the bars represent the monthly average mean number of metacercariae.

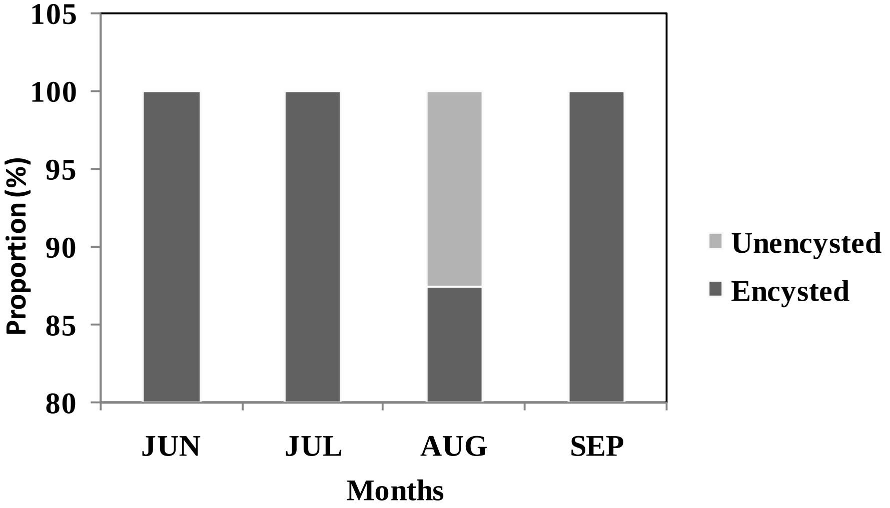

Figure 10. Monthly proportions (%) of encysted and unencysted metacercariae in *Helobdella stagnalis.*

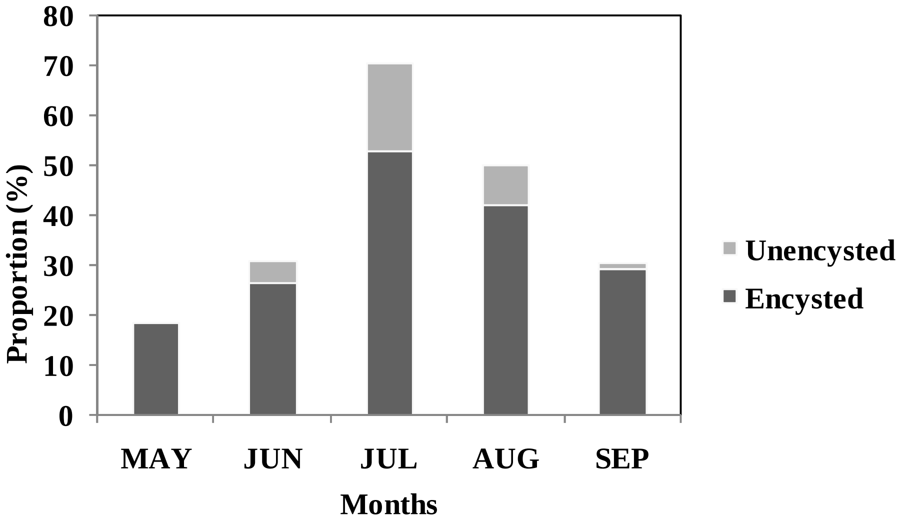

Figure 11. Monthly proportions (%) of encysted and unencysted metacercariae in *Erpobdella octoculata*

3.3 Experimental exposure

Nine individuals (90%) of the initially infected leeches of the two species showed new infections after an experimental exposure while four individuals (40%) of the initially uninfected leeches and all controls (100%) did not show new infection (Table 3). Analysis of variance indicated that initial infection had an effect on parasite numbers after exposure such that they were higher in leeches infected already before the exposure (Table 4 and Figure 9). There was no difference in susceptibility between the two leech species (ANOVA, F=0.416, df=1, P=0.528) (Table 4). However, there was a significant interaction between the factors (AVOVA, F=4.658, df=1, P=0.048) (Table 4) probably because of the difference in abundance of metacercariae between the treatments in *Helobdella stagnalis* (Table 4 and Figure 9).

Table 3. Intensity of *Australapatemon sp.* metacercariae in experimentally exposed *Helobdella stagnalis* and *Erpobdella octoculata* individuals after three months.

Species	Previous infection (Yes/No)	Intensity of previous infection	Intensity after three months	New infection
H. Stagnalis	No	0	12	12
	No	0	8	8
	No	0	0	0
	No	0	14	14
	No	0	0	0
	No	0	0	0
	Yes	6	15	9
	Yes	3	19	16
	Yes	1	57	56
	Yes	4	4	0
Control	Yes	1	1	0
	No	0	0	0
	No	0	0	0
	No	0	0	0
	No	0	0	0
	No	0	0	0
	No	0	0	0
	No	0	0	0
	No	0	0	0
	No	0	0	0
E. octoculata	No	0	17	17
	No	0	10	10
	No	0	20	20
	No	0	0	0
	No	0	29	29
	Yes	1	12	12
	Yes	2	12	35
	Yes	1	35	27
	Yes	1	27	18
	Yes	1	18	14
Control	Yes	5	5	0
	No	0	0	0
	No	0	0	0
	No	0	0	0
	No	0	0	0
	No	0	0	0
	No	0	0	0
	No	0	0	0
	No	0	0	0
	No	0	0	0
	No	0	0	0

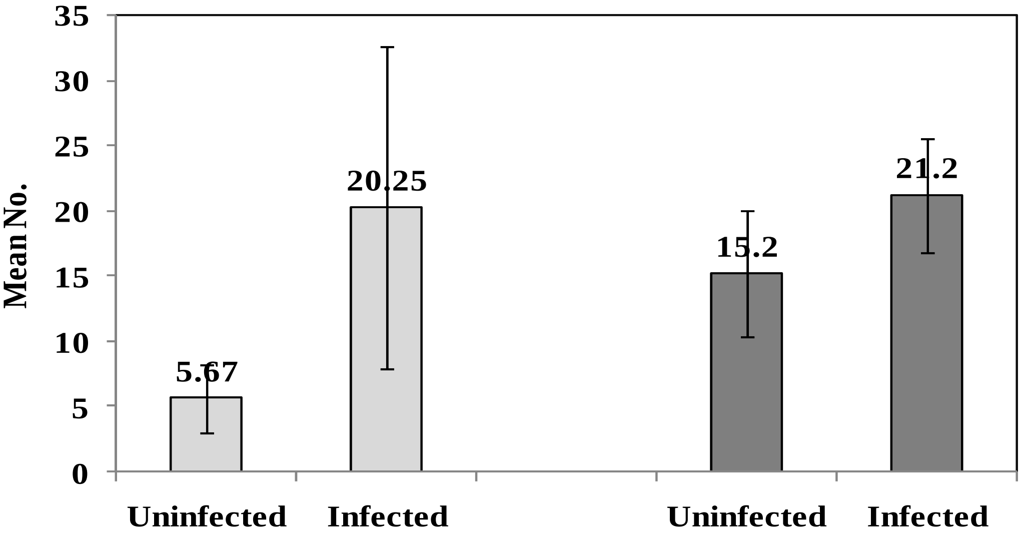

Figure 9. Average mean number of *Australapatemon sp.* metacercariae (± S.E) in *Helobdella stagnalis* and *Erpobdella octoculata* after three months of experimental exposure. White (*H. stagnalis*) and grey (*E.* octoculata). Error bars represent the standard deviation of the average mean number of metacercariae in initially infected and uninfected leeches after three months of exposure. Values above the bars indicate the average mean number of metacercariae in exposed infected and uninfected leeches after three months.

Table 4. Result of ANOVA on numbers of *Australapatemon sp.* metacercariae in *Helobdella stagnalis* and *Erpobdella octoculata* after experimental exposure. Leech species and initial infection status (uninfected / infected) were used as fixed factors. Initial number of parasites was used as a covariate.

Source	df	Mean square	F	Sig.
Initial	1	904.1222	6.83	0.02
Species	1	55.126	0.416	0.528
Infection	1	1421.7888	10.741	0.005
Species * infection	1	616.533	4.658	0.048
Error	15	132.371		

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4 DISCUSSSION

4.1 Parasite seasonality in *Valvata macrostoma* (first intermediate host)

4.1.1 Temperature

The total prevalence of *Australapatemon sp*. cercariae in *Valvata macrostoma* from the two study sites was very low. *Australapatemon sp*. cercariae were found only in snails from the shallow site and their prevalence varied between the months, even though the difference between the shallow and deep site was statistically insignificant. These results were similar to those of Faltýnková *et al*. (2008) who reported a similar pattern of prevalence from the same two sites i.e. no *Australapatemon sp.* cercariae in snail samples from the deep site. Generally, the production of cercariae occurs throughout the open water period (from June to September), but this is not constant, it varies from one month to the other. In some trematodes (flukes), temperature has an obvious and pronounced effect on a significantly important step of cercariae emergence and transmission (Poulin 2006). At higher temperatures cercariae output and emergence from mollusc, trematode first intermediate host and transmission is enhanced (Lo & Lee 1996, Umadevi & Madhavi 1997, Mouristen 2002, Poulin 2006, Poulin & Mouristen 2006) whereas, at lower temperatures little or no emergence and transmission occurs. Enhanced cercariae production at higher temperatures may probably be an outcome of increased metabolic activity of the host and the greater energy this may make available to the parasite (Poulin 2006). Thus this seasonal variation in the prevalence of infection observed could be attributed to fluctuations in environmental temperature. Worth mentioning also is the fact that many parasites spend a part of their life stages outside the host, their development and survival depend on abiotic factors in their environment (Kovats *et al*. 2001, Kutz *et a*l. 2005). Amongst them, temperature plays a major role in creating parasite seasonality since it has an effect on parasite transmission and occurrence which can be impaired at low temperatures in vertically transmitted parasites (Kelly *et al*. 2002). In Northern latitudes, parasite dynamics are focused on the summer months where favourable temperatures permits parasite transmission and development, with shorter winter periods there is little or no transmission of parasites due to low temperatures. This is the same situation in this study.

The striking difference in the prevalence of *Australapatemon sp*. cercariae in *Valvata macrostoma* between these two study sites despite their close proximity (ca 50-60 m) to each other could be attributed to some ecological processes. The distribution/frequency of visits of the final hosts, the birds (probably ducks), disseminating the eggs and habitat characteristics and preference by the snails. Thus parasite eggs which must be eaten by these snails are found in greater numbers in the shallow site where birds spend most of their time. This indicates that the definitive host of *Australapatemon sp*. shows some spatial differences in their distribution. Indeed, some authors have reported that the distribution of the final hosts releasing parasite eggs affects the spatial distribution of infections among molluscs in both the marine and freshwater habitat (Robson & Williams 1970, Esch & Fernández 1994, Sapp & Esch 1994). The particular bird definitive host species of *Australapatemon sp.* and their distribution is not known. However, these reasons may still not be sufficient to provide a comprehensive explanation of the complete absence of *Australapatemon sp*. from the deep site. Perhaps there is something to do with the occurrence of second intermediate hosts (leeches) as well.

4.1.2 Host generations

There was an enormous difference in the total proportion of *Valvata macrostoma* collected between the two sites though the sites were close to each other, 75.1% were collected from the shallow site and 24.9% from the deep site. These results were not different from those found by Faltýnková *et al*. (2007) who carried out a comprehensive study on the spatiotemporal structure of trematode communities in *V. macrostoma* from May to October from the same lake and same sampling sites. They collected 75.9% snails from the shallow site and 24.1% from the deep site. This difference in snail proportions between the two sites might be due to snail's preference of certain sites where conditions are near the best like the shallow site which was soft and had aquatic plants, this thus shows a high preference of the snails to certain habitats. Glöer (2002) reported that *V. macrostoma* can be found inhabiting a range of ecosystems like fluvial flats in rivers, marshy littoral areas of lakes, small water bodies with abundant aquatic vegetation and temporary waters. Snail life-history parameters and some other physical factors like water depth and light intensity (Fernández & Esch, 1994) might have been responsible for the lower snail proportion in the deeper site. These results are in accordance with those of other authors working on trematodes of other mollusc (Rohde 1981, Fernández & Esch 1991b, Curtis & Hurd 1983 and Sousa 1993). However, it is not possible to compare population densities exactly between these two sites because different collection methods were used in shallow site (grap sampling and hand-picking) and deep site (grap sampling), it was not really a quantitative sampling.

Aside temperature fluctuations influencing parasite seasonality, the life cycle of the hosts, *Valvata macrostoma* snails, plays an important role as well. These results revealed a seasonal variation in the proportion of *V. macrostoma* snails collected from June to September from the shallow site which was higher in July (34.6%) and lower in September (22.8%). In June the production of *Australapatemon sp*. cercariae was high, they were produced solely by the overwintered "old" snails that were most probably infected in autumn of the previous year. In July, some overwintered adults were still producing cercariae but also some overwintered snails that were not yet infected might have been infected in early June when water temperature was favourable. The infections that occurred in May-June developed and became visible in July. In August and September the prevalence declined markedly, probably because during these months young snails started to appear. The majority of these young snails were not infected or had no visible infections yet, moreover, the water temperature started to decrease in August-September thus, cercariae production decreased. The older generations and infected snails started to die during this time, since *V. macrostoma* quite often dies after reproduction.

4.2 Prevalence and abundance of *Australapatemon sp*. metacercariae in *Helobdella stagnalis* and *Erpobdella octoculata* (second intermediate hosts).

4.2.1 Differences between the two leech hosts

Erpobdella octoculata and *Helobdella stagnalis* regularly occurred in the same places and usually under the same stones, they however differed from each other in relation to population proportion (*H. stagnalis* 59% and *E. octoculata* 41%), as well as in some ecological parameters like life history, parental care and feeding strategies. *E. octoculata* was heavily infected, bigger in size and did not show parental care (Kutschera & Wirtz 2001). *H. stagnalis* was less heavily infected, smaller in size and showed a high level of parental care

(Kutschera & Wirtz 2001). The infection of *Australapatemon sp.* in these two leech species was not uniform throughout the sampling months, it showed seasonal variation in prevalence and abundance which was higher in *E. octoculata* and lower in *H. stagnalis*. *E. octoculata* started to produce cocoons in June-July and the young hatched from these in July and August. In May-June only the overwintered "old" leeches were present, new recruitment started in July-August. In May the prevalence of infection was low since the parasites occurred only in overwintered "old" leeches that were probably infected in autumn the previous year and the parasites developed to be visible in May to June. In June the prevalence was higher compared to May, parasites appeared mainly in overwintered "old" leeches but also new infections started to appear (see Figure 11). Probably some uninfected overwintered adults were infected in May which became visible in June. In July the prevalence was highest compared to all the other months, here the parasites appeared mainly in overwintered adults and/or some juveniles (Figure 11). Infections that occurred in June when water temperature was favourable for parasite occurrence and transmission became visible in July. In August and September the prevalence declined, old generations started to die since generally some adult *E. octoculata* die after brooding, many juveniles now made up the population, and the majority of them were not infected. Some heavily infected leeches probably died after they have been exploited by the parasite leading to their death.

In H*elobdella stagnalis,* cocoon-and-young carrying leeches appeared in June and free-living juveniles appeared in July-September. In May, June and July parasites occurred only in overwintered "old" leeches (no new infection, see Figure 10) that were most probably already infected in autumn of the previous year. In August there was "old "and "new" infection (Figure 10), parasites occurred in overwintered leeches or juveniles and probably some previously uninfected overwintered adults got new infections within May-June which became visible in August. In September no new infection appeared (see figure 11) and the prevalence rate declined, the "old" generation started to die after brooding. The population was mainly made up of juveniles with very low infection; eventually the unencysted metacercariae were not visible yet. Host leeches reproduction and life cycle, time of cercariae shedding from snails and transmission from the first intermediate host (*V. macrostoma*) jointly play a role in the seasonality of these trematodes.

4.2.2 Seasonality in the abundance of the parasite

Absolute mean number of *Australapatemon sp.* metacercariae in the two leech species varied within the months with a higher abundance in August and lower in May in both leech species. This could be explained by the fact that in May the parasites occurred only in the overwintered "old" leeches whereas, in August parasites occurred in overwintered leeches and newly infected leeches. Infections that occurred in May-June became visible in August. Another possible explanation for this increase in mean number of metacercariae could be that, initial infection rendered the leech more vulnerable for further infection. The mean number of metacercariae was noticeably higher in *Erpobdella octoculata* (15.58±2.74) compared to *Helobdella stagnalis* (0.55±0.05) resulting in an enormous difference in infection intensity between individuals of the two species. The difference in metacercariae abundance between the two leech species could be attributed to the differences that exist between them, difference in sizes, feeding behaviour and activity (Faltýnková *et al*. unpublished data). *E. octoculata* is bigger and more active (Faltýnková *et al*. unpublished data), it can easily come into contact with parasites thereby increasing their chances of infection compared to *H. stagnalis* which is smaller and has a sit and wait strategy.

Normally, in nature parents showing parental care are expected to be more stressed and heavily infected than parents not showing any level of parental care. Many findings have supported this fact, it is very costly to take care of offsprings (Hasu *et al*. 2006). This is because parental care compromises foraging ability with more hunting time required and brooding parents suffer reduced success rates in pursuit of prey when eggs or juveniles are attached on the ventral surface; food uptake is therefore reduced resulting in reduced growth during brooding period. However, contrarily to this expectation, *H. stagnalis* showing the most developed level of parental care involving brooding eggs and young, with direct feeding of young were the least infected in this study. On the other side, experimental results of this study revealed that both leech species can become equally infected; there was no difference in susceptibility between the two species (Table 5). Reproduction cycle with the experimental results has revealed that, the difference in infection between these two leech species it is not the question of evolutionary specificity (genetical), but ecological differences that must exist between these two leech species. The experimental order of *H. stagnalis* exploitation by *Australapatemon sp*. was different from that found in the natural habitats, in a natural multi-species leech community the highest abundance of *Australapatemon sp*. was found in *E. octoculata*. This clear difference between the pattern of leech host utilization in the natural habitats and that determined experimentally in this study is very interesting. This suggest in some way that inborn differences in the susceptibility of various leech species and individuals to *Australapatemon sp*. cercariae infection do not reveal the most essential determinant of the degree to which each leech species may become parasitized in the natural environment but ecological differences that exists between the two species. *E. octoculata* is very active (Faltýnková *et al*. unpublished data) and thus more likely to be exposed and easily comes into contact with the parasites than *H. stagnalis* which has a sit and wait strategy. In the nature both species showed different levels of infections with *E. octoculata* being the most heavily infected but from the experimental results there was no difference in susceptibility between both species.

4.2.3 Parasite aggregation

Generally parasites aggregate within available hosts, but this is not constant. It varies with time and space since the processes that lead to it are not constant. The number of cercariae shed and the infective stages varies in space and time, therefore equally susceptible hosts exposed to infection will end up having different numbers of parasites. Parasite aggregation has an observable effect on parasite ecology in that the intensity of intraspecific competition for space and food will not be the same for all individuals in a population but proportional to infrapopulations size. May (1977) proposed that parasite aggregation may bring about stabilization of the host-parasite relationship, and Kennedy (1975) suggested that since aggregation occurs only within few individual hosts at different levels, only few heavily infected individuals of a host population usually dies. There is no experimental evidence to support these facts.

Australapatemon sp. metacercariae became aggregated in 9.9% individuals of *E. octoculata* that contained 88.6% of the total metacecariae where each leech contained > 50 metacercariae. In the other host, *H. stagnalis,* no aggregation was found as most leeches had just 0-7 metacercariae. These differences within members of *E. octoculata* could be as a result of size differences. The bigger "old" leeches have been more exposed to parasites which have accumulated over time than in the smaller "younger" leeches. Also differences that exist between the host individuals contribute to parasite aggregation in some leeches and not in others, some hosts are good at preventing parasite first establishment or at doing away with them immediately after establishment or soon after (Poulin 2007). Initial infection might also increase the chances of further infection.

5 CONCLUSION

It is noticeable in this study that both prevalence and intensity of *Australapatemon sp.* metacercariae infection was higher in *E. octoculata* and lower in *H. stagnalis* in the nature whereas results of the laboratory study indicated that both leech species showed a similar degree of susceptibility to the parasite. There is no clear explanation available what is primarily responsible for producing the observed pattern of leech second intermediate host utilization in the natural environment. It is suggested that it might be due to ecological differences that exist between the two species. Taking into account the behavioural differences of these leech species, it is conceivable that the pattern of host utilization observed in natural habitats may be a product of differing degrees of contacts between the cercariae and the two leech species. A relatively high degree of contact would be expected to occur between cercariae and *E. octoculata*, the bigger and most active of the two species and a much lower degree of contact between the cercariae and *H. stagnalis*, the smaller and less active species (Faltýnková *et al* unpublished data). This study is one of the few ones where these two leech species have been recorded as second intermediate host of *Australapatemon sp*. Other studies have revealed these two leech species as second intermediate hosts of other trematode species (Spelling & Young 1986, McCarthy 1990).

In this study the infectivity of *Australapatemon sp*. to *Valvata macrostoma* was very low, this is similar to the results obtained by Faltýnková *et al*. (2008). It is not known if it is due to spatial distribution of definitive host or on the infection status of the second intermediate host or of the definitive host from the past year. For effective transmission parasitized leeches (second intermediate host) must be consumed by the definitive host. But these leeches spend most of their time under the stones and only come out to feed during night or evening hours. At this time the birds are no longer flying over the lake, and within the day it is not so evident that these birds can go under the stones or substrates (leech habitat of choice) searching for these leeches to feed on. So the probability for the birds to consume infected leeches is really low leading to fewer numbers of infected birds.

RECOMMENDATIONS FOR FURTHER RESEARCH

This study is one of the few ones that has reported *Helobdella stagnalis* and *Erpobdella octoculata* to be second intermediate hosts of *Australapatemon sp.* after Faltýnková *et al*. (2008) recorded *Valvata macrostoma* as the first intermediate host. It will be of interest to parasitologists, evolutionalists and others sooner or later to know the complete life cycle of *Australapatemon sp*. Trematodes of the genus *Australapatemon* are known to parasitize birds, especially ducks (Niewiadomska 2002) and European leeches especially *E. octoculata* are food items of ducks (Kuffl 1974/1975). For further research on *Australapatemon sp*., it would be of interest to know its definitive host.

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