Master's thesis

The effect of temperature on cercariae production of two *Rhipidocotyle* trematodes parasitizing freshwater mussel, *Anodonta anatina.*

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ABSTRACT

Two bucephalid trematodes, *Rhipidocotyle campanula* and *R. fennica* are known to infect the duck mussel, *Anodonta anatina*. The infection will lead to decrease growth, reproduction and survival of *A. anatina*. Given the important role of temperature on cercariae production, a vital component of the parasite's transmission and life cycle success, the effect of temperature on cercariae production of two *Rhipidocotyle sp*. in their molluscan host, *Anodonta anatina*, was investigated. Mussels were collected from the Rivers, Haajaistenjoki and Kuusaankoski, marked and allocated to three temperature treatments—high, intermediate and low. Between May 31-October 28, 2011, clams were individually monitored every third week for cercarial emergence in the laboratory. Cercariae production was temperature-dependent but differential. *R. campanula* emergence started in late May and *R. fennica* in July. In the River Haajaistenjoki, in high temperature, average annual *R. fennica* production was higher (17000 larvae per mussel) than *R. campanula* (3000 larvae per mussel), but the opposite in low temperature (600 vs. 1300 larvae). Similar results were found from the River Kuusaankoski. Results suggest that *R. campanula* would be better adapted to low temperatures while *R. fennica* should have an advantage in high temperature habitats.

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TIIVISTELMÄ

Rhipidocotyle fennica ja *R. campanula* -loiset (Trematoda, Bucephalidae) käyttävät pikkujärvisimpukkaa *Anodonta anatina* ensimmäisenä väli-isäntänään. Losinta johtaa isäntäsimpukan alentuneeseen kasvuun, lisääntymiseen ja elossapysymiseen. Loisen transmission avaintekijä, kerkaria-toukkien tuotanto simpukkaisännässä, voi olla altis lämpötilan muutosten vaikutukselle. Tästä syystä ennustetun ilmaston lämpenemisen voidaan olettaa aiheuttavan muutoksia loisten esiintymisrunsaudessa ja levinneisyydessä sekä tätä kautta simpukkaisännän populaatioissa. Tämän työn tarkoituksena oli tutkia lämpötilakäsittelyjen vaikutusta kahden trematoda-loisen, *Rhipidocotyle fennica* ja *R. campanula*, kerkariatoukkatuotantoon vuorokausi- ja vuositasolla. Simpukat kerättiin Laukaan Kuusaankoskesta ja Vieremän Haajaistenpurosta keväällä 2011, kuljetettiin Konneveden tutkimsasemalle, merkittiin yksilöllisesti ja arvottiin kolmeen lämpötilakäsittelyyn; alhainen, keskimääräinen ja korkea lämpötila, jossa simpukoiden kerkariatuotanto arvioitiin joka toinen viikko lokakuun lopulle saakka. *Rhipidocotyle campanula* –lajilla toukkatuotanto alkoi jo toukokuussa, mutta *R. fennica* –lajilla vasta heinäkuussa. Korkeassa lämpötilassa *R. fennica* –lajin toukkatuotanto oli selvästi korkeampi (17000 toukkaa per simpukka) kuin *R. campanula*lla (3000 toukkaa per simpukka) , mutta alhaisessa lämpötilassa tilanne kääntyi päinvastaiseksi (600 / 1300 toukkaa). Samanlainen tulos saatiin myös Kuusankosken simpukoille. Tulosten perusteella voidaan ennustaa *R. fennica* –lajin hyötyvän ilmaston lämpenemisestä enemmän kuin toisen lajin. Kokeen aikana suurin kuolevuus havaittiin korkeassa lämpötilassa sekä *R. campanula* –lajin loisimissa simpukoissa sekä simpukoissa, joissa esiintyivät molemmat loiset.

Contents

1 INTRODUCTION

1.1 Freshwater bivalve mussels (Bivalvia: Unionidae)

Unionidae play an important ecologic role within their ecosystems. Not only do they purify aquatic systems and improve water quality through their filter feeding (Welker and Walz 1998), they also influence nutrient turnover through the decomposition of detritus and keep bacterial and planktonic populations under control (Pusch *et al*. 2001, Hwang *et al*. 2004). They produce easily assimilated-food sources for benthic invertebrates and fishes (Howard and Cuffey 2006), and serve as host to many aquatic parasites, especially as first intermediate host for fish parasites, such as digenean trematodes (Taskinen *et al*. 1991, Poulin and Cribb 2002, Howard and Cuffey 2006). In addition, they serve as food to many species of wildlife and man, (Dudgeon *et al*. 2006) and are used as response systems to monitor water quality (Englund and Heino 1994, Sures 2004, Aldridge *et al*. 2007).

The family Unionidae, commonly referred to as pearly mussels, naiads or Unionids, has members occurring in Europe, Asia and Africa but the most diversity is in North America (Grizzle and Brunner 2009) with approximately 286 species (Turgeon *et al*. 1998, Jennings 2000). Unionids thrive in tropical to temperate climates and occur in freshwater sources such as lakes, streams and rivers with coarse substrates like sand or gravel (Smith 2001), but not in high mountain lakes maybe due to lack of proper fish hosts for the glochidia or poor nutrient supply (Smith 2001). Unionidae are different from other bivalves because of their unique life cycle including a parasitic larval stage, glochidium, requiring a fish host. Sexes are usually separate, although few species are hermaphrodites which sometimes may occur or be induced by low population density (Bauer and Wachtler 2001). Unionids are long-lived with an average exceeding 10 years (Smith 2001), sedentary and capable of a restricted form of locomotion. Unfortunately, they occupy one of the most unstable habitats on earth thus are often among the first to decline in population under unfavourable environmental conditions (Babarro *et al*. 2002, Lydeard *et al*. 2004). Nowadays, many species of freshwater unionid mussels have decline worldwide in terms of their population sizes (Lydeard *et al*. 2004, Strayer 2008), with about 126 species on the International Union for Conservation of Nature red list in 2007 due to anthropogenic disturbance, pollution and climate change. Thus, unionids are among the world's most imperiled freshwater organisms (Lydeard *et al*. 2004). Considering the important role unionids play, their extirpation may lead to lose of a variety of associated organisms and severe impoverishment of one of our richest component of aquatic biodiversity.

Anodonta anatina is a common and abundant freshwater clam in northern Europe. It matures between 2-4 years of age and reproduces every year attaining a maximum life span of about 15 years (Negus 1966, Haukioja and Hakala 1978a). From the beginning of July to mid-August, like other Unionidae glochidia larvae are developed in the outer gill blades of the females (Jokela *et al*. 1991) where they are stored and maintained over winter to be released the next spring (Negus 1966, Haukioja and Hakala 1978a, Richard *et al*. 1991). The larvae are obligate ectoparasites on a wide range of host fish spectrum (Jokela *et al*. 1991, Wächtler *et al*. 1994). They are released from fish host after several weeks to assume benthic life. *A. anatina* also serve as first intermediate host of two *Rhipidocotyle species* (Taskinen *et al*. 1991).

1.2 Bucephalid trematodes

Studies on the ecology, seasonality and life cycle stages of bucephalid digeneans have so far received little attention, despite the fact that parasite ecology has been the center of attention of many studies over the past few decades (Esch and Fernández 1993, Bush *et al*. 1997). The only existing literature is related to the occurrence and seasonality of two species, *Rhipidocotyle fennica and R. campanula*, in their freshwater bivalve and fish hosts in Finland (Taskinen *et al*. 1991, 1994; Taskinen and Valtonen 1995). Buccephalid trematodes, like other digenean trematodes, is an important parasite group with a complex life cycle usually requiring more than one host for its completion. Their larvae, metacercariae and adult worm parasitize a wide range of hosts ranging from mussels through fishes to birds and mammals (Grizzle and Brunner 2009). There exists age long host-parasite relationship between mussels and bucephalid trematodes (Bauer 1997), as they occupy and co- inhabits in the littoral and sub littoral zones of the freshwater ecosystem. The coexistence and host-parasite relationship are centered on benefits for the parasite and harm for the host, (Taskinen 1998). The devastating damage caused by bucephalid trematodes on unionid mussels is sterilization of their hosts (Taskinen *et al*. 1997).

Rhipidocotyle campanula and *Rhipidocotyle fennica* are species of bucephalid trematodes; their infective larval stage (cercariae) develops asexually in *A. anatina* which serves as their first intermediate host (Taskinen *et al*. 1991, Gibson *et al*. 1992). This parasitic larvae infestation may lead to decrease in growth rate, reproductive output and even castration of *A. anatina* (Taskinen and Valtonen 1995, Gangloff *et al*. 2008) as they replace the host gonads with sporocyst or fibrosis. Both *Rhipidocotyle species* occur as metacercariae in the cyprinid fish, *Rutilus rutilus*, which is the second intermediate host although each species shows penetration site preference on *R. rutilus*. *R. campanula* cercaria attaches to the gill while *R. fennica* cercaria attaches to the fins of *R. rutilus* (Taskinen *et al*. 1991). In the definitive host, *R. campanula* occurs as adult worm in European perch, *Perca fluviatilis* while *R. fennica* occurs as adult worm in pike, *Esox lucius* (Taskinen 1992). The adult worms reproduce sexually in the definitive host to release eggs that hatch into miracidia larvae in water and find mussels. Miracidia undergo development to mother and daughter sporocyst which produces cercaria that is shed into the water (Figure 1).

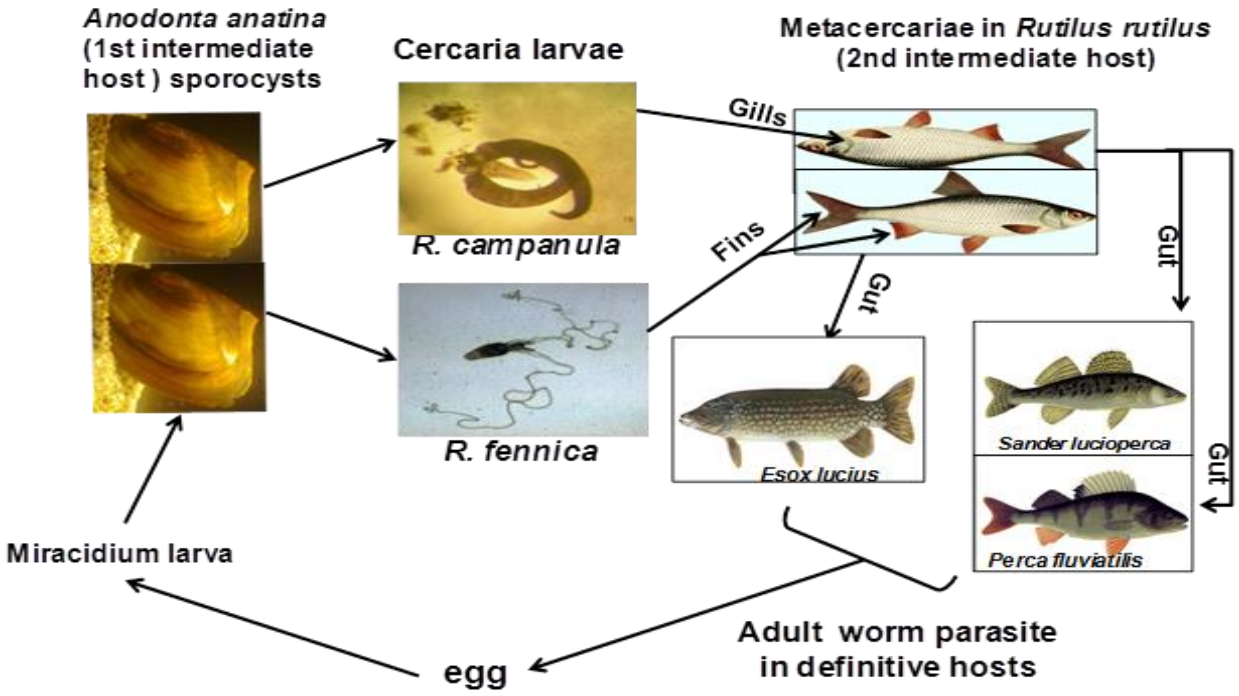

Figure 1. Life cycle of *Rhipidocotyle* trematodes parasitizing freshwater mussel, *Anodonta anatina* (Taskinen 1992).

1.3 Climate change and parasitism

Climate models have predicted small increase in average temperatures for many parts of the world. Considering the vital role of parasites and pathogens, understanding the potential impacts of climate change in general and global warming in particular on parasitism, disease dynamics as well as host populations and communities should be of major concern. Climate change will affect parasite species directly (Harvell *et al*. 2009), through enhance development, emergence, transmission and decrease survival rate in response to increase temperature. For example, increasing water temperature has been shown to increase the incidence of some fish pathogens while the cold-water diseases may even suffer from elevated temperatures (Karvonen *et al*. 2010). Thus, the predicted global warming may increase or decrease local abundance of the parasites and cause geographic range extension or reduction of parasites with possible consequences for host population and community dynamics. Therefore, the intensity of parasitism and local abundance of parasites can be significantly regulated by climatic or environmental conditions (Cattadori *et al*. 2005, Poulin & Mouritsen 2006) with potentially important repercussions for host individuals, populations, communities and ecosystems (Kutz *et al*. 2005, Harvell *et al*. 2009). Moreover, the more complex the parasite's life cycle is, the more likely it is that it will be affected by changes in climate (Marcogliese 2008).

1.4 Temperature and cercariae production

Among the climatic variables that affect parasites and host-parasite interactions, temperature is often considered the most important abiotic parameter because it strongly affects parasites at all life-cycle stages (Chubb 1979), though the effects of temperature may be modified by other key climate variables. Temperature not only affects parasite directly, but also acts as a stressor on hosts through weakening of hosts' defense, increase in susceptibility thereby leading to increased parasite-induced mortality of hosts and possible extinction (Harvell *et al*. 2002, 2009). An important factor for reproductive accomplishment of trematodes is the production of cercariae and this is mainly regulated by temperature (Pflüger *et al*. 1984, Taskinen 1998, Slenning 2010). The effect of temperature-mediated changes in cercarial output varies within and among parasite species. Usually, increasing temperature increases numbers of parasite infective stages in a system through increased production within, and emergence from the first intermediate hosts (Poulin 2006, Marcogliese 2008). In trematodes for example, cercariae shedding rate is strongly positively related to temperature and a larger pool of parasite infective stage is expected in a warmer world (Poulin 2006).

1.5 Aims, question and hypothesis

The aim of this study was to investigate the effect of temperature on daily and annual cercariae production of two trematode species, *Rhipidocotyle campanula* and *R. fennica* in their molluscan host, *Anodonta anatina* (Unionidae). Considering the important role of *Rhipidocotyle species* and as potential threat to unionid mussels, it is important to assess their performance especially in the face of radical anthropogenic changes associated with increasing temperature.

Study question: Is parasite cercariae production affected by water temperature?

Hypothesis: Cercaria production increases as a function of water temperature.

2 MATERIALS AND METHOD

2.1 Study site

Freshwater bivalve clams were collected from two sites in Finland. A total of 281 *Anodonta anatina* were collected by snorkeling from the littoral sites 1.5–2 m of depth from the River Kuusaankoski (Laukaa, Central Finland), latitude 62^{o}46 ´N, and longitude 25^{o}95 ´E on the 17th of May 2011. On 22nd of May 2011, 290 clams were collected by snorkeling at 0.5–1 m of depth from the River Haajaistenjoki (Vieremä, Northern Savo), latitude 63^{o}63 ´N, longitude 26^{o}99 ´E. Temperature was 9 ^{o}C at both sites at the time of collection. All clams were transported live to Konnevesi Research Station where they were monitored individually for cercarial emergence. The study sites differ in their depths, sizes, latitudes and infection rates (see Results). The River Kuusaankoski flows from Vatianjärvi to Saravesi—being a big river at lower part of "Saarijärven reitti" river system, belonging to Kymijoki drainage. River width at the site of collection, downstream from the actual Kuusaankoski Rapids and railroad bridge, is 110 m. Current velocity is substantial so that a diver can work efficiently only close to the shore line. Bottom sediment is sandy with boulders. Bottom quality of the River Haajaistenjoki is quite similar to that of the River Kuusaankoski, but more clayish. The River Haajaistenjoki is a small creek, 2–3 m wide with the maximum depth of 1.0 m in pools,

flowing from Ala-Haajainen Lake to Joutsenjoki which flows to Porovesi (close to the City of Iisalmi), belonging to Vuoksi drainage.

2.2 Monitoring of clams for cercarial emission

Each of 571 *A. anatina* collected was marked with an individual population-specific code (to allow repeated counts of the number of cercariae released by individual clams over time) on the shell using a drill, and the shell morphological measurement (length, height and width) taken using digital caliper (±1) at the beginning and at the end of the study. Average length ± SE for mussels from the River Haajaistenjoki was 61.4 ± 0.6 mm (range: 33-86.8 mm, median: 61 mm) and from the River Kuusaankoski, 78.1 ± 0.6 mm (range: 51.7-101.7 mm, median: 78.3 mm. From 31st of May to 27th of June 2011, clams were kept in 2 flow-through holding tanks supplied with natural incoming filtered lake water so that clams from each site were placed into respective tanks. At the end of the third monitoring, i.e. June 27, 2011, clams were randomly assigned to three temperature treatments; high, intermediate and low, such that clams from both populations and of all sizes were equally represented into all tanks. There were two replicate tanks for each treatment totaling six tanks. High temperature treatment was achieved by keeping the mussels in outside tanks. Therefore, high temperature treatment was more variable compared to intermediate and low temperature treatments that were stable as the tanks were kept indoors. Intermediate temperature treatment was established by heating the regular incoming water by 2–3 ^{o}C and low temperature treatment was achieved by supplying the tanks with natural incoming water from the hypolimnion of Lake Konnevesi. Water supply into high temperature treatment tanks was from the littoral zone of Konnevesi Lake, whereas for the intermediate and low temperature tanks, it was from the deeper source further away from the shore. 5 cm sand was added to each tank bottom. Temperature was recorded approximately after every four hours using submersible temperature loggers placed in one replicate tank for each treatment throughout the monitoring period (Figure 2). Water flow was adjusted so that it was equal in all treatments. However, in the high temperature treatment the pumping system was not effective enough so that the water flow was lower than in the other treatments (Table 1).

Between May 31 and October 28, 2011, every third week, clams were individually placed in 3 l transparent containers (length 26.5 cm, breadth 19 cm and height 13.6 cm) filled with 2 l filtered incoming water kept overnight in respective temperature treatment rooms (to achieve temperature conditions approximate to that of holding tanks). Clams in one replicate tank from each treatment were set for monitoring either between 0800 and 1000 hours or between 1600 and 1800 hours, these procedures were also applied when counting the numbers of cercariae released. After 24 hours, number of cercariae shed by each clam into water was identified and counted visually or with a microscope when necessary. For each temperature treatment, different rooms were used so that temperature conditions approximated those in respective holding tanks. Light conditions most especially were set to correspond with natural rhythm because the cercarial production of *Rhipidocotyle species* is diurnal periodically (Taskinen *et al*. 1991). After each monitoring, clams were returned to their respective holding tanks. The total number of monitorings was 12.

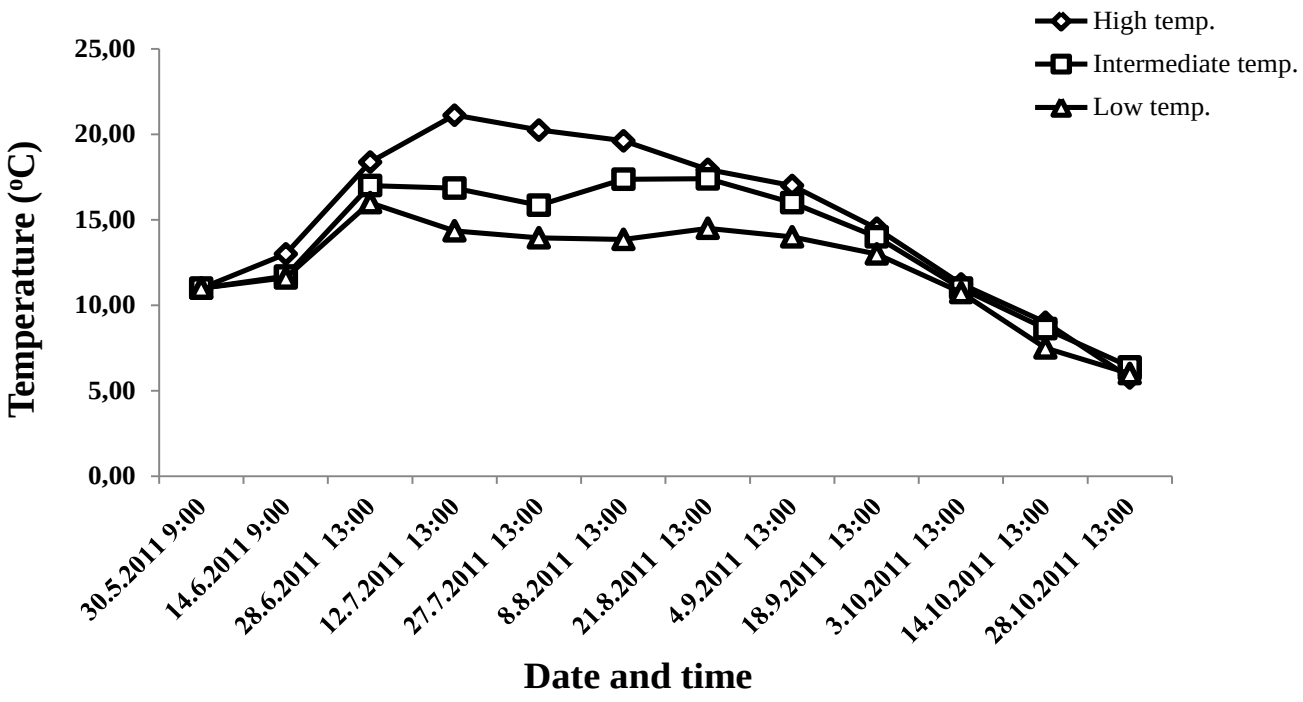

Figure 2. Temperature record by loggers (°C) against date and time. The dates represent monitoring days, the points in the figure represents the water temperature on the corresponding dates and time.

Table 1. Water flow rate in the six experimental tanks.

Treatments	Tanks	Water flow rate (L/min)
Low temperature	1	4.7
	2	9.6
Intermediate temperature	1	8.0
	2	10.9
High temperature	1	5.3
	2	5.3

2.3 Data analysis

The data were analyzed with PASW Statistics 18. Mean daily cercarial production for 2-weeks monitoring period was achieved by multiplying the respective daily production by 14. Total average annual cercariae production was analyzed by adding together results of the 2-week cercariae productions throughout the monitoring period. Using analysis of variance (ANOVA), differences in the mean daily cercaria production between temperature treatments and clam populations were analyzed. In the same way, also the differences in the annual cercariae production were analyzed. Tukey test was further used to determine the pair wise differences between time points and treatments. Mann-Withney U-test was used to test for the earlier start of cercariae production between treatments.

3 RESULTS

3.1 Proportion of clams shedding cercariae

The proportion of clams shedding *Rhipidocotyle campanula* and *R. fennica* cercariae in all treatments was higher in the River Haajaistenjoki (12.4 % vs. 19.3 %) compared to the Kuusaankoski (5.3 % vs. 18.1 %) respectively. This difference between both populations was statistically significant (χ^2=4.73, df= 1, *P*= 0.030). Twelve individual clams shed both parasite species in the River Haajaistenjoki and none in the River Kuusaankoski. Overall, 104 *Anodonta* clams shed cercaria larvae in the River Haajaistenjoki against 66 in the River Kuusaankoski (Table 2).

Table 2. Numbers of River Haajaistenjoki and River Kuusaankoski clams in different temperature treatments and numbers of clams shedding *Rhipidocotyle campanula* (RC), *R. fennica* (RF) and clams shedding both cercariae (proportion % given in parentheses).

	No. of clams	Proportion of clams shedding cercariae (%)		
		RC	*RF*	*RC+RF*
Haajaistenjoki				
High temp.	96	8(8.3)	31(32.3)	9(9.4)
Intermediate temp.	97	10(10.3)	19(19.6)	1(1)
Low temp.	97	18(18.6)	6(6.2)	2(2.1)
Total	290	36(12.4)	56(19.3)	12(4.1)
kuusaankoski				
High temp.	93	2(2.2)	37(39.8)	0
Intermediate temp.	93	4(4.3)	14(15.1)	0
Low temp.	95	9(9.5)	0	0
Total	281	15(5.3)	51(18.1)	0

3.2 Survival rate of shedding and non-shedding clams

The average number of *Anodonta anatina* that shed and those that did not shed but survived throughout the experiment varied between populations. Survival rate of clams that shed *Rhipidocotyle* cercariae larvae was low in the River Haajaistenjoki compared to the River Kuusaankoski. Similarly, same result for non-shedding clams, survival rate was lower in the River Haajaistenjoki than in the River Kuusaankoski. Overall, in both populations, survival rate of non-shedding clams was higher compared to the shedding clams (Table 3).

However, survival rate between temperature treatments, statistically showed no significant difference (χ^2= 0.63, df=1, *P*>0.10).

Table 3. Survival rates of shedding and non-shedding *Anodonta anatina* throughout the experiment. N represents the numbers of shedding and non-shedding clams, N_S represents numbers of shedding clams that survived throughout the experiment and N_{S1} represents the numbers of non-shedding clams that survived throughout the experiment. The proportions of N_S and N_{S1} clams given in % parentheses.

	R. campanula (RC)			*R. fennica* (RF)			R.C + R.F			Total Shedding			Total Non-shedding		
	N	N_s	%	N	N_s	%	N	N_s	%	N	N_s	%	N	N_{s1}	%
Haajaistenjoki															
High temp.	8	1	12.5	31	8	25.8	9	2	22.2	48	11	22.9	48	33	68.8
Intermediate	10	6	60	19	9	47.4	1	0	0.0	30	15	50	67	46	68.7
Low temp.	18	12	66.7	6	4	66.7	2	2	100	26	18	69.2	71	48	67.7
Total	36	19	52.8	56	21	37.5	12	4	33.3	104	44	42.3	186	127	68.3
Kuusaankoski															
High temp.	2	1	50	37	13	35.1	0	0		39	14	35.9	54	35	64.8
Intermediate	4	3	75	14	11	78.6	0	0		18	14	77.8	75	50	66.7
Low temp.	9	6	66.7	0	0		0	0		9	6	66.7	86	70	81.4
Total	15	10	66.7	51	24	47.1	0	0		66	34	51.5	215	60	72.1

3.3 Mean daily cercariae production

3.3.1 *Rhipidocotyle campanula*

The mean daily number of *R. campanula* cercariae shed per mussel varied considerably from one monitoring session to another. In the River Haajaistenjoki, *R. campanula* cercariae emergence started in late May before clams were assigned to temperature treatments. Afterwards, mean daily number of cercariae produced from an average shedding clam increased gradually with highest values in late July in both high and intermediate temperatures, about 63 larvae at 22.2 ^{o}C and 67 larvae at 16.5 ^{o}C respectively. Highest value in low temperature was 28 larvae at 16 ^{o}C in early September, decreasing thereafter with no cercariae emergence in October in any treatments (Figure 3).

In the River Kuusaankoski similarly, cercariae emergence started in late May. The mean daily *R. campanula* production was highest in late July in all treatments with about 20 larvae at 22.2 ^{o}C in high, 20 larvae at 16.5 ^{o}C in intermediate and 41 larvae at 13 ^{o}C in low temperatures treatment. Lowest values were achieved in early August in high temperature, mid-August in intermediate temperature and early October in low temperature during which no cercaria was shed (Figure 4).

3.3.2 *Rhipidocotyle fennica*

Mean daily *R. fennica* cercaria production also varied, and emergence in both populations only started in early July in high and intermediate temperature, about 43 days later than that of *R. campanula.* From the River Haajaistenjoki, daily number of cercariae increased from 0 in late June at 21.3 ºC to highest productions of 600 larvae at 19.1 ºC in early August in high temperature, 120 larvae at 17.6 ºC in early September in intermediate temperature and 45 larvae at 10.7 ºC in early October in low temperature (Figure 4). In early October, daily cercariae production declined to 0 in high and intermediate temperature treatments at 5.9 ºC and 8.4 ºC respectively. However in low temperature treatment, *R. fennica* larvae production started declining to 0 larvae at 6.9 ºC (Figure 5).

In the River Kuusaankoski, no clam shed *R. fennica* in low temperature. Highest mean values were in early August, about 600 larvae at 19.1 ºC in high temperature and mid-September, about 120 larvae at 15.7 ºC in intermediate temperature. *R. fennica* mean daily production decreased thereafter with no shedding in early October at 10.7 ºC and late October at 7ºC in high and intermediate temperature respectively (Figure 6).

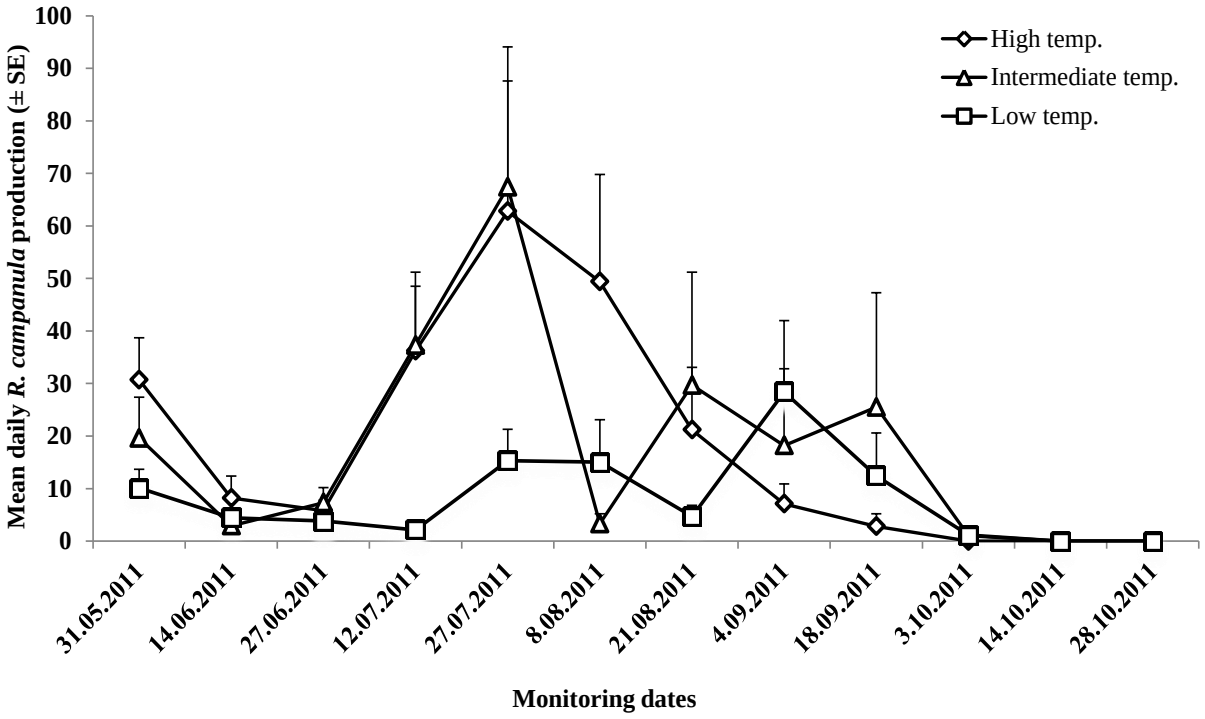

Figure 3. Mean daily (±SE) *Rhipidocotyle campanula* cercariae production per mussel in the River Haajaistenjoki from 31.05–28.10.2011 at three different temperature treatments. Error bars represent the daily standard error of the daily average numbers of *R. campanula* cercariae shed.

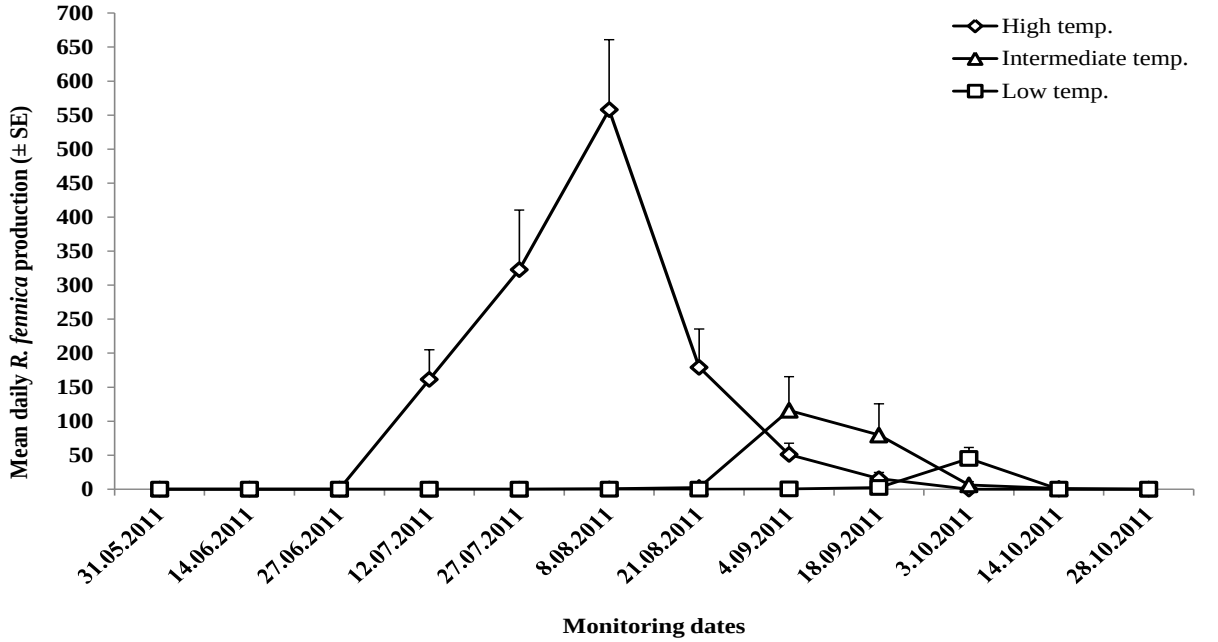

Figure 4. Mean daily (±SE) *Rhipidocotyle campanula* cercariae production per mussel in the River Kuusaankoski from 31.05–28.10.2011 at three different temperature treatments. Error bars represent the daily standard error of the daily average numbers of *R. campanula* cercariae shed.

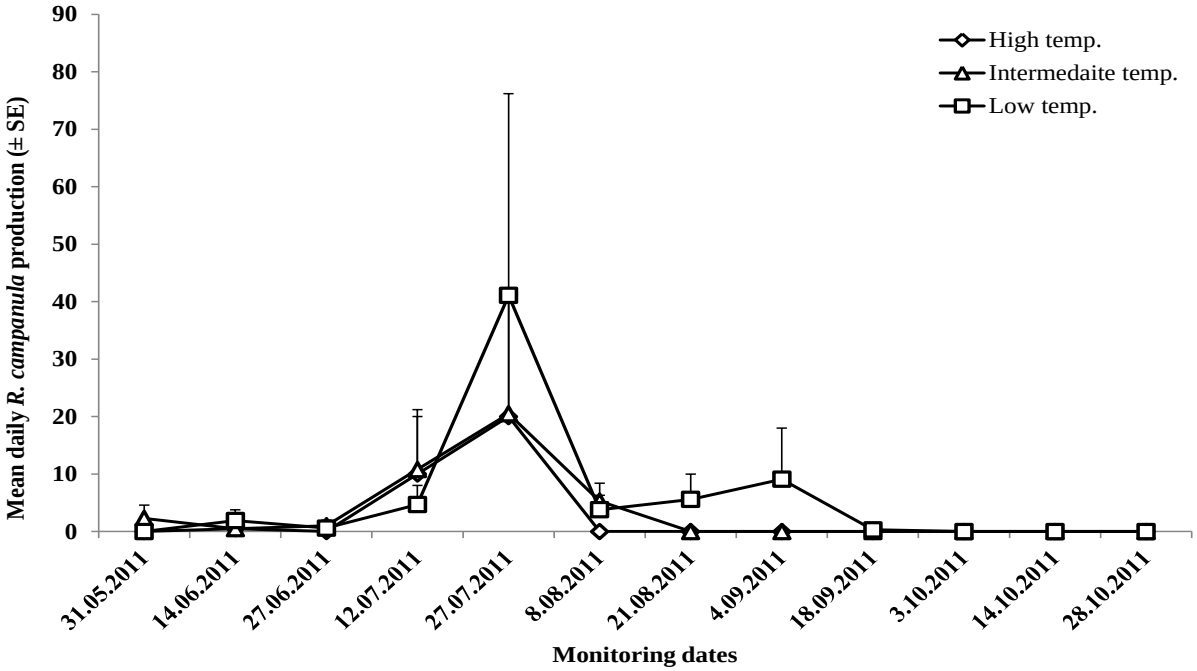

Figure 5. Mean daily (±SE) *Rhipidocotyle fennica* cercariae production per mussel in the River Haajaistenjoki from 31.05–28.10.2011 at three different temperature treatments. Error bars represent the daily standard error of the daily average numbers of *R. fennica* cercariae shed.

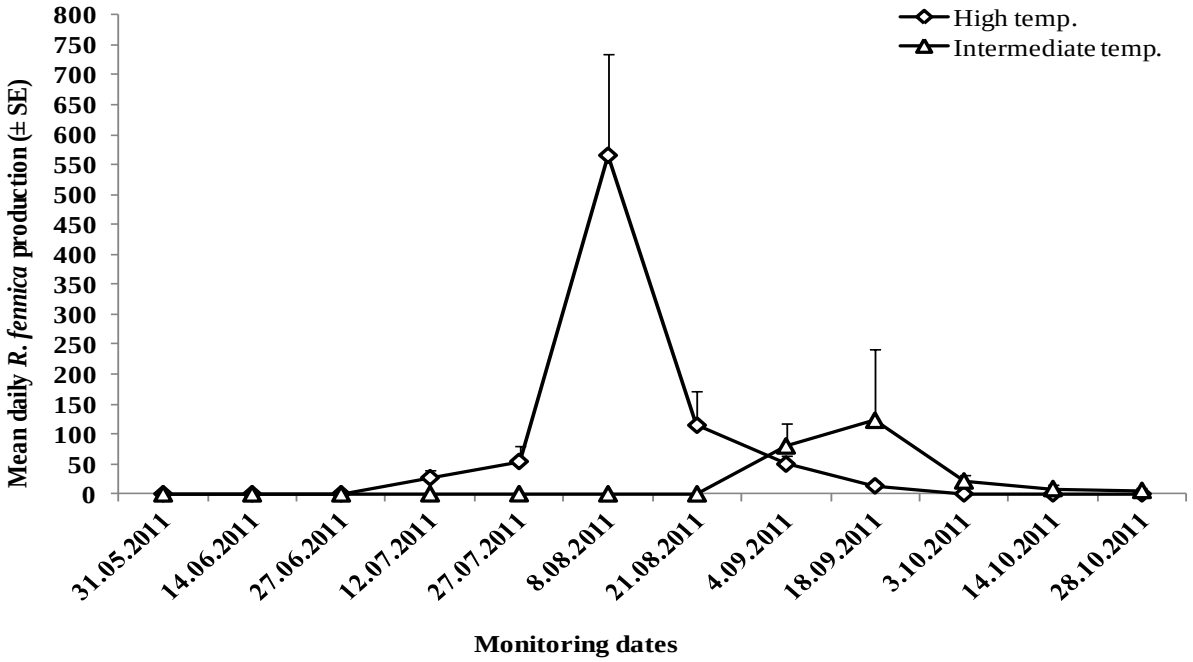

Figure 6. Mean daily (±SE) *Rhipidocotyle fennica* cercariae production per mussel in the River Kuusaankoski from 31.05–28.10.2011 in two different temperature treatments. Error bars represent the daily standard error of the daily average numbers of *R. fennica* cercariae shed.

3.4 Annual cercariae production

3.4.1 The River Haajaistenjoki

The average annual number of cercariae produced by both parasite species varied between treatments and between the parasite species. Average annual cercariae output of *R. campanula* was about 3000 vs. 2900 vs. 1300 in high, intermediate and low temperatures respectively (Figure 7). Analysis of variance (one-way ANOVA) after log (x+1) transformation showed that the annual cercariae produced by *R. campanula* differed significantly in different temperature treatments ($F_{2, 45}$=3.666, P= 0.033). Further, Tukey tests showed that the average annual cercariae produced by *R. campanula* differed significantly between high and low temperatures, but intermediate did not differ from any other (Figure 7). Average annual cercariae output of *Rhipidocotyle fennica* was about 17000 vs. 3000 vs. 600 larvae in high, intermediate and low temperatures respectively (Figure 7). One-way ANOVA results after log (x+1) transformation revealed a very high significant difference in average annual number of *R. fennica* produced between all treatments ($F_{2, 63}$=313.34, P<0.001). Tukey tests further showed that all treatments differ from each other.

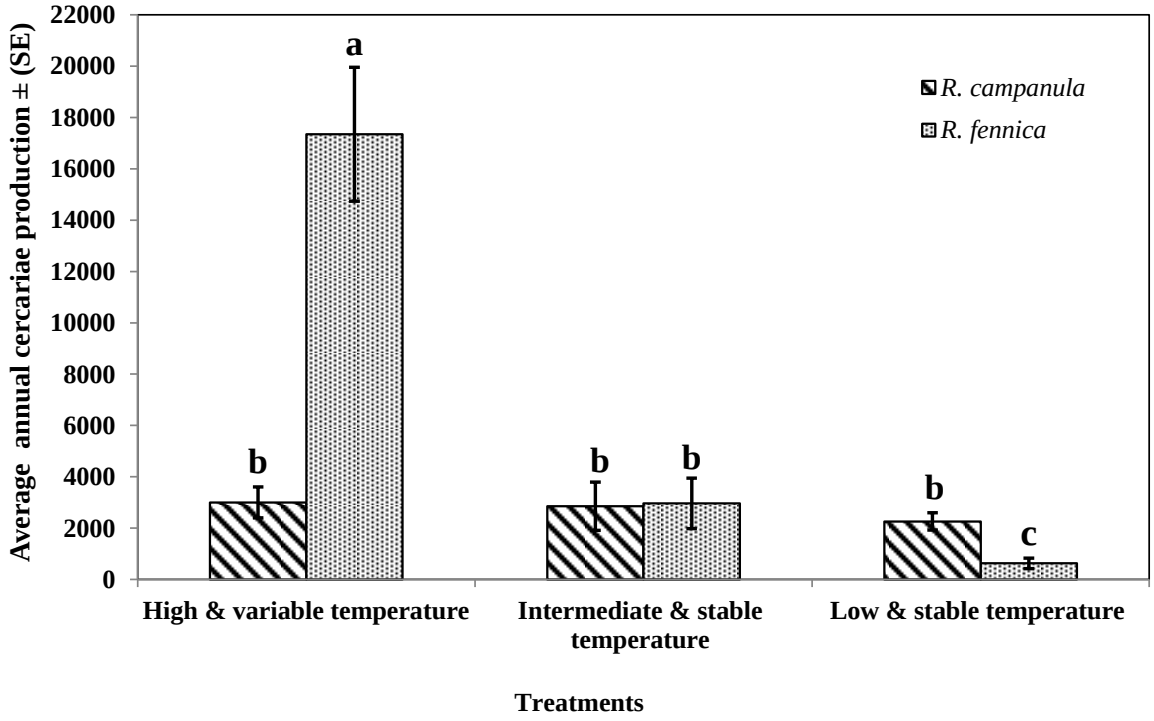

Figure 7. Average annual cercariae production from 31.05–28.10.2011 by *Rhipidocotyle campanula* (±SE) and *R. fennica* (±S.E) from the River Haajaistenjoki in different temperatures. Different letters above the bars indicate statistically significant difference in pairwise comparison (Tukey test, $P<0.005$).

3.4.2 The River Kuusaankoski

In the River Kuusaankoski, the difference between average annual *R. campanula* cercariae production in all temperature treatments was not significant (ANOVA, $F_{2,\ 12}=0.154$, $P=0.859$) with annual mean values of about 420 vs. 550 vs. 870 in high, intermediate and low temperature treatments respectively (Figure 8).

Similarly, average number of cercariae produced by *R. fennica* was about 11000 vs. 3400 larvae in high and intermediate temperature treatments respectively, this difference was highly significant (ANOVA, $F_{1,\ 49} = 5.507$, $P = 0.008$). There was no *R. fennica* production in low temperature treatment.

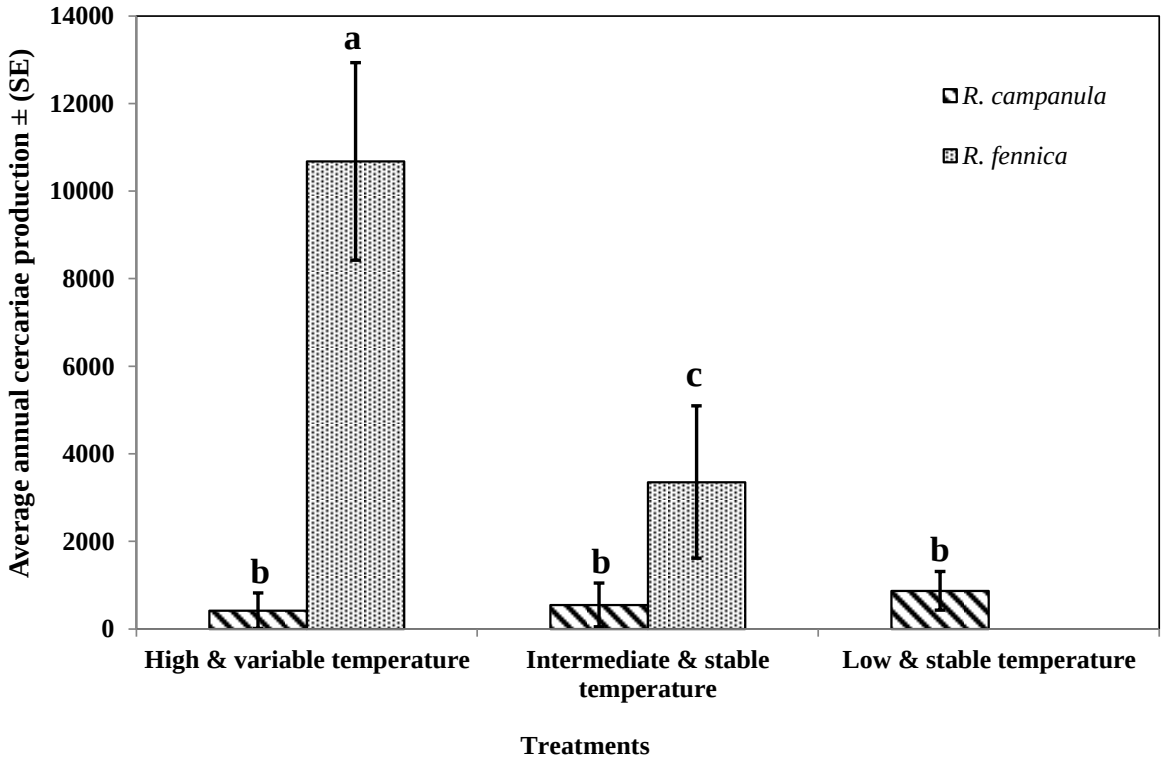

Figure 8. Average annual cercariae production from 31.05–28.10.2011 by *Rhipidocotyle campanula* (±S.E) and *R. fennica* (±S.E) from the River Kuusaankoski in different temperature treatments. Different letters above the bars indicate statistically significant difference in pairwise comparison (Tukey test, $P<0.005$). There was no mussel shedding *R. fennica* in Low and stable temperature treatment.

3.5 Timing and length of cercariae emergence

Rhipidocotyle campanula cercaria larvae emergence started in late May in both populations and ended between early October in high temperature and mid-October in low and intermediate temperatures in the River Haajaistenjoki and between early August and October in the River Kuusankoski. *R. fennica* cercaria emergence on the other hand started in early July in both high and intermediate temperature treatments in all populations but in low temperature in the River Haajaistenjoki it started in early September while in the River Kuusaankoski, *R. fennica* did not start at all. For clams that shed both *Rhipidocotyle* species in the River Haajaistenjoki, emergence started late July in high temperature, early August and early September in intermediate and low temperature treatments respectively and ended in early September in high and intermediate temperature treatments respectively, but not until mid- September in low temperature treatment. No clam shed both parasite species in the River Kuusaankoski (Table 4).

The length in days of *Rhipidocotyle species* shedding in infected clams varied between treatments and populations. In the River Haajaistenjoki, *R. campanula* cercariae production lasted for 126 days in high temperature, 137 days in both intermediate and low temperature treatments respectively. However, for *R. fennica*, larvae production lasted for 84 days in high,

109 days and 95 days in intermediate and low temperature treatments respectively. Similarly in the River Kuusaankoski, *R. campanula* cercariae shedding lasted for 56 days in high, 83, days and 112 days in intermediate and low temperature treatments respectively. For *R. fennica* cercariae shedding also lasted for 84 and 109 days in high and intermediate temperature treatments respectively, but in low temperature, there was no *R. fennica* shed (Table 4). In clams that shed both parasite species, larvae production lasted for 40 days in high, 28 days in intermediate and 15 days in low temperature treatment only in the River Haajaistenjoki.

Table 4. Dates of first emergence, last emergence and length of shedding of *Rhipidocotyle species* in *A. anatina* from both populations. *RC = R. campanula* and *RF = R. fennica*.

	No. of clams shedding		First emergence of cercariae		Last emergence of cercariae		Length of cercariae shedding (days)	
	RC	*RF*	*RC*	*RF*	*RC*	*RF*	*RC*	*RF*
Haajaistenjoki								
High temp.	8	31	May 31	July 12	October 3	October 3	126	84
Intermediate temp.	10	19	May 31	July 12	October 14	October 28	137	109
Low temp.	18	6	May 31	Sept. 4	October 14	October 14	137	95
Kuusaankoski								
High temp.	2	37	June 14	July 12	August 8	October 3	56	84
Intermediate temp.	4	14	May 31	July 12	August 21	October 28	83	109
Low temp.	9	6	June 14	Did not start at all	October 3	Did not start	112	Did not start

4 DISCUSSION

4.1 Effect of temperature on cercariae output from clams

Our results showed that the mean number of cercariae produced per *Anodonta* clam per day as well as annually was highly variable. Our hypothesis that increase in temperature will lead to increase in cercarial output was supported only partly. As predicted, both the total average annual reproductive output of *R. fennica* cercariae and proportion of *Anodonta* clams shedding *R. fennica* were higher in all populations in response to increased temperature, same as has been reported in many studies investigation on temperature-mediated cercariae production; and a significant opposite in low temperature in both populations as well. Contrarily, *R. campanula* did not respond to increased temperature in the same manner. Rather, higher mean annual *R. campanula* cercariae output in response to increased temperature was observed only in the River Haajaistenjoki and same response to decreased temperature in the River Kuusankoski. These results are similar to those of Koprivnikar & Poulin (2009a) who studied variations in cercarial emergence of two trematodes, *Maritrema novaezealandensis*

(Microphallidae) and *Acanthoparyphium sp*. (Echinostomatidae) which both uses the intertidal mudsnail *Zeacumantus subcarinatus* as first intermediate host, in response to increased temperature. These snails were collected from different sites with different latitudes. They found increased *Acanthoparyphium sp*. cercariae production at warmer temperatures and the reverse with *M. novaezealandensis* cercariae production which also differed among study sites.

This observed variation in *Rhipidocotyle sp.* cercariae output across population, with greater production in the River Haajaistenjoki could simply be the result of differences in biological characteristics and genetic drift occurring in isolated populations, or it could be due to parasite or clam adaptation to local climatic conditions or differences in latitude from where the clams were collected. This shows that different trematode species in different populations will be impacted differently by temperature. Also, the variation across treatments with higher productions in high temperature could be due to increased host metabolic activity which in turn makes greater energy available to the parasite to exploit. However, increased cercarial output is not accounted for only by host metabolism but other factors that act alongside with it to determine how many cercariae are released (Poulin 2006). Worth mentioning is the fact that, increase temperature enhance cercarial development and accumulation in host (Poulin 2006, Poulin and Mouristen 2006) with demonstrably elevated cercarial emergence into external environment. Temperature-mediated increase in cercarial output is more substantial compared to results expected from basic physiological processes (Poulin 2006).

The average annual *R. fennica* production of 9000 vs. 11200 cercariae, and *R. campanula* 800 vs. 2300 larvae in the Rivers, Kuusankoski and Haajaistenjoki respectively was lesser compared to 291000 reported by Taskinen (1998) who studied cercariae production of *R. fennica* kept in the field from the River Kuusankoski. The present study was carried out under laboratory conditions. Shostak and Esch (1990) and (Keas and Esch 1997), also reported differences in cercariae production between trematodes under natural conditions and in the laboratory with higher productions under natural conditions. Laboratory conditions usually do not represent natural conditions as there maybe lesser resources or food available (Keas and Esch 1997), and resource limitation may affect cercarial output (Loker 1983). The overall average annual cercaria output from both populations by *R. fennica* was higher than *R. campanula*. This large difference probably reflects differences in the phylogenetic constraints of each host-parasite relation. *R. fennica* cercariae are smaller, short-lived and drift passively whereas *R. campanula* is larger, long lived and swim actively (Taskinen *et al*. 1991, Gibson *et al.* 1992). Thus, the production of cercariae by *R. fennica* may be less costly than *R. campanula*.

The mean daily cercariae production also varied with greatest *R. campanula* output in late July and *R. fennica* in early August to early September when temperature was highest. These results were different compared to (Taskinen *et al*. 1991, 1994, 1998) who reported higher values in both species with peak value in late August for *R. fennica* when highest temperature was recorded. Interestingly, lower values compared to those of this study were reported in *Fasciola hepatica-Lymnea truncatula* (Hodasi 1972). This difference in daily cercariae production between parasite species reveals that constraints on cercarial output can vary among trematode species (Galaktionov and Dovrovolskij 2003) and that temperature of maximal or minimal production is specific for each trematode. These results further indicate that trematodes are sensitive to variations in daily temperatures, thus may benefit from increased temperature by increasing cercarial emergence although this increase may not be

continuous. It may take the form of brief stops when a relatively high thermal limit is attained, and when output is already very high. Thus, it becomes difficult for the parasite to increase its output even if the temperature increases. Variability in the seasonal development of *Rhipidocotyle* cercariae stages especially miracidia availability during late summer and autumn, at which time clams are infected (Taskinen *et al*. 1991), could account for the daily fluctuations observed in *Rhipidocotyle* / *Anodonta* association. New infections or cercarial production may stop or decrease at low temperatures but development of sporocyst or rediae stages still continues (Galaktionov and Dovrovolskij 2003) and when water temperature rises, these new infections develop and grow fast, hence increase cercarial emission. This increase in cercarial production in this case is a consequence of the clearance of extensive accumulation of sporocyst/rediae not necessarily direct influence of higher temperature (Galaktionov and Dovrovolskij 2003, Poulin 2006).

4.2 Early emergence of *Rhipidocotyle campanula* and higher output of *R. fennica*.

Despite the earlier emergence of *R. campanula* cercariae before *R. fennica* cercariae, the mean total number of *R. fennica* cercaria produced was higher than *R. campanula* cercariae in both populations in this present study. Having known that increase in water temperature has been one of the major factors that trigger cercariae production in shedding clams, the early emergence of *R. campanula* cercariae and overall higher production of *R. fennica* cercariae at the end of the study is quite interesting. One would expect *R. campanula* that started earlier to increase production progressively as water temperature increases. Interestingly, *R. fennica* that started late shed highest number of cercariae. Jokela *et al.* (1993) have reported that *R. campanula* has a different overwintering strategy with cercariae being more readily available for shedding already in the spring. Another probable explanation could also be that *R. campanula* has a faster stage along its life cycle that enhances earlier completion which could be responsible for its early emergence. Taskinen *et al*. (1991) also reported that the sporocyst of *R. campanula* was found throughout the year in Lake Kuivasjärvi in 1989. This study did not investigate sporocyst in *A. anatina*, however availability of the sporocyst of *R. campanula* year round could possibly aid in the early emergence of the cercariae larvae as the cercariae larvae develop from sporocyst stage (see Figure 1). *R. fennica* on the other hand started late but produced highest number of cercariae. Studies and literatures have reported that, a host becomes more susceptible to parasitic exploitation when its immune defense is weak (Taskinen *et al*. 1991, Harvell *et al*. 2002), hence paving way for parasite proliferation in a weak host. This could probably the case for *R. fennica* cercariae shedding in this study.

4.3 Survival rate

In this study, uninfected clams had highest survival rate and clams shedding both parasite species with least survival rate, this is expected because the former is free from exploitation while the latter is subjected to exploitation from two different parasite species. The survival rate of clams shedding *R. campanula* cercariae was higher than *R. fennica* in both populations. Even as the experimental clams in this present study were naturally infected, owning to the fact that temperatures acts as a stressor and accelerates cercariae production in infected clams as previously discussed, the rate of exploitation of the host resources could be low by *R. campanula* and probably high by *R. fennica*, thus subjecting the host to further stress and subsequent death. Taskinen *et al*. (1994) reported that *R. fennica* cercariae has been found to use energy reserves of *A. piscinalis* meant for reproduction instead of the

maintenance energy, infected clam whose energy reserves are indiscriminately used up could become weak and imminent death. This could probably be the reason for the differences in the survival rates of the two *Rhipidocotyle* species.

Further, competition for food and position of the clams in the holding tanks are also important for their survival. Though the incoming water had a steady flow, there could be limited inflow of phytoplankton and other microscopic organisms that serve as food since they are filter feeders. Overcrowding of clams in the holding tanks could possibly give preference of survival to stronger ones in filtering the water for their food, thus subjecting the weaker ones to starvation and imminent mortality.

Handling stress during every third week monitoring could also have an impact on the survival of clams. The mussels have been described to be fragile and less tolerant to stress, this is probably one of the reasons why they are good candidate for biomonitoring (Eglund and Heino 1994). Mortality was high in August in high temperature than in others. Marcogliese (2008) reported that infected intermediate hosts are faced by mortality when the temperature favours the infective stage of parasites. Poulin (2006) also reported that various laboratory studies have revealed that the exposure of second intermediate hosts to many cercariae induces higher mortalities better than on field situation. This present study was carried out under laboratory condition and this could probably affect the survival of the clams in different temperatures.

5 CONCLUSIONS

Temperature strongly and differentially impacted *Rhipidocotyle species*/*Anodonta anatina* association. The large variation for *R. campanula* cercariae reproductive output across temperature in both population will likely rule out this general view regarding response of trematodes to increase temperature. Temperature-mediated cercariae shedding in our study was found to be species specific. Emergence of *R. campanula* started already in late May, whereas for *R. fennica* not until July. The mean period of cercarial production was about 60 days longer in *R. campanula* than in *R. fennica*, giving probably an advantage for *R. campanula* in northern areas where short summer is limiting occurrence of the parasite. In high temperature, the total annual cercariae production was clearly higher for *R. fennica* than for *R. campanula*, but the opposite was found in low temperature. With respect to this study we can predict that *R. campanula* should be more northernly distributed as it can be better adapted to low temperatures in terms of length of cercarial emergence while *R. fennica* should have an advantage in high temperature habitats or areas in terms of the number of cercariae produced. Hence, it is difficult to generalize the potential impacts of elevated temperature on cercariae shedding and subsequent parasite transmission, considering that populations in different localities will be expected to show different responses to changes in temperature.

Apart from the morphological differences in *Rhipidocotyle species*, little is known about the possible reason/s for the earlier emergence of *Rhipidocotyle campanula* before *R. fennica.* Hence this can be opened for future research as this study is the first to investigate *R. campanula* cercariae production in different temperature regimes in two rivers from different geographical locations in Finland.

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