Temperature-dependent transmission and virulence of two *Rhipidocotyle* species parasitising a molluscan host

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Abstract

Background
It has been suggested that global warming may increase both the transmission rate and the virulence of parasites. To test this hypothesis, we investigated the temperature-dependent transmission (measured as annual cercarial release) of two closely related, sympatric trematode species, *Rhipidocotyle fennica* and *R. campanula* and the molluscan host (*Anodonta anatina*) survival.

Methods
Freshwater clam hosts, *A. anatina,* from two different populations, were exposed throughout the annual cercarial shedding period between May and October to high (max. 24 °C), intermediate (max. 20 °C) and low (max. 17 °C) temperatures, which paralleled the current temperatures occurring within the distributional range of study species in Finland (60-68°N). Clams were individually monitored for cercarial release and survival at 14-day intervals.

Results
The proportion of clams shedding cercariae and the total annual cercarial output of *R. fennica* per cercariae-shedding clam increased significantly with temperature, resulting in a 200-500 fold higher annual cercarial output at a high temperature compared with a low temperature. However, these cercarial shedding traits were unaffected by temperature in *R. campanula*. Annual cercarial output of *R. fennica* was higher than that of *R. campanula*, but, only in the high temperature treatment. Regardless of the cercarial-shedding status (shedding or not), clam mortality was higher at high temperature, although, the mortality of cercariae-shedding clams was remarkably higher. The shedding of *R. campanula* cercariae was associated with a higher clam mortality than the shedding of *R. fennica*.

Conclusions
The present results 1) indicate interspecific differences in transmission with respect to thermal conditions, 2) emphasise possible between-species differences in the effects of the projected climate warming, 3) indicate that virulence associated with cercarial shedding increases with temperature, 4) suggest that *R. fennica* should be relatively more prevalent in southern areas

and *R. campanula* in northern areas of the current high latitude region, and 5) suggest that the predicted future climate warming should favour *R. fennica* more than *R. campanula*.

Key words: *Anodonta anatina,* Bucephalidae, cercarial production, climate change, temperature, transmission, Trematoda, Unionidae, virulence.

BACKGROUND

Global warming is expected to have a profound influence on parasites and host-parasite relationships. It has been predicted that, along with increasing temperatures, both the transmission rate and the virulence (the parasite-induced host mortality) of parasites will increase [1-3]. The anticipated effects of climate change include geographical range expansion or the range shift of parasites, and, if other factors remain constant, an increase in local prevalence and abundance of parasitism, as a consequence of higher mean and maximum temperatures and the associated longer warm season annually [1-3]. The warming is projected to be the strongest at high latitudes of the globe [4-6].

Trematodes are an important group of parasites, interacting within different trophic levels during their complex 2-3 host life cycle. They can have important roles in the function of aquatic ecosystems. For example, the annual production of free-swimming trematode cercariae was greater than the bird biomass in coastal estuaries [7]. Trematodes also include species that cause major veterinary or health problems [8]. Cercarial release from the molluscan host is one of the key factors of trematode transmission success. In temperate and cold climatic zones, the cercarial release of trematodes from the molluscan hosts primarily takes place during the warm summer months [9, 10]. This suggests that environmental temperature is an important determinant of cercarial emergence. Indeed, in experimental studies, cercarial release has been shown to increase with rising temperature [11, 12]. As reviewed by [13], a 10 °C rise in temperature causes, on average, an 8-fold (up to 200-300 fold in some species) increase of cercarial production in snail hosts. However, the relationship between temperature and cercarial emission is not straightforward, because neutral or even slightly negative temperature effects have also been reported [13, 14]. More recently, the results of Morley and Lewis [15] and Studer and Poulin [16] indicate that thermal effects on cercarial emergence are complex, depending on the host-parasite system, temperature range, acclimation and latitude.

The importance of studying the temperature-cercarial shedding relationship at high latitudes has been highlighted in previous papers [15, 16], but, to our knowledge, experimental temperature manipulations have not been performed in relation to high latitude (> 60°) host-trematode associations. Thus, the aim of the present study was to investigate the effect of temperature on cercarial release and virulence (parasite-induced mortality) of two closely related, sympatric trematodes, *Rhipidocotyle campanula* and *R. fennica* (Bucephalidae), in their first intermediate molluscan host, *Anodonta anatina* (Bivalvia, Unionidae) in a northern latitude. In addition to sharing the same first host, these parasites also have the same second intermediate host, the cyprinid fish *Rutilus rutilus* [17, 18]. We used three temperature levels

covering the range of summer temperatures (15-25 °C) currently occurring in the distributional range of *A. anatina* at 60°-68°N in Finland.

Thus, our hypothesis was that the transmission, measured as annual cercarial production, of *R. fennica* and *R. campanula* would increase with temperature, whereas the survival of the host *A. anatina* would decrease, as their metabolic rate is expected to increase with temperature. Furthermore, as Jokela et al. [19] found *Rhipidocotyle* infection to increase the mortality of *A. anatina* under stress, our hypothesis was that the lowest host survival would be among those clams shedding cercariae at high temperature.

METHODS

Study species

The first intermediate host, *Anodonta anatina,* is a common, long-lived (> 10 years) and abundant freshwater bivalve in Europe. It is mature at 2-4 yr of age and can grow up to 12 cm in length [20, 21]. During July, female *A. anatina* develop glochidia larvae in the outer gill blades, where they are stored and maintained over winter to be released the following spring [22-24]. Before the start of their benthic life, the glochidia are ectoparasitic on a variety of freshwater fish species [22, 25].

The parasites, *Rhipidocotyle campanula* and *R. fennica* use *A. anatina* as their first intermediate host where the miracidium larvae develop into sporocysts. Branching sporocyst tubules of *Rhipidocotyle campanula* and *R. fennica* invade mainly the gonads of the freshwater bivalve clam *A. anatina* [17, 24, 26] producing asexually large numbers of free-swimming cercarial larvae. Emerged cercariae are transmitted to the second intermediate host, the cyprinid fish *Rutilus rutilus*, where they encyst as a metacercaria, and subsequently pass to the final host, the predatory fishes *Perca fluviatilis/Stizostedion lucioperca* and *Esox lucius*, respectively, in which the worms mature and produce eggs [17, 18]. In natural *Anodonta* populations, the prevalence of infection by *R. campanula* is usually less than 10% [17, 26], whereas that of *R. fennica* can be up to 50 % [27]. Both parasite species have been linked to the decreased growth, survival and reproduction of *A. anatina* [19, 21, 26, 28]. The mean (± s.e.) annual cercarial output of *R. fennica* was previously estimated to be 290,000 ± 26,000, with a maximum production of 440,000 cercariae y^{-1} [10].

Clam collection

A. anatina individuals were collected by snorkelling from the River Kuusaankoski (62°25'N, 26°00'E) and the River Haajaistenjoki (63°63'N, 26°99 'E) in southern Finland on May 17, 2011 and May 22, respectively (water temperature was 9 °C at both sites). At the Konnevesi Research Station, University of Jyväskylä, clams were individually marked and measured (shell length, height and width) on the date of collection and at the end of the study. Average shell length (± s.e.) in clams from the River Haajaistenjoki and River Kuusaankoski was 61.8 ± 0.6 mm (range 33.0 - 92.6 mm, n = 290) and 77.8 ± 0.6 mm (range 51.7 - 101.7 mm, n = 281), respectively. There was no length-difference between clams allocated to different temperature

treatments (see below) ($F_{1, 565}$ = 0.040, P = 0.961) and there was no interaction between population and treatment ($F_{1, 565}$ = 0.728, P = 0.484) in terms of clam length.

From the date of collection until June 25, clams were established in the laboratory in two 163 l tanks (48 x 60 x 70 cm) under flow–through conditions (i.e. allowing a continuous flow of new water) with one population per tank. Each tank was filled with 5 cm of sand on the bottom and supplied with running water (10 l min^{-1}) from the hypolimnetic zone (9 m depth) of Lake Konnevesi. Water temperatures in both tanks were the same throughout this period and ranged from 10.5 °C on May 31 to 11.7 °C on June 25 (Fig. 1).

Temperature treatments

From June 25, 2011, clams were randomly assigned to treatment groups of high, intermediate and low temperature; two replicate tanks per treatment. Clams from both populations and from all size groups were allocated evenly among the six tanks (48 River Haajaistenjoki clams per tank and 47 River Kuusaankoski clams per tank). The temperature range used in the three treatments (Fig. 1), were chosen to cover the natural variation experienced throughout the distribution area of *A. anatina* in Finland between 60° to 68°N, with the maximum summer temperatures varying from about 23° to 15°C, respectively. From June 25 to October 28 (when experiment was terminated because cercarial shedding had stopped practically in all treatments), the average water temperatures from June 25 to October 28 were 18 °C (range 7–24 °C), 15 °C (range 6.6–20 °C) and 13 °C (range 6–17 °C) in high, intermediate and low temperature treatments, respectively. The maximum daily water temperature was attained on July 27 in high temperature (24 °C) and, on September 4 in the intermediate (20 °C) and low temperature treatments (17 °C).

Anodonta clams are filter feeders utilising phytoplankton, bacteria and fine organic particles [28], thus a continuous flow of lake water was necessary to provide the clams with food. To create the high temperature treatment, the tanks were placed in an outside shelter and supplied with running water from the littoral zone (< 2m depth) of Lake Konnevesi. Low and intermediate temperature tanks were kept indoors and supplied with the colder hypolimnetic water and heated hypolimnetic water from Lake Konnevesi, respectively. Due to logistic constraint, some differences between the treatments, in addition to temperature, could not be avoided. A submersible temperature logger, was placed in one replicate tank per treatment from June 25 to October 28 to measure water temperature every 4 h. In the high temperature treatment, the clams were subject to a larger daily fluctuation of temperature than the intermediate or low temperature (Fig. 1), as the littoral water and outdoor tanks were used. The indoor tanks were illuminated by artificial lights, however, the photoperiod was set to correspond with that outside. The outdoor tanks received natural light and the large shelter above the tanks provided effective cover against sunlight. However, during each 24 h cercarial release monitoring, similar artificial light was used for all clams to provide equal light conditions (see below). Water flow was set to be higher in the intermediate and low temperature tanks (10 l min^{-1}) than in the high temperature (5 l min^{-1}) to compensate for the likely higher food density in the high temperature tanks that received littoral water, as compared to the intermediate and low temperature tanks.

Cercarial emergence

The number of cercariae released per *A. anatina* was counted at 14-day intervals over a period of 20 weeks between 31 May and 28 October, 2011, during a total of 12 monitoring sessions. Each clam was placed individually into a 4 l transparent plastic box (length 26.5 cm, width 19 cm and height 13.6 cm) filled with 2 l of filtered lake water. Host mortality was assessed at this stage. After 24 h, clams were removed and shed cercariae were identified, following Taskinen et al. [17], and counted visually (when numbers were low) or from a 50 ml subsample with a microscope (when cercarial numbers were high). The temperature prevailing in each temperature treatment was maintained during monitoring – if necessary, a temperature regulated room was used. Similar artificial lights were used for all clams and light conditions were set to correspond with natural day length and rhythm, because the cercarial release of *Rhipidocotyle* species is diurnal [17]. The experiment was terminated on October 28, 2011. At that point cercariae emergence from *A. anatina* had ceased practically in all treatments. For *R. fennica* and *R. campanula*, the seasonal cercarial shedding period has been reported to occur between late May and early October [10, 24, 27].

Data analysis

Cercarial output for each 14-d interval was estimated/calculated by multiplying the cercariae release on each monitoring day by 14. To determine the total annual cercarial output, cercarial productions for all 14-d intervals were summed. All statistical analyses were performed using PASW Statistics 18. Clams that did not shed cercariae (except for the survival analysis), and those that shed both *R. campanula* and *R. fennica* cercariae (double infected), as well as those that were infected by *Phyllodistomum* sp. were not included in the analyses. Before the final analyses, data from two replicate tanks were combined, as prior analyses revealed no differences between replicates for any measured variables. Means are given with ± 1 standard error (s.e.).

χ^2-tests were used to compare the proportions of clams shedding *R. fennica* and *R. campanula* for the different temperature treatments. To compare temperature treatments and populations with regard to the log-transformed total annual cercarial output, 2-way ANOVA was applied for *R. campanula* and *R. fennica* with treatment and population as fixed factors.

To compare the two parasite species, the treatments were analysed separately to satisfy ANOVA assumptions. Total annual cercarial output was used as the response variable, while parasite species (*R. campanula*, *R. fennica*) and population (River Kuusaankoski, River Haajaistenjoki) were the fixed factors. To account for multiple tests, the Bonferroni correction was applied to p-values when analysing differences between parasite species. Whenever ANOVA indicated significant effect of temperature, the differences between treatments were analysed with Tukey's Post Hoc tests.

Survival differences with respect to cercarial-shedding status (shedding or not) were studied using logistic regression, with survival (survived throughout the experiment vs. did not survive) as the dependent variable and temperature treatment (TREAT), clam population (POPU) and cercarial-shedding status (CERC) as independent categorical covariates. The logistic regression model that best described the data was searched using an automated forward stepwise model construction procedure of PASW utilising likelihood ratio

significance tests for evaluating each explaining term, i.e. the three categorical covariates as well as all their possible interactions. It is worth noting that it was not possible to compare the survival of uninfected and infected clams, since the infection status of the dead individuals was not always clear.

Clams growth was analysed using 2-way ANOVA with population and temperature treatment as fixed factors and shell length increment from the start to the end of the experiment as the dependent variable; only the non-cercariea-shedding clams were included, since *Rhipidocotyle* infection reduces the growth of *A. anatina* [28].

RESULTS

Cercarial release

The total shedding period by *R. fennica* from the first to the last observation of emergence lasted for 10–16 weeks in high and intermediate temperature treatments, respectively, and for 4–6 weeks at low temperature. In contrast, that of *R. campanula* ranged from 14 to 18 weeks at low temperature, (which was remarkably longer than that for *R. fennica*), and it varied from 8 to 18 weeks at high and intermediate temperatures. The difference in the proportion of clams shedding *R. fennica* cercariae between high and intermediate temperatures was significant (χ^2-test, $p < 0.008$ in both rivers), as was that between high and low temperatures (χ^2-test, $p < 0.001$ in both rivers) and between the intermediate and low temperatures (χ^2-test, $p < 0.013$ for both rivers). Thus, the proportion of clams from both populations shedding *R. fennica* increased remarkably with temperature (Table 1A). In contrast, there were no differences in the proportion of clams shedding *R. campanula* cercariae between the three treatments, either in the River Haajaistenjoki (χ^2-test, $p = 0.315$) or the River Kuusaankoski (χ^2-test, $p = 0.765$) (Table 1A) material.

The effect of temperature on the total annual cercarial output of *R. fennica* was statistically significant but not of *R. campanula* (Table 2). The total cercarial output of *R. fennica* clearly increased with increasing temperature (Fig. 2), being 30 and 27 times higher at high compared to low temperature in both River Haajaistenjoki and River Kuusaankoski clams, respectively (all paired differences were significant using the Tukey's post hoc tests), whereas the annual cercarial output of *R. campanula* was constant with respect to temperature (Fig. 2).

Interspecific differences in annual cercarial output were highly significant at the high temperature, but not for the other temperature treatments (Table 3). In the high temperature treatment, the annual cercarial output of *R. fennica* was as much as 18 times that of *R. campanula* (Fig. 2). The effect of population and the treatment × population interaction , were not significant under any treatment (Table 3), suggesting that the annual cercarial output within each treatment group was independent of the population, and that the treatment specific effects for both parasite species were identical in both populations.

If host density is constant, the number of cercariae produced at the population level is a product of the mean annual cercarial output clam^{-1} and the proportion of shedding clams. Consequently, the estimated annual cercarial output of *R. fennica* at the population level at the high temperature was, respectively, 179 and 518 times higher than that at the low temperature

in both the River Haajaistenjoki and River Kuusaankoski clams (Table 4). In contrast, for these two populations, the estimated annual cercarial output of *R. campanula* at the high temperature was 1.8 and 9.8 times lower than that at the low temperature, respectively (Table 4).

Survival and growth of clams

When analysing the survival of the clams, the logistic regression model that best fits the data included the terms treatment (TREAT) (change in log likelihood of the model if term removed = 18.193, df = 2, $P < 0.001$) and cercarial production status (CERC) (change in log likelihood if term removed = 10.580, df = 2, $P = 0.005$) (Table 5). Within the term TREAT, the contrast that compared survival at high versus low temperature was significant with an odds ratio value of 0.538 (Table 5), indicating that clam survival at high temperature was only close to half that at low temperature (see also Table 1B). Within the term CERC, the contrast, comparing non-shedding clams to *R. fennica* shedding individuals, was not significant, whereas the contrast that matched non-shedding clams against *R. campanula* shedding individuals was significant (Table 5). This indicates that clam survival was lower among *R. campanula* shedding clams (but not among those shedding *R. fennica*) than among non-shedding individuals (see Table 1B). The odds ratio value suggests that the probability of survival throughout the experiment was 1.63-fold in non-shedding clams as compared to *R. campanula* shedding clams. Although the TREAT × CERC interaction was significant (change in log likelihood of the model if term removed = 11.768, df = 4, $P = 0.019$), none of the contrasts comparing temperature treatments / shedding groups were statistically significant *per se*. The proportion of surviving individuals among clams shedding *R. fennica* and *R. campanula* decreased significantly from 75 and 68 % to 31 and 18 %, respectively, from low to high temperature (χ^2 test, $P = 0.015$ and $P = 0.005$, respectively), while the respective decrease among the non-shedding clams was from 75 to 68 % (χ^2 test, $P = 0.227$) (populations combined). Thus, the TREAT × CERC interaction was probably due to a higher decrease of survival at high temperature in individuals shedding cercariae than in non-shedding ones.

The growth of *A. anatina* was not affected by temperature treatment (2-ANOVA, $F_{2, 221} = 0.526$, $P = 0.592$), population ($F_{1, 221} = 0.025$, $P = 0.873$) or by their interaction ($F_{2, 221} = 1.667$, $P = 0.191$). The mean shell length increment of the clams that did not release cercariae during the experiment, was 0.2 ± 0.1, 0.4 ± 0.3 and 0.1 ± 0.1 mm in high, intermediate and low temperatures (populations combined), respectively, equalling 0.1-0.7 % of their initial length.

DISCUSSION

Analyses related to the effects of global warming on host-parasite relationships predict (i) an increase in transmission and virulence of the parasites and (ii) species-specific variation (i.e. context dependency) in the transmission success of the parasites [1-3]. The results of the present study mainly support these predictions. The transmission of *R. fennica*, measured as the annual cercarial output, was remarkably higher at a high temperature compared with a low temperature treatment. However, that of *R. campanula* was unaffected by temperature

treatments, suggesting a fundamental interspecific difference in the temperature–cercarial shedding relationship between these two species. In addition, regardless of the cercarial-shedding status (shedding or not shedding), clam mortality was significantly higher at high temperature, although, the mortality of cercariae-shedding clams was considerably higher, which supports the prediction that parasite virulence increases with increasing temperature.

The contrasting temperature response in annual cercarial release of *R. fennica* and *R. campanula* suggest that the effect of climate warming on these two closely related, sympatric trematodes species, even though they utilise the same molluscan host and are transmitted to the same second intermediate host, may be remarkably different. The predicted climate warming of 2 to 7° C by 2080s in these high latitudes [30] should clearly favour *R. fennica* more than *R. campanula*. The present results also allow us to predict that the current latitudinal distribution of the species may change. If transmission of the other life cycle stages would remain the same, *R. fennica* should be relatively more prevalent in southern areas and *R. campanula* in northern areas of the current study region.

It would be tempting to suggest that the good performance of *R. campanula* in the low temperature treatment would be an adaptation to northern, high latitudinal conditions with low summer temperatures. However, the geographical distribution of this species in Europe does not support this view. Both *R. campanula* and *R. fennica* occur as far south as the Ukraine [17, 26, 31, 32]. The northernmost finding of bucephalid trematodes is that of *R. campanula* in Lake Kuivasjärvi, Finland (65 °N) [27], but a detailed mapping of occurrence and abundance of *R. fennica* and *R. campanula* in northern regions is required. It is also difficult to explain the observed difference between these *Rhipidoctyle* species in terms of transmission. Although the definitive hosts of the species differ (*E. lucius* and *P. fluviatilis* / *S. lucioperca*, respectively) [17, 18], it is not known how transmission between the various life cycle stages might explain the observed result. It is also worth noting that both *R. fennica* and *R. campanula* are restricted to only *A. anatina* as their first intermediate host in Finland [17, 18].

The total annual production of *Rhipidocotyle* cercariae measured in field conditions yielded clearly higher numbers than in the present study [10]. A plausible reason for this difference could be a resource limitation in the present study, which was carried out under laboratory conditions. This was indicated by the lower survival of cercariae-shedding clams (48.3-80.0 %, Table 1) and a lower growth (less than 1 %) in this experiment compared to 91.7 % survival and 3.4 % growth under field conditions [10, 28]. However, even though there were differences between the treatments in terms of water flow and water source (littoral or hypolimnetic), the equal growth of non-shedding clams in the different temperature treatments suggests that the treatments did not differ in terms of the abundance of food resources. The tanks also differed with respect to light conditions and temperature fluctuations. High temperature tanks kept in an outdoor shelter were subject to a diurnal temperature fluctuation and natural light, whereas the intermediate and low temperature tanks were located in an indoor tank hall and illuminated with artificial light, although the photoperiod rhythm was equal in all treatments. Even though we cannot completely rule out confounding factors other than temperature, we do not believe that differences in water and light source, or temperature fluctuation, would likely explain the observed contrasting responses in the cercarial release of *R. fennica* and *R. campanula* between the temperature

treatments. New infections, via miracidia from unfiltered lake water, were unlikely due to the seasonal maturing of *Rhipidocotyle* trematodes in late autumn [17]. Thus, the present results should reliably indicate the temperature responses of *R. fennica* and *R. campanula* in a long term experiment – an attribute that differentiates this study from most of the temperature/cercarial shedding studies that have been completed within relatively short period (days or weeks).

Many parasites display greater virulence (i.e. parasite-induced host mortality) at higher temperature [33]. In the present study, the mortality of cercariae-shedding clams was remarkably greater at higher temperature, indicating that virulence associated with cercarial shedding increases with temperature. Jokela et al. [34] have demonstrated that the mortality of cercarial-shedding snails is higher than that of non-shedding snails under stress condition. This is not surprising because cercarial production is very demanding and costly for the host. The intensive production of cercariae is usually accompanied by excessive depletion of host energy reserves [35] and excessive tissue damage or even death of the hosts [36]. Moreover, the mortality of mollusc host has been reported to be highest during the cercarial shedding period [37]. The synergistic effect of cercarial shedding and increasing temperature is important to the molluscan host. The shedding of *R. campanula* cercariae induced higher host mortality than the shedding of *R. fennica* in the present study. This is in accordance with the previous result that infection by *R. campanula* increased the mortality of *A. anatina* under stress more than infection by *R. fennica* [19]. Furthermore, higher virulence was associated with the release of *R. campanula* cercariae even though the numbers of cercariae released were much higher in *R. fennica*. This can probably be explained by the more intensive host use by *R. campanula* in terms of the number of parasite sporocysts in the host clam, by a more frequent invasion of vital organs by sporocysts of *R. campanula*, as well as by the seasonal timing of cercarial emergence by *R. campanula* to a period that is more harmful for *A. anatina*, as compared to *R. fennica* [24].

Trematode parasites are a ubiquitous part of freshwater and marine food webs. These worms may account for a remarkable proportion of the biomass and production in an aquatic ecosystem [7, 38] and thus play important roles in aquatic food webs and energy transfer [39]. This is also true for *Rhipidocotyle* species. Cercariae of *R. fennica* may occur in densities of 1,000 larvae m^{-3} [10], *Rhipidocotyle* infections in the fish second intermediate host may have prevalences up to 95 % [40], and an infection with *Rhipidocotyle* parasites may sterilise *A. anatina* and reduce clam growth [21, 28]. Therefore the present study contributes to our understanding and helps with prediction of the effects of climatic warming on parasitism by species of *Rhipidocotyle*. Moreover, the current results does not completely support the prediction that climate change will generally benefit parasites, as warming was associated with an increased cercarial output only in the case of *R. fennica*, but completely support the complexity of climate change–parasitism interplay [1-3, 41, 42].

Although, these results indicate a clear increase in cercarial output by *R. fennica* at higher temperatures, further studies examining the survival and infectivity of these parasites in relation to temperature would provide a better understanding of the transmission of *Rhipidocotyle* species from the first to the second host. Our study focused on cercarial emergence from the clam host, which is an important feature in the transmission dynamics of

infection in trematode life cycle, however, cercarial survival and infectivity, both of which are temperature-dependent are also crucial to the transmission process of *Rhipidocotyle* parasites. Cercarial survival generally decreases with increasing temperature (McCarthy 1999, Mouritsen 2002), likely owing to a faster depletion of their energy reserves, and cercarial infectivity increases with temperature, at least up to an optimum temperature (McCarthy 1999, Studer *et al*. 2010). Therefore, increased cercarial emergence and lengthening of the seasonal duration of larval release with increasing temperature and the associated longer thermal growing season, will not necessarily translate into increased transmission success if the cercariae have lower survival and infectivity at high temperature. Further studies on the effects of clam exposure to higher temperatures than those used here will be needed to better understand how *Rhipidocotyle spp.-A. anatina* system will respond to predicted temperature rise in Finland.

COMPETING INTERESTS

Both authors in this research paper declare that they have no competing interests.

AUTHOR'S CONTRIBUTIONS

JMC and JT contributed equally to the planning and implementation of the study, including the writing. Both authors read and approved the final manuscript.

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Table 1. Non-shedding and cercariae-shedding *Anodonta anatina* clams, and their survival at different temperatures. A - Numbers and proportions (%) of *A. anatina* clams that did not release cercariae (non-shedding), released *R. campanula* or *R. fennica* cercariae. B - Numbers and proportions of individuals which survived among the respective categories over a period of 20 weeks.

	A. Number of clams		B. Number of clams surviving	
	R. Haajaiste.	R. Kuusaan.	R. Haajaiste.	R. Kuusaan.
High temperature				
Non-shedding	46 (52.9%)	53 (57.0%)	32 (69.6%)	35 (66.0%)
R. fennica	33 (37.9%)	37 (38.8%)	9 (27.3 %)	13 (35.1%)
R. campanula	8 (9.2%)	3 (3.2%)	1 (12.5%)	1 (33.3%)
Total	87 (100%)	93 (100%)	42 (48.3%)	49 (52.7%)
Intermediate temp.				
Non-shedding	66 (68.8%)	74 (79.6%)	46 (69.7%)	51 (68.9%)
R. fennica	19 (19.8 %)	13 (14.0%)	10 (52.6%)	11 (84.6%)
R. campanula	11 (11.5%)	6 (6.5%)	6 (54.5%)	4 (66.7%)
Total	96 (100%)	93 (100%)	62 (64.6%)	66 (71.0%)
Low temperature				
Non-shedding	69 (72.6%)	85 (89.5%)	46 (66.7%)	69 (81.2%)
R. fennica	6 (6.3%)	2 (2.1%)	4 (66.7%)	2 (100%)
R. campanula	20 (21.1%)	8 (8.4%)	14 (70.0%)	5 (62.5%)
Total	95 (100%)	95 (100%)	64 (67.4%)	76 (80.0%)

Table 2. Differences between treatments in the total annual cecarial output. 2-way ANOVA statistics for the effect of temperature treatment and clam population on (log-transformed) the annual cercarial release of *R. fennica* and *R. campanula*. Statistically significant effects are marked with an asterisk*.

Parasite	Factor	Statistics	*P*
R. fennica	Treatment	$F_{2, 104} = 36.386$	< 0.001*
	Population	$F_{1, 104} = 0.428$	0.514
	Treatm. x Pop.	$F_{2, 104} = 3.887$	0.024*
R. campanula	Treatment	$F_{2, 50} = 1.057$	0.355
	Population	$F_{1, 50} = 11.421$	0.002*
	Treatm. x Pop.	$F_{2, 50} = 5.227$	0.009*

Table 3. Interspecific difference in the total annual cercarial output. Results of 2-way ANOVA testing the effect of parasite species (*R. fennica* vs. *R. campanula*) and clam population on the annual cercarial release in each temperature treatment. Statistically significant effects are marked with an asterisk*.

Treatment	Factor	Statistics	*P*
High temperature	Species	$F_{1, 77} = 9.501$	0.003*
	Population	$F_{1, 77} = 1.125$	0.292
	Species x Pop.	$F_{1, 77} = 0.699$	0.416
Intermediate temp.	Species	$F_{1, 45} = 3.118$	0.081
	Population	$F_{1, 45} = 0.255$	0.616
	Species x Pop.	$F_{1, 45} = 2.037$	0.160
Low temperature	Species	$F_{1, 32} = 1.316$	0.260
	Population	$F_{1, 32} = 0.197$	0.660
	Species x Pop.	$F_{1, 32} = 0.026$	0.873

Table 4. Annual cercarial output at the population level. The number of cercariae shed at the populations level is a product of the mean numbers of cercariae released annually, the proportion of cercariae-shedding clams and the calculated total annual cercarial output per 1000 clams (with 95 % confidence interval in parentheses) based on the proportion of cercariae-shedding clams and the mean number of cercariae shed per clam during the experiment.

	Cercariae individual^{-1}	% shedding	Cercariae population^{-1}
Rhipidocotyle fennica			
River Haajaistenjoki			
High temperature	28266 (11922-36611)	37.9 (28.5-48.4)	10713 (3398-17720)
Intermediate temp.	4004 (1945-6063)	19.8 (13.1-28.9)	792 (255-1752)
Low temperature	947 (36-1858)	6.3 (2.9-13.1)	60 (1-243)
Total	17492 (11771-23213)	20.9 (16.5-26.0)	3656 (1942-6035)
River Kuusaankoski			
High temperature	15617 (10249-20985)	39.8 (30.4-49.9)	6216 (3116-10472)
Intermediate temp.	5346 (1241-9451)	14.0 (8.4-22.5)	748 (104-2126)
Low temperature	585 (0-1411)	2.1 (0.6-7.4)	12 (0-104)
Total	12471 (8347-16596)	18.5 (14.4-23.5)	2307 (1202-3900)
Rhipidocotyle campanula			
River Haajaistenjoki			
High temperature	2005 (470-3539)	9.2 (4.7-17.1)	184 (22-605)
Intermediate temp.	3483 (1384-5582)	11.5 (6.5-19.4)	401 (90-1083)
Low temperature	1535 (857-2213)	21.1 (14.1-30.3)	324 (121-671)
Total	2181 (1459-2902)	14.0 (10.4-18.6)	305 (152-540)
River Kuusaankoski			
High temperature	371 (0-1624)	3.2 (1.1-9.1)	12 (0-15)
Intermediate temp.	672 (0-1614)	6.5 (3.0-13.4)	44 (0-216)
Low temperature	1366 (342-2390)	8.4 (4.3-15.7)	115 (15-375)
Total	946 (402-1489)	6.1 (3.8-9.5)	58 (15-141)

Table 5. Logistic regression statistics of clam survival during the experiment. Categorical variables explaining survival were temperature treatment (TREAT; high, intermediate and low temperature), cercarial shedding status (CERC; non-shedding, shedding *R. campanula* and shedding *R. fennica*) and clam population (POPU; River Haajaistenjoki and River Kuusaankoski). TREAT contrasts survival at high temperature to that at intermediate or low temperature. CERC contrasts survival of non-cercarial-shedding clams to that of *R. campanula*-shedding and *R. fennica*-shedding individuals.

Best model	ß ± s.e.	Wald	df	*P*	Odds ratio
TREAT		14.763	2	0.001	
High vs. Intermediate temp.	-2.50 ± 0.20	1.561	1	0.212	0.78
High vs. Low temperature	-6.20 ± 0.24	6.779	1	0.009	0.54
CERC		10.041	2	0.007	
Non-shedding vs. *R. fen.*	0.03 ± 0.24	0.020	1	0.888	1.03
Non-shedding vs. *R. cam.*	0.49 ± 0.25	3.828	1	0.050	1.63
TREAT x CERC		10.862	4	0.028	
Variables not in the best model		Score	df	*P*	
POPU		3.005	1	0.083	
POPU x TREAT		1.217	2	0.544	
CERC x POPU		2.123	2	0.346	
CERC x POPU x TREAT		5.236	4	0.264	

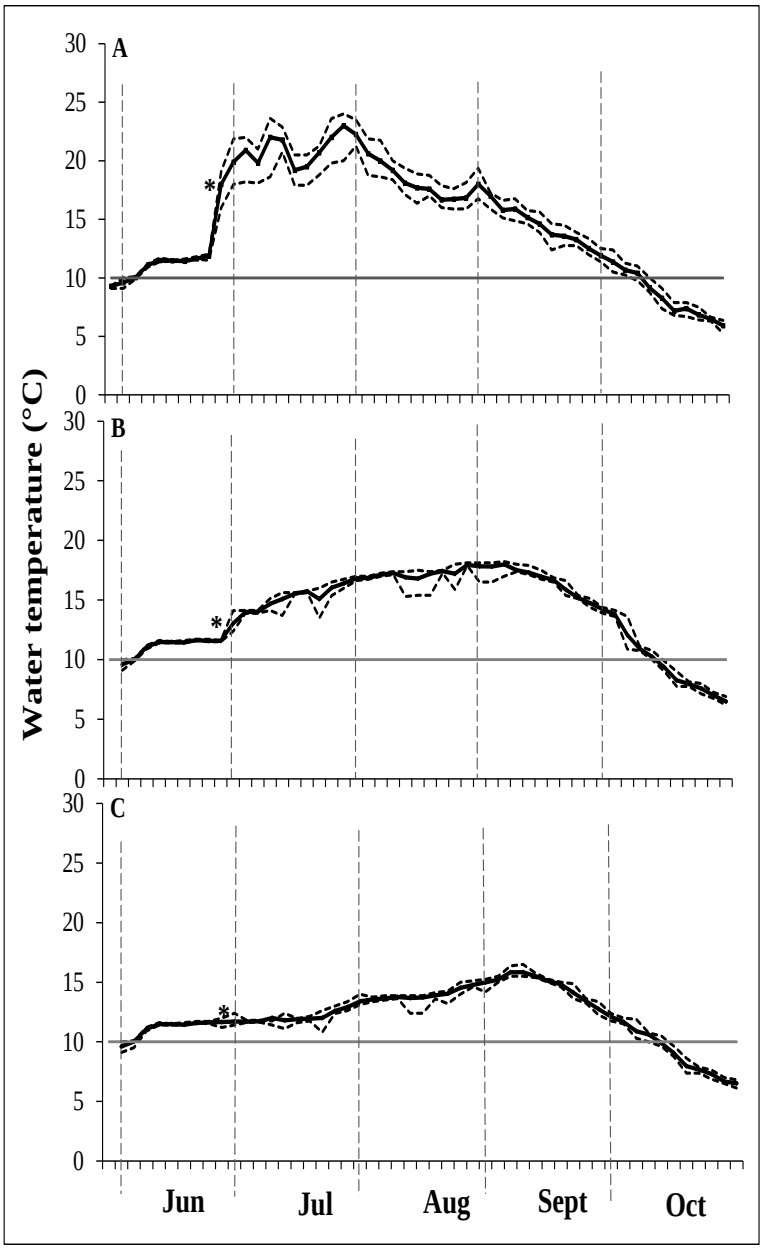

Figure 1. Water temperature profile in the three temperature treatments from June 25 to October 28. A – high temperature. B – intermediate temperature. C – low temperature. A submersible temperature logger, was placed in one replicate tank per treatment from June 25 to October 28 to measure water temperature every 4 h. Average (solid line), minimum and maximum (dotted lines) temperatures for every third day experienced by clams. Asterisks represents the day when clams were assigned to the different temperature treatments.

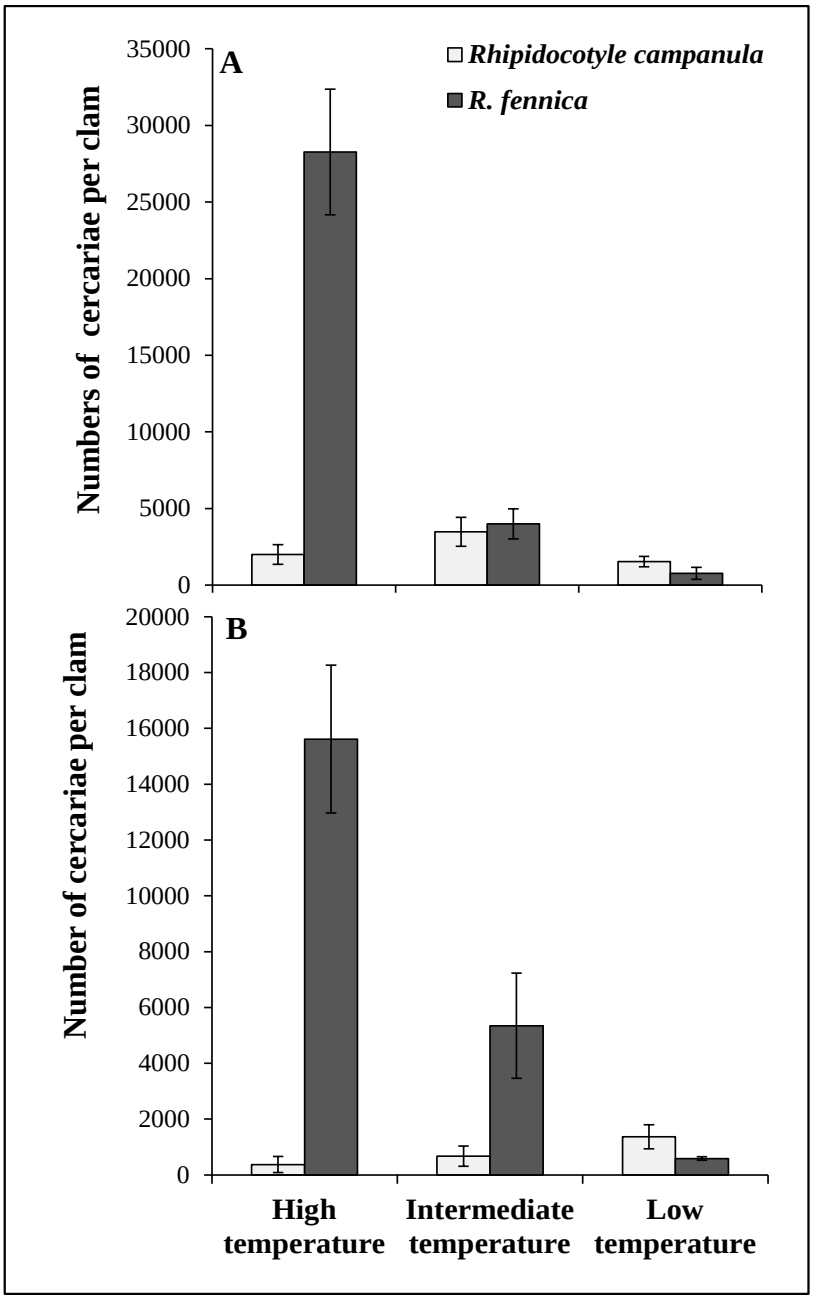

Figure 2. Total annual cercarial output (mean ± s.e.) of *R. campanula* and *R. fennica* at different temperatures. A – total annual average cercarial output from the molluscan host, *Anodonta anatina,* originating from the River Haajaistenjoki. B - total annual average cercarial output from *A. anatina,* originating from the River Kuusaankoski. For numbers of clams, see Table 1A.