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Allee effect in a manipulative parasite within poikilothermic host under temperature change

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Abstract

Temperature and intraspecific competition are important factors influencing the growth of all organisms, including parasites. The temperature increase is suggested to stimulate the development of parasites within poikilothermic hosts. However, at high parasite densities, this effect could be diminished, due to stronger intraspecific competition. Our study, for the first time, addressed the joint effects of warming and parasite abundances on parasite growth in poikilothermic hosts. The growth of the common fish parasite larvae (trematode *Diplostomum pseudospathaceum*) within the rainbow trout at different infection intensities and temperatures (15°C and 18°C) was experimentally investigated. The results showed that temperature was positively correlated with both parasite infection success and growth rates. The growth rates increased much more compared to those in many free-living poikilothermic animals. Atypically for a majority of parasites, *D. pseudospathaceum* larvae grow faster when abundant (Allee effect). The possible causes for this phenomenon (manipulation cost sharing, etc.) are discussed in this study. Importantly, limited evidence of the interaction between temperature and population density was found. It is likely that temperature did not change the magnitude of the Allee effect but affected its timing. The impact of these effects is supposed to become more pronounced in freshwater ecosystems under current climate changes.

Introduction

Recent global climate changes have garnered much attention due to their impact on free-living and parasitic animals both at individual and population levels (Harvell *et al.*, 2002; Walther *et al.*, 2002). Temperature is a powerful factor affecting parasite transmission in aquatic ecosystems (Marcogliese, 2001, 2016). It positively influences the growth of parasite larvae in poikilothermic hosts (reviewed in Chubb, 1979, 1980; Barber *et al.*, 2016). Temperature effects can be modified by the within-host factors controlling parasite development, among which the density of parasite infrapopulation is of particular importance (Holmes, 1961; Poulin, 1994; Brown, 1999; Kuris, 2003; Parker *et al.*, 2015). For example, many parasites grew slower when abundant due to ‘crowding effect’, i.e. stronger intraspecific competition for resources (Read, 1951; Bush and Lotz, 2000; Parker *et al.*, 2015; Fong *et al.*, 2017). At high infrapopulation densities, crowding could diminish the stimulating effect of warming on parasite growth (parasites may benefit less from higher temperatures than in smaller infrapopulations). Therefore, opposite impacts of temperature and parasite density on growth of parasites could be expected, but to our knowledge, there is lack of quantitative experimental studies on the combined effect of these factors.

However, temperature and parasite density do not necessarily influence parasite growth in opposite directions. In a few studies of trophically transmitted, manipulating, small-sized trematode larvae, the ‘crowding effect’ was not detected or even a positive density-dependence was reported (Weinersmith *et al.*, 2014; Gopko *et al.*, 2017*a*), probably due to a weak competition for resources and cooperation between conspecifics sharing costs of host manipulation and/or defence against host’s immunity (Weinersmith *et al.*, 2014; Gopko *et al.*, 2017*a*). Therefore, growth of such parasites can potentially be jointly stimulated by higher temperature and parasite densities. The positive density-dependent growth can be considered as an example of a component Allee effect, defined as ‘a positive relationship between any component of individual fitness and either numbers or density of conspecific’ (Stephens *et al.*, 1999). This is because metacercariae size/growth rate is tightly connected with the shorter maturation time/probability of successful transmission to the next host in trematodes. The component Allee effect refers to a certain fitness-related trait whose mean (*per capita*) value increases with population size, in contrast to the demographic Allee effect, which is observed at the level of overall population dynamics (Stephens and Sutherland, 1999; Stephens *et al.*, 1999; Angulo *et al.*, 2018).

Numerous field studies concern seasonal dynamics of different parasitic worms (Chubb, 1977, 1979, 1980). Growth rates of macroparasite larvae in poikilothermic hosts are suggested to increase with temperature, similarly to free-living organisms (Schmidt-Nielsen, 1997). It has

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been supported by *in vitro* studies on Cestoda plerocercoids (Wikgren, 1966; Sinha and Hopkins, 1967) and *in vivo* experiments on larvae of different helminths within invertebrate hosts (Chubb, 1980; Tokeson and Holmes, 1982; Lv *et al.*, 2006; Studer *et al.*, 2010). However, for parasite larvae in vertebrate poikilotherms (e.g., fish), experimental evidence of faster growth at higher temperatures is still scarce (reviewed in Chubb, 1979, 1980; Voutilainen *et al.*, 2010; Macnab and Barber, 2012; Franke *et al.*, 2017). In most of the studies, the growth of larvae was assessed by the presence of certain developmental stages and the size of larvae was measured (if measured) only at the end of experiments, while data on growth rates were not provided. The only exception is the study of trematode growth under low temperatures (10–15°C) (Voutilainen *et al.*, 2010), while information about growth rates of parasite larvae within the fish hosts at higher temperatures is absent. Further investigations of temperature effects on parasite growth are important for predicting the consequences of climate change for parasite transmission (Marcogliese, 2001, 2016; Cable *et al.*, 2017).

In this study, we aimed to experimentally study the growth of trematode larvae at different parasite densities and temperature conditions. As a host–parasite study system, we chose rainbow trout infected with the eye fluke *Diplostomum pseudospathaceum*, a common fish parasite that impairs vision and alters fish behaviour in natural freshwater ecosystems and fish farms (Karvonen *et al.*, 2004; Seppälä *et al.*, 2004; Mikheev *et al.*, 2010; Gopko *et al.*, 2015, 2017*b*). This study system shows the evidence for positive effect of both temperature (Voutilainen *et al.*, 2010) and infrapopulation density (Gopko *et al.*, 2017*a*) on parasite growth. *Diplostomum* metacercariae are interesting objects to study the influence of temperature on host–parasite interactions since they localize in eye lenses, where the confounding effect of host immunity is minimal (Dittmar *et al.*, 2014) or absent (Höglund and Thuvander, 1990; Wegner *et al.*, 2007).

We hypothesize that: (1) both temperature and parasite' infrapopulation density have a positive influence on the growth of *D. pseudospathaceum* metacercariae; (2) a positive interaction between these two factors exists.

Materials and methods

Study objects

Experiments were conducted at the Konnevesi Research Station (University of Jyväskylä) in July–August 2019. Rainbow trout, *Oncorhynchus mykiss*, was used as the host for *D. pseudospathaceum*. Young-of-the-year rainbow trout, free of macroparasites, were obtained from a fish farm and acclimated to laboratory conditions (temperatures 14–15 °C) in 165 L flow-through tanks filled with water from Lake Konnevesi for 2 weeks. Cercariae for infection were obtained from six infected pond snails *Lymnaea stagnalis* (the first intermediate host of *D. pseudospathaceum*) collected from Lake Konnevesi. The procedures of snail maintenance in the laboratory, identification, and counting of cercariae were similar to those described previously (e.g., Gopko *et al.*, 2017*a*, 2017*b*). All cercariae used for infection were not older than 5 h.

Experimental design

Fish were placed in two 165 L tanks (132 fish in each) at acclimation temperatures (15 °C) and exposed to low and high doses of cercariae (120 and 250 cercariae/fish, respectively) for 30 min without water flow (Seppälä *et al.*, 2004). These exposure doses were chosen to get a larger variation in the infection intensities and were similar to the ones used earlier (Karvonen *et al.*, 2004; Seppälä *et al.*, 2004; Gopko *et al.*, 2017*a*, 2017*b*). Infection intensities (4–94, mean = 36 metacercariae ind^{-1}) were similar to that of the natural ones (Shigin, 1986; Valtonen and Gibson, 1997; Valtonen *et al.*, 1997). The same mixture of cercariae obtained from six snails was used to infect all fish.

After exposure, the fish were placed in eight identical flow-through tanks (31–35 fish in each), and two temperature treatments were set up (with two replicates for each of the two infection doses). Four tanks were heated to 18°C, while other four were kept at 15°C. Heating started 1 h after the exposure and took 9 h to reach the target water temperature. Then, during the whole experiment (17 days), the average temperature (±S.D.) was 15.1 ± 0.54°C and 18.0 ± 0.54°C in non-heated and heated tanks, respectively. Fish were fed *ad libitum* with food flakes. Water temperature and oxygen concentration were measured three times a day.

Maintenance temperatures were similar to summer temperatures in Lake Konnevesi (Kuha *et al.*, 2016) and temperate lakes in general (mean ± S.E. = 16.8 ± 0.52°C), calculated using the 'laketemps' package (Sharma *et al.*, 2015; Gopko *et al.*, 2020). The temperature in our heated tanks was in the range of growth temperatures (18–19°C) of rainbow trout (Coutant, 1977; Eaton *et al.*, 1995), and the oxygen saturation was kept high (>86%).

To study metacercariae abundances and sizes, we dissected fish on the 12th and 17th day after the exposure (half of the fish were randomly chosen from each tank each time). Metacercariae of *D. pseudospathaceum* stop growing on reaching maturation (Parker *et al.*, 2015), which takes about a month at 18°C in salmonids (Sweeting, 1974; Shigin, 1986); therefore, we tried to catch the period of active growth for dissections. Fish were killed with an overdose of MS 222 (Sigma Chemical Co., St Louis, USA), weighed and measured (fork length). In each dissection, fish were killed simultaneously (120 fish), stored at 4°C, and examined in a balanced order from different tanks during a period of 2 days. The number and length of metacercariae in eye lenses were estimated under the microscope Olympus SZX12 (magnifications ×40 –×90). Metacercariae for measurements were chosen randomly. The first ten individuals from the central upper part of the microscopic field were measured clockwise in each eye. When metacercariae moved, their maximum lengths in the stretched state were measured.

Data analysis

Metacercariae size

Given that the body temperature of the fish host reflects water temperature, we calculated the thermal constant (Q_{10}) for metacercariae growth for a 5-days period between the 12th and 17th day post-infection, using the Van't Hoff formula (Schmidt-Nielsen, 1997):

$$Q_{10} = \left(\frac{k_1}{k_2}\right)^{10/(T_1 - T_2)}$$

where k_1 and k_2 are growth rates at temperatures 1 (18 °C) and 2 (15 °C), respectively.

We used 'lmerTest', 'ggplot2', 'lme4', and 'gridExtra' R packages in data analysis (Bates *et al.*, 2015; Wickham, 2016; Auguie, 2017; Kuznetsova *et al.*, 2017). To study the influence of temperature and metacercariae densities on the metacercariae growth, we used linear mixed models, where tank, fish, and eye IDs were nested random factors, while metacercariae age (12th *vs* 17th day post-infection), temperature treatment, fish weight, within-eye infrapopulation size and double interactions were included as fixed factors. We did not consider higher-order interactions because of their complicated interpretability and lack of *a* *priori* hypotheses (but see below the triple interaction Time × Temperature × Infection intensity). Then, the model was simplified using a ‘step’ tool (Kuznetsova *et al.*, 2017; R Core Team, 2019). The resulted model included all main effects we were interested in and the interaction between metacercariae age and temperature. The response variable was log-transformed.

We plotted within-eye infection intensity *vs* length of metacercariae separately for each treatment. The parasite numbers for each eye were considered separately and were not summarized because metacercariae sizes depends on metacercariae abundance in the respective eye (Gopko *et al.*, 2017*a*). The overlapping data points were showed as proportionally larger ones. Pearson’ correlation coefficients were also added to the plots for illustrative purposes. These correlations, in contrast to the stricter mixed-model, do not account for potential non-independence; however, intraclass correlation was quite low (0.10 at the highest level). Degrees of freedom in all mixed models were obtained using Satterthwaite’s approximation (Kuznetsova *et al.*, 2017).

We also aimed to test whether there was an interaction between temperature and infrapopulation size, i.e. a modifying influence of temperature on the relationship between the length of parasites and infrapopulation size. However, these modifying effects could change with time, and, indeed, when the triple interaction Time × Temperature × Infection intensity was added to the model, it was significant (Estimate = −0.0082, S.E. = 0.0028, df = 38.7, $t = -2.926$, $P = 0.0036$). Since triple interactions are hardly interpretable, we checked the interaction between temperature and infection separately for both time points. We fitted two models similar to that described above; all variables were the same excluding the time of the dissection, and all interactions between the fixed factors were included. Then, we simplified them as described above. The exclusion of random effects was suppressed. Both resulted models included temperature and infection intensity as predictors. The model for the 17th day also included the interaction between these variables.

Infection intensity

We started with a linear model that considered fish weight as a continuous predictor, and the temperature treatment and metacercariae age as factors of interest. Tank ID and infection dose (high/low) were also included in the initial model as factor variables along with double interactions. Then, we simplified the model. The most parsimonious model was the following: log (Infection intensity) ~ Weight + Temperature + Metacercariae age + Infection dose + Temperature × Weight + Infection dose × Temperature. Model residuals were checked for normality visually on QQ-plots and formally checked using Shapiro–Wilk’ test ($W = 0.99$, $P = 0.22$).

Variation in metacercariae size

Our initial linear mixed model included a log-transformed coefficient of variation (CV) in metacercariae size for each eye as a dependent variable, Fish ID nested within Tank ID (random factors), metacercariae age and temperature treatment (fixed factors), and a mean metacercariae length within the eye as a continuous variable. Based on the visual inspection of the data, we concluded that a three-way interaction between the predictor variables is likely and included all interactions in the initial model. However, neither fish nor tank ID explained the substantial amount of variance; therefore, we continued with a simple linear model without random factors. Triple interaction was significant as expected (Estimate ± S.E. = 0.16 ± 0.034, $t_{206} = 4.80$, $P < 0.0001$). We split our data into two parts as per the age of metacercariae (12th and 17th day post-infection) to get more interpretable models. These models included the same predictors as the initial model, and fish weight and infection intensity were added as covariates. We started with the models that included all double interactions and then simplified them. Finally, we created a separate model with Metacercariae age, temperature treatment, and their interaction as predictors, and tank ID as a random factor.

Fish body condition

We used GLM ANOVAs to check whether temperature treatment and Tank ID influenced the condition (Fulton’ condition factor) of fish. In both cases, we added the time of dissection (12- or 17-day post-infection) as an additional factor.

Results

Metacercariae growth

There was a positive relationship between number and size of *D. pseudospathaceum* metacercariae within the eye (Table 1, Fig. 1). The positive density-dependent growth was seen in all treatments excluding the 15 °C treatment on 12th day post-infection, where the relationship was slightly non-significant (Fig. 1).

The temperature had a substantial positive influence on the growth of metacercariae. Metacercariae at 18°C were larger than at 15 °C (Fig. 2, Table 1). The 3 °C higher temperature led to 2-fold increase in the size of metacercariae in 17 days (mean ± S.D. sizes at 15 °C and 18 °C: 221 ± 32 and 460 ± 58 μm, respectively). The difference in parasite sizes grew with time, i.e. the interaction between temperature and time was significant (Fig. 2). The largest metacercariae (maximal length 757 μm, mean ± S.D. 460 ± 58 μm) were observed in the heated tanks at the end of the experiment. Average growth rates of metacercariae between 12th and 17th day constituted 14 and 35 $\mu\text{m day}^{-1}$ at 15 °C and 18 °C, respectively. The thermal constant calculated from these growth rates was very high ($Q_{10} = 21$).

Models fitted separately for the 12- and 17-day time points also showed that parasites grew faster at higher temperature and abundances (Table 1). However, the interaction temperature × infrapopulation size was significant only at 17th day. Under higher temperature, the influence of infrapopulation size on metacercariae growth was still positive though less pronounced than under lower temperature. Effects of fish weight and its interactions with other predictors were not significant in all the tested models and were, therefore, excluded from the final models.

Infection intensity

As expected, infection dose influenced the infection intensity positively (Table 2). More interestingly, fish in the high-temperature treatment were more infected compared to the low-temperature treatment (Table 2, Fig. 3). Holding other variables constant, the mean infection intensity at 18°C was 16 additional eye flukes higher than at 15°C. There was a positive relationship between fish weight and infection intensity. With 1 g increase in fish weight, the mean infection intensity increased roughly by one metacercaria (Fig. 3). There was no difference in the parasitic load in fish dissected on 12th and 17th day post-infection (Table 2).

Variation in metacercariae size

After the simplification, the model for 12th day post-infection included the temperature treatment, metacercariae size (centred) within the fish eye, their interaction and tank ID as predictors, while the model for the 17th day post-infection included only metacercariae size as a predictor. On the 12th day post-infection,

Table 1. Influence of predictors (metacercariae age, within-eye abundance, temperature) on the size of *D. pseudospathaceum* metacercariae estimated from the GLMMs

	Estimate	S.E.	Df	*t*-value	*P* value
(A) *Model for data from both 12 and 17 d time points*					
Metacercariae age (17 days)	0.3679	0.0182	233.78	20.22	<0.00001
Temperature (Heating)	0.5916	0.0277	9.27	21.38	<0.00001
N of metacercariae/eye	0.0047	0.0007	261.77	6.38	<0.00001
Metacercariae age × Temperature	0.1463	0.0261	228.28	5.61	<0.00001
(B) *Models fitted separately for 12 and 17 d time points*					
12 days					
Temperature (heating)	0.5916	0.0354	5.65	16.74	0.00001
N of metacercariae/eye	0.0048	0.001	191.6	5.054	<0.00001
17 days					
Temperature (heating)	0.8232	0.0426	195.89	19.30	<0.00001
N of metacercariae/eye	0.0078	0.0018	184.32	4.42	0.00002
N of metacercariae × temperature	−0.0050	0.0022	199.29	−2.28	0.0235

(A) Results of a model, where parasites measured on the 12th and 17th day post-exposure were taken together. (B) Results of the models fitted separately for the 12th and 17th day post-exposure (are presented since there was a marginally significant triple interaction between metacercariae age, infrapopulation size and temperature)

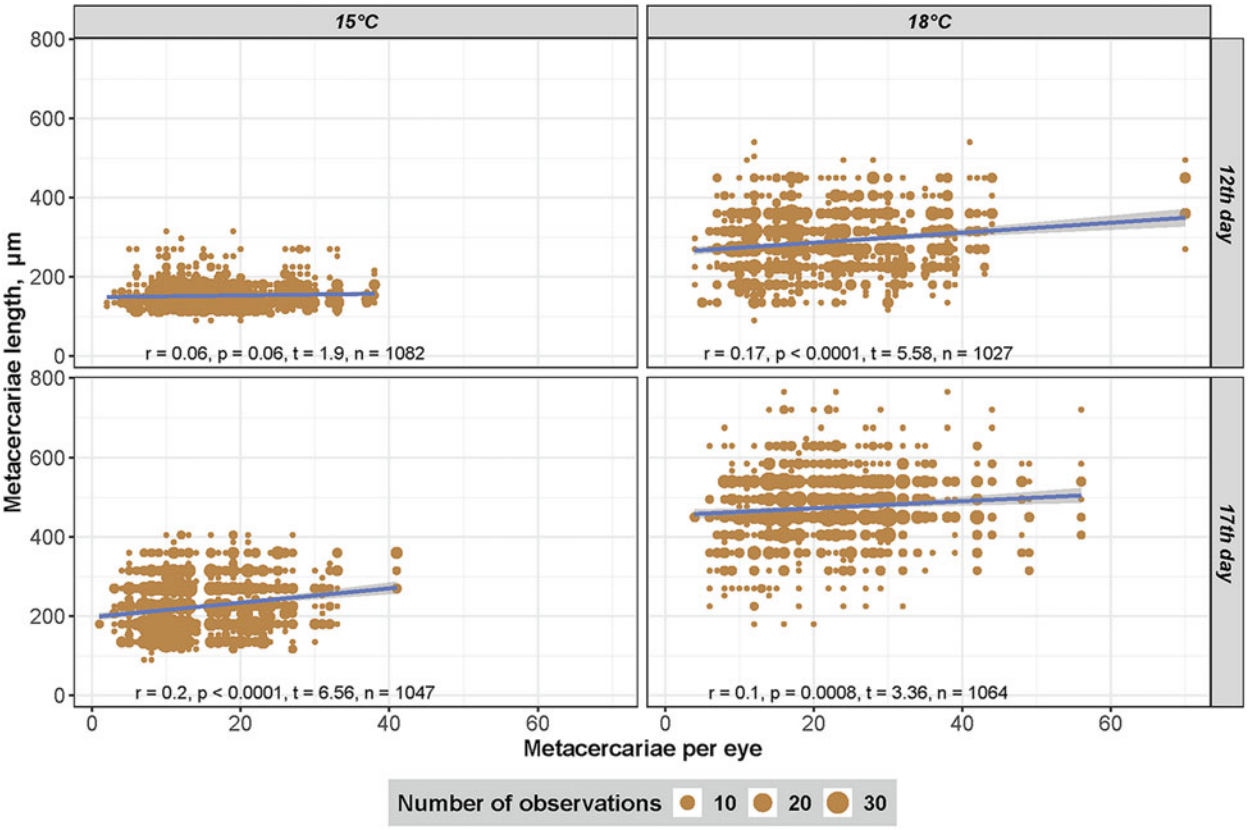

Fig. 1. Within-eye infection intensity *vs* metacercariae size taken separately for each metacercariae age × temperature treatment. The shaded area represents the 95% confidence intervals.

the main effect of metacercariae size was positive (Estimate ± S.E. = 0.14 ± 0.03, $t_{107.73} = 4.53$, $P < 0.0001$), reflecting the increase in CV with the mean metacercariae length under lower temperature. However, a significant interaction between the temperature and metacercariae size indicated that this effect disappeared at 18 °C; the relationship was even slightly negative (Estimate ± S.E. = −0.16 ± 0.03, $t_{103.00} = -5.05$, $P < 0.0001$; Fig. 4a). On the 17th day, there was a negative correlation between metacercariae size and size variability at both temperatures (Estimate ± S.E. = −0.15 ± 0.02, $t = -9.84$, $P < 0.0001$; Fig. 4b).

In the model, where Metacercariae age, Temperature treatment were the predictors, there was a significant positive influence of both main effects on the CV of metacercariae size (Estimate ± S.E. = 0.37 ± 0.047, $t_{209.84} = 7.96$, $P < 0.0001$ and Estimate (17 days) ± S.E. = 0.34 ± 0.050, $t_{19.74} = 6.72$, $P < 0.0001$). However, the interaction between variables was also significant (Estimate ±

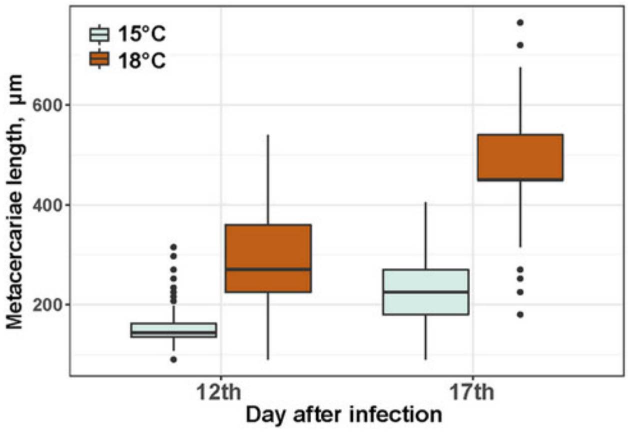

Fig. 2. Difference in metacercariae sizes at different temperatures. 'Boxes' represent a median with the interquartile range (IQR), whiskers extend from the highest to lower values within 1.5*IQR.

Table 2. Effect of metacercariae age, fish weight, temperature, infection dose on the infection intensity of *D. pseudospathaceum* in rainbow trout estimated from the GLMM

	Estimate	S.E.	*t*-value	*P* value
Metacercariae age (17 days)	−2.858	1.665	−1.72	0.088
Temperature (heating)	15.829	2.296	6.89	<0.00001
Fish weight	0.928	0.265	3.51	0.0006
Infection dose (low)	−19.419	2.276	−8.53	<0.00001
Infection dose × temperature	−6.465	3.288	−1.97	0.051

Data for 219 fish were used

S.E. = −0.77 ± 0.067, $t_{210.07} = -11.38$, $P < 0.0001$). Variation in sizes was higher in the heated treatments on the 12th day than in the non-heated treatments, but the relationship was inverse on the 17th day (Fig. 4c).

Effects of temperature and parasite density on fish condition

There was no influence of temperature, time, or interaction between these factors on fish condition ($F_{1,\ 215} < 3.41$, $P > 0.07$ in all cases). Fish also did not differ in Fulton' condition factor between the tanks on 12th and 17th day post-infection ($F_{1,\ 215} < 3.46$, $P > 0.06$ for all main effects and the interaction). Fish body condition was found to be slightly positively correlated with metacercariae numbers ($r_s = 0.171$, $P = 0.008$) and did not correlate with metacercariae sizes ($r_s = 0.058$, $P = 0.374$), so there was no indication of negative effect of the infection on the host.

In most of the tanks, fish mortality was low (share of dead individuals <6%), but in three heated tanks from different infection treatments mortality, was rather high (15–22% by the end of the experiment). Meanwhile, in the last heated tank, only 3% of fish died, calling into question that mortality simply increased with temperature. In addition, the temperature did not influence fish condition and did not exceed maximum growth temperatures for rainbow trout (Coutant, 1977; Eaton *et al.*, 1995). The majority of fish died in the first week, and we did not find any obvious signs of concomitant infections. Dead individuals did not differ by body condition from fish that remained alive in the respective tanks and were dissected on the 12th day post-exposure ($F_{1,\ 55} = 1.3$, $P = 0.28$). The log-transformed infection intensities were also similar (interaction infection dose × fish status (dead/alive), $F_{1,\ 70} = 2.0$, $P = 0.16$, ns). Therefore, it is unlikely that selective mortality influenced our results.

Discussion

Our study provides the first quantitative data about the joint effect of temperature and parasite density on the growth of parasite larvae. Both heating and larger infrapopulation size stimulated the growth of *D. pseudospathaceum* metacercariae in the rainbow trout. There is no surprise in the increase of growth rates under higher temperature, but the extent of this increase ($Q_{10} = 21$) is unusual. The observed positive density-dependence of parasite growth (Allee effect) supports the results of earlier studies of this system (Gopko *et al.*, 2017*a*), contrary to the common expectation of resource competition in parasites (Read, 1951; Fong *et al.*, 2017), including trematode metacercariae (Sandland and Goater, 2000; Brown *et al.*, 2003; Fredensborg and Poulin, 2005; Saldanha *et al.*, 2009; Stumbo and Poulin, 2016).

Surprisingly, there was a negative interaction between temperature and infrapopulation density of parasites – at higher temperature, the positive effect of infrapopulation size on parasite growth was less pronounced. However, this interaction was significant only at the later time point (17th day post-infection) when metacercariae were the largest and variation in their sizes decreased. This can be probably explained by growth arrest at larval maturity to avoid overexploitation and/or mobilization of host responses (Parker *et al.*, 2015) – under high temperatures and infrapopulation densities, parasites slowed down growth earlier because they almost reached their maximal size. It is likely that warming did not change the Allee effect but affected its timing, making the effect evident earlier. Thus, at 15°C, the Allee effect was observed not until 17th day, while at 18°C, it became obvious at 12th day.

Stimulating effects of heating on development of parasite larvae were found in a variety of helminths within poikilothermic hosts (review by Chubb, 1979, 1980; Tokeson and Holmes, 1982; Lv *et al.*, 2006; Studer *et al.*, 2010; Voutilainen *et al.*, 2010; Macnab and Barber, 2012; Franke *et al.*, 2017) and can be explained by increased metabolic rates. These effects are important for parasite transmission because warming shortens the period when parasite larvae are non-infective (Barber *et al.*, 2016). During this period, if the fish host of *D. pseudospathaceum* metacercariae is consumed by any predator, the parasites will perish. Immature metacercariae can overwinter in the fish and continue growing in the next warm season (Valtonen and Gibson, 1997; Hakalahti *et al.*, 2006). Therefore, the infection of the definitive host can be postponed, especially in boreal aquatic environments (Hakalahti *et al.*, 2006). Metacercariae of *D. pseudospathaceum* need about 700–1000 degree-days to reach infectivity in salmonids (Sweeting, 1974; Shigin, 1986), but these assessments are based on larvae morphology and were not tested experimentally. In our experiment, metacercariae grew up to the sizes (average 460 ± 58 μm) similar to the maximum values (ranging from 390 to >500 μm) reported for *D. pseudospathaceum* (Niewiadomska, 1986; Shigin, 1986; Voutilainen *et al.*, 2010) in 306 degree-days. However, it does not necessarily guarantee their maturity. To avoid confusion, it is important to note that, in the cited studies, other species names were used instead of *D. pseudospathaceum* (*D. spathaceum, D. chromatophorum, D.* spp. in Sweeting (1974), Shigin (1986), Voutilainen *et al.* (2010), respectively). However, the fact that parasites were obtained from *L. stagnalis* snails, which are typically infected by *D. pseudospathaceum* (Rellstab *et al.*, 2011), indicates that these studies most likely dealt with *D. pseudospathaceum*.

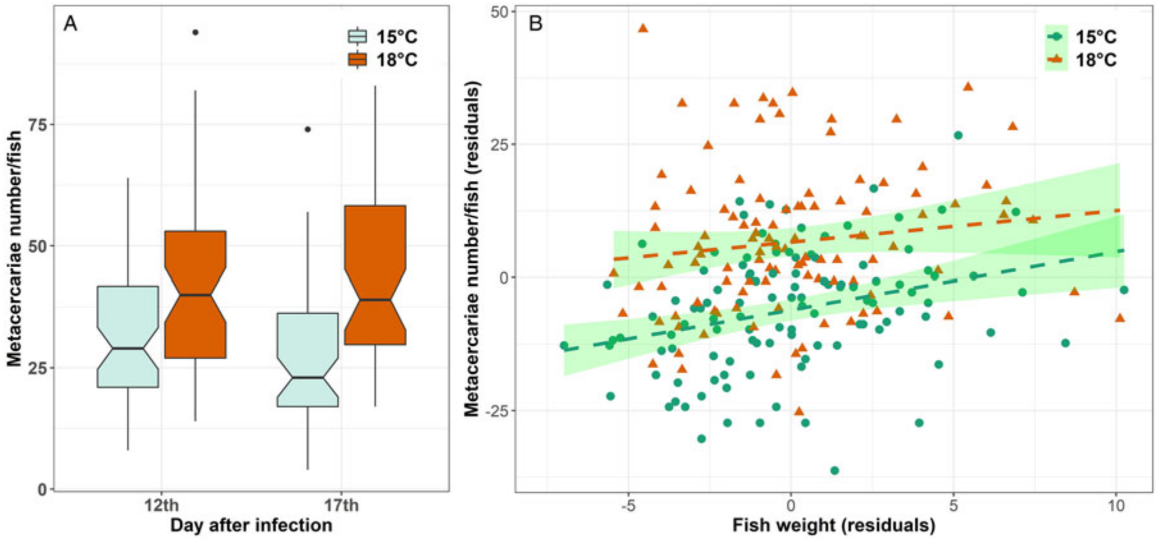

Fig. 3. Difference in infection intensities at different temperatures (A). The relationship between fish weight and infection intensity taking into account different infection doses is presented in the residual plot (B). 'Boxes' represent a median with the IQR, whiskers extend from the highest to lower values within 1.5*IQR, the shaded area represents the 95% confidence intervals.

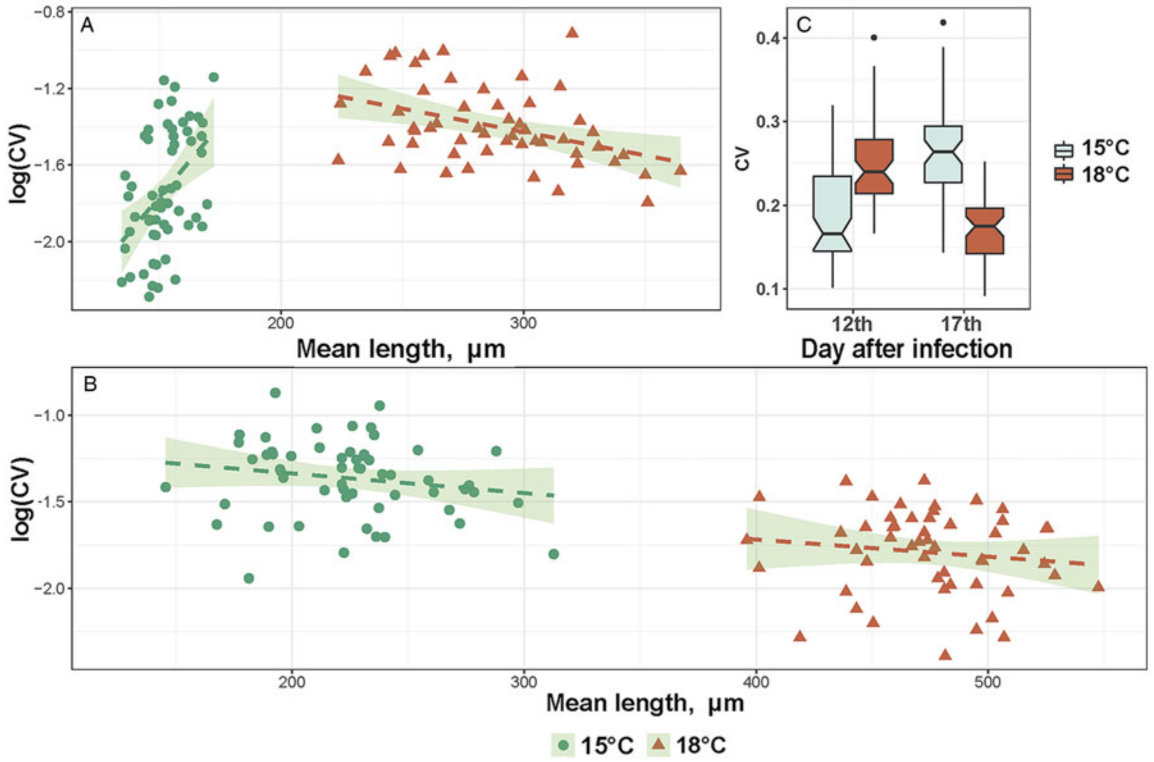

Fig. 4. Mean metacercariae length *vs* log(CV) of metacercariae length in fish dissected on the 12th (A) and 17th (B) day after infection. (C) Variability of metacercariae length in fish from different temperature treatments. The shaded area represents the 95% confidence intervals.

We showed that even a small (3°C) increase in water temperature led to 2.5-fold rise in growth rates of *D. pseudospathaceum* and eventually (17 days post-infection) resulted in the twice larger size of metacercariae. The thermal constant calculated for growth rates of metacercariae was much higher ($Q_{10} = 21$) than expected following the Q_{10}-rule, predicting a 2 to 3-fold rise in metabolic rates of poikilothermic animals for every 10°C (Schmidt-Nielsen, 1997). This could mean that small and actively growing immature metacercariae are much more sensitive to temperature shifts than their hosts and many other poikilothermic organisms. However, it may not be necessarily so, since the thermal constant was initially formulated for relatively simple chemical processes and the values obtained in biological systems often differ (Vernberg, 1968). Information about thermal response in helminths is controversial, varying from being extraordinarily high (cercariae: Poulin, 2006) to slightly elevated (cestode plerocercoids: Sinha and Hopkins, 1967) and limited response (cercariae and miracidia: Morley, 2012; Morley and Lewis, 2013; Marcogliese, 2016; adult trematodes: Vernberg, 1968). This can be explained not only by differences between parasite species, strains, and life stages but also by acclimation conditions and temperature history of the host (Paull *et al.*, 2015).

Our results showed a positive relationship between temperature and infection intensity, similarly to the results of previous studies of *D. pseudospathaceum* (Lyholt and Buchmann, 1996; Gopko *et al*., 2020). This cannot be a result of temperature-dependent exposure rate since the temperature was increased only after the exposure to cercariae, but it indicates that the number of parasite larvae that reached eye lenses after penetration into the host was higher under higher temperature. It could be explained by the immediate suppression of fish immune response under warming (Dittmar *et al*., 2014), which increases chances of parasites to reach the eye lens safely. In the eye lens, parasites are inaccessible for the host immunity (Höglund and Thuvander, 1990; Wegner *et al*., 2007) and, therefore, the temperature–host immunity interaction unlikely influences their development. Since temperature in the heated treatments was set up before (~9 h post-infection) the parasites possibly reached the eye lens (~15 h at 15°C) (Lyholt and Buchmann, 1996), parasites could be less influenced by the immune system on their way to the final localization in the heat-stressed hosts.

Variation in sizes of *D. pseudospathaceum* larvae increased under warmer conditions at early growth stages (on 12th day post-infection), but this pattern disappeared later, on 17th day, when metacercariae approached their maximum size. Expression of the possible initial inter-clonal differences in the growth rates could be promoted by heating and reduced later, as the growth of the most developmentally advanced larvae was arrested (Parker *et al*., 2015). Therefore, temperature can potentially influence the growth variation and the size structure of metacercariae infrapopulations, but it is unclear whether this influence is direct or mediated by metacercariae development.

In general, an increase in environmental temperature may benefit the completion of the life cycle in *D. pseudospathaceum*, similarly to other parasite species (Barber *et al*., 2016). In addition to faster development of parasite larvae, warming may promote the release of infective stages, enhance their infectivity for a short period (Poulin, 2006) and alter biology of the hosts, making them more accessible to parasites (Hakalahti *et al*., 2006; Barber *et al*., 2016).

The positive correlation between the average size of *D. pseudospathaceum* metacercariae and their abundance in fish eye (Allee effect) found in our study contradicts the large majority of the studies that showed the ‘crowding effect’ (Read, 1951) in parasite infrapopulations (Holmes, 1961; Jones and Tan, 1971; Yao *et al*., 1991; Poulin *et al*., 2003; Fong *et al*., 2017), including studies on trematode metacercariae (Sandland and Goater, 2000; Brown *et al*., 2003; Fredensborg and Poulin, 2005; Saldanha *et al*., 2009) and in particular, diplostomids (Stumbo and Poulin, 2016). Situations when the presence of conspecifics does not influence parasite fitness or even benefits them may happen when parasite–host size ratio is small, infection intensity is low, in manipulative parasites with similar transmission goals or when some form of public goods is produced (Poulin, 1994; Brown, 1999; Kuris, 2003; Bashey, 2015). However, experimental evidence of a such positive density-dependence relationship in macroparasites is, to our knowledge, limited to a previous study on the same host–parasite system (Gopko *et al*., 2017*a*). A field study of trematode *Euhaplorchis californiensis* infecting the California killifish also reported a possible mild positive density-dependent growth in metacercariae (Weinersmith *et al*., 2014).

The Allee effect found in these trematode species can be explained by similar reasons. First, their metacercariae are small relative to the fish host, suggesting no resource limitation (Weinersmith *et al*., 2014; Gopko *et al*., 2017*a*), which typically restricts the growth of larger parasites (nutrients, physical space, attachment sites) (Holmes, 1961, 1962; Heins *et al*., 2002; Parker *et al*., 2015). When abundant, metacercariae of *D. pseudospathaceum* may feed more effectively because of more perforations in the eyeball covers created during the penetration, which leads to a higher amount of tissue fluid entering the eye. It is unlikely that metacercariae in our study were involved in the strong exploitative competition since positive density-dependence remained even when metacercariae reached sizes close to their maximum. In addition, parasite densities and sizes did not correlate with fish body condition; therefore, there is a lack of obvious signs of host depletion by the parasite.

The second possible explanation of the Allee effect in these trematodes is their ability to manipulate host behaviour and share the manipulation costs among metacercariae (Weinersmith *et al*., 2014; Gopko *et al*., 2017*a*). Immature *D. pseudospathaceum* larvae manipulate fish behaviour to suppress the predation risk for the host fish, while mature larvae enhance it (Mikheev *et al*., 2010; Gopko *et al*., 2015, 2017*b*) similarly to mature metacercariae of *E. californiensis* (Lafferty and Morris, 1996).

The suppression of defensive behaviour in fish infected by mature *D. pseudospathaceum* larvae was supposed to be caused by vision deterioration due to cataract formation (Karvonen *et al*., 2004; Seppälä *et al*., 2005, 2012). However, it could not explain manipulations of small immature metacercariae (Gopko *et al*., 2015) before the start of active cataract formation, which typically begins after the full maturity of the parasite (Karvonen *et al*., 2004; Seppälä *et al*., 2005) and parasite manipulations at low infection intensities. Altering host vision by diel migrations of metacercariae, similarly to diplostomid *Tylodelphys* sp., actively moving to certain sites within vitreous humour of fish eye (Stumbo and Poulin, 2016) is unlikely to be possible for *D. pseudospathaceum* metacercariae inhabiting much denser lens matrix. Therefore, we suggest that another mechanism of manipulation, e.g., the release of chemicals by parasites or by-products of infection also could be important in *D. pseudospathaceum*, similarly to many other parasites (Adamo, 2012). The fact that the extent of behavioural changes caused by immature *D. pseudospathaceum* metacercariae does not increase with infection intensities also supports this assumption (Gopko *et al*., 2015). The manipulations may be costly (if special chemicals are released) and the total cost of manipulation is likely to be constant in *D. pseudospathaceum*; therefore, parasites may benefit from cost sharing when abundant (Gopko *et al*., 2015). We suggested that immature eye fluke metacercariae can cooperate with conspecifics and, when abundant, invest less energy in host manipulation but more in growth (Gopko *et al*., 2017*a*). Sharing costs of defence against host immune system is unlikely to be important for *Diplostomum* larvae because they are unaffected by the host’ immunity in the fish eye (Höglund and Thuvander, 1990; Wegner *et al*., 2007). For this reason, the found Allee effect cannot be explained by individual differences in host susceptibility to parasites, which are supposed to influence parasite growth in other hosts (Weinersmith *et al*., 2014).

To our knowledge, the present study together with the previous one on this object (Gopko *et al*., 2017*a*) provides the first experimental evidence of positive density-dependent growth in macroparasites. The results on *E. californiensis* (Weinersmith *et al*., 2014) were based on field data therefore, the influence of previous life history on hosts and parasites cannot be disregarded in that study. Since the infection intensities in our experiment were similar to the natural intensities (Shigin, 1986; Valtonen and Gibson, 1997; Valtonen *et al*., 1997), we suggest that positive density-dependent growth in this trematode species could also take place in natural conditions. We suppose that *D. pseudospathaceum* provides a useful study system for investigation of ‘pure’ costs of parasite manipulation because the costs of defence against the host immunity can be ignored here. Evidence of manipulation costs remains elusive, although indirect indications are presented in a few studies (reviewed in Gopko *et al*., 2017*a*, Hafer-Hahmann, 2019).

In conclusion, the obtained results demonstrate that the moderate temperature increase influences important traits of individual parasites and their infrapopulations. Under higher temperature, *D. pseudospathaceum* infects fish more successfully and grows faster, which most likely increases parasite transmission success in freshwater ecosystems. Higher densities of parasites within the eye stimulate parasite development (Allee effect), in contrast to almost all other host–parasite systems. Importantly, our study provided the first data on the joint effect of temperature and infrapopulation densities on parasite growth. Warming did not substantially change the magnitude of Allee effect but affected its timing. The impact of these effects on natural freshwater ecosystems is likely to become more pronounced under current climate changes. These effects might take place also in fish farming, where relatively high parasite abundances often occur.

Data. The data that support the findings of this study are openly available in 'figshare' at https://doi.org/10.6084/m9.figshare.14184770.v1.

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