Experimental infection of Arctic charr *Salvelinus alpinus* (L.) with the cercariae of diplostomid eye flukes *Diplostomum* spp.

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Abstract

The aim of the present study was to quantify the relative importance of dose and exposure time in the experimental infection of Arctic charr with the cercariae of *Diplostomum* spp. To reach the aim, 15-month-old Arctic charr (n = 19) were exposed singly to 100-3300 parasite cercariae (range) for 110-1225 seconds (range) at 17 °C. The number of parasites located themselves in the fish eye lenses as a result of the experimental infection correlated positively both with the number of parasite cercariae in the exposure (dose) and the exposure duration (time). A linear model having the number of parasites located in the fish as dependent variable and dose and time as independent variables explained 85% of the original variation in the dependent variable. Adding fish size as an independent variable did not improve the model's explanatory power. To conclude, the outcome of the experimental infection of Arctic charr with *Diplostomum* spp. is predictable, when experimental conditions are standardized and the parasite challenge is well-controlled. The present results can serve as a reference for any experimental infection of Arctic charr with *Diplostomum* spp.

Introduction

Studying infectivity of parasites requires experimental infections of hosts. The outcome of an experimental infection depends on several factors, which can be categorized in the two groups: the factors which are related to the specimens and the factors which are related to the experimental conditions. The first group refers mainly to age, size, and "physiological quality" of the host and the parasite (Karvonen et al., 2003; Seppälä et al., 2007; Voutilainen et al., 2010a) together with geographical origins (allopatric or sympatric) of the host and the parasite (Voutilainen et al., 2009a), as well as to possible previous contacts between the host individuals in question with the parasite species in question (Karvonen et al., 2005). The second group consists of temperature, the number of parasites per host in the exposure (dose), and duration of the exposure (time) as the main factors (Stables and Chappell, 1986; Lyholt and Buchmann, 1996; Voutilainen and Taskinen, 2009). The last two (dose and time) can be combined to the strength of parasite challenge.

Diplostomatid parasites include several ubiquitous species which use lymnaeid snails

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as first and fresh- and brackish water fishes as second intermediate hosts. Final hosts of diplostomatids are fish-eating birds, such as gulls. Metacercariae of *Diplostomum spathaceum* and *Diplostomum pseudospathaceum* establish themselves in fish eye lenses and cause cataract by their movements and metabolic excretions (see Karvonen, 2011 for a review). *Diplostomum spathaceum* and *D. pseudospathaceum* have been widely used in many experiments carried out to study infectivity of the parasites in salmonid fish (Betterton, 1974; Stables and Chappell, 1986; Karvonen et al., 2003, 2005; Voutilainen and Taskinen, 2009; Voutilainen et al., 2010a) and to find out effects of parasitic infection on fish behavior (Seppälä et al., 2005), oxygen consumption (Voutilainen et al., 2008), growth (Voutilainen et al., 2010b), and mortality (Larsen et al., 2005). The aim of the present study was to quantify the relative importance of dose and exposure time in the experimental infection of a salmonid fish, Arctic charr *Salvelinus alpinus* (L.), with the cercariae of the diplostomid eye flukes, *Diplostomum* spp.

Materials and methods

Parasite

Diplostomum spp. cercariae were obtained from the infected snails *Lymnaea stagnalis* (Gastropoda: Lymnaeidae), which are known to act as the first intermediate hosts for *Diplostomum* spp., and specifically for *D. pseudospathaceum* (Niewiadomska et al., 1997). *Lymnaea stagnalis* (n = 75) were sampled by two wading persons from the littoral zone of Lake Ylä-Enonvesi located in eastern Finland. The specific sampling site (Pirttilahti) has been described in more detail by Voutilainen et al. (2009b). The hatchery (Saimaa Fisheries Research and Aquaculture) which provided the fish, receives water for its rearing tanks from the same lake. After the sampling, the snails were placed in a large bucket filled with water from their home lake and transported to the laboratory during the same day.

At the laboratory, the snails were placed singly in 500 ml transparent plastic beakers filled with de-chlorinated tap water for overnight and the shedding of *Diplostomum* spp. cercariae from infected snails was stimulated by raising the water temperature from 15 to 20 °C within 12 h (see Lyholt and Buchmann, 1996). Next morning, a 10 ml water sample was pipetted from each beaker for stereomicroscopic (MZ95, Leica Microsystems, Heerbrugg, Switzerland) inspection, which revealed that cercariae of *Diplostomum* spp. had emerged into the water from 10 out of 75 snails. The 10 infected snails were removed from the beakers and placed in a 10 l bucket filled with tap water at 20 °C (see Seppälä et al., 2007) in which shedding of cercariae continued. After 6 h, the snails were removed from the bucket and the density of *Diplostomum* spp. cercariae in the bucket was estimated. Firstly, cercarial suspension in the bucket was gently stirred with a scoop. Secondly, 10 × 10 ml water samples were pipetted from the bucket and the number of *Diplostomum* spp. cercariae in these samples was counted using a stereomicroscope. The density of parasites in the bucket was estimated to be 20 ± 7 cercariae ml^{-1} (mean ± S.D.).

The parasites were identified to genus according to morphology and size of cercariae (Niewiadomska, 1986; Larsen et al., 2005). In addition, the species of the snail host was taken into account in the parasite identification. Traditionally, diplostomatid fish eye flukes infecting the

great pond snails *L. stagnalis* have been classified as *D. pseudospathaceum* and those infecting *Radix balthica* as *D. spathaceum* (Niewiadomska, 1986). Recent studies on the genome of the genus *Diplostomum*, however, have revealed that the parasites establishing themselves in fish eye lenses include more species than previously have been thought and that some of the species seem to have very high host specificity (Rellstab et al., 2011). The parasite classification in the present study was due to practical purposes and the main aim was to gather *Diplostomum* spp. cercariae having as similar morphology and physical quality as possible, not to identify the parasites at the species level.

Fish

Arctic charr representing the landlocked Lake Saimaa population were supplied by the Saimaa Fisheries Research and Aquaculture (Finnish Game and Fisheries Research Institute) located at Enonkoski, southeastern Finland. There the fish were maintained in filtered water (filter size 25 μm) to prevent the supply of parasites in maintaining tanks from the lake water, which would have caused uncontrolled contacts between the fish and parasites. Altogether 21 15-month-old Arctic charr [fresh mass (FM) 52.91 ± 21.84 g, total length (TL) 18.8 ± 2.7 cm, mean ± S.D.] from one family originating from a second-generation hatchery population were used in the experimental infection carried out at a laboratory of University of Eastern Finland, Joensuu in August 2007.

Experimental infection

To infect Arctic charr with *Diplostomum* spp., the fish were placed singly in 2 l transparent glass beakers filled with de-chlorinated tap water at 17 °C. The temperature was chosen on the basis of previous studies carried out on salmonid fish (Stables and Chappell, 1986; Lyholt and Buchmann, 1996). The decided number of parasite cercariae was then pipetted from the "parasite bucket" into each beaker in a random order. The number of cercariae was estimated from previously measured parasite density in the bucket. The fish was removed from the infection beaker when the planned challenge time had passed. The dose-time combinations used in the experiment were: 100 cercariae for 875 sec and 925 sec, 200 cercariae for 575 and 625 sec, 400 cercariae for 1175 and 1225 sec, 900 cercariae for 275 and 325 sec, 960 cercariae for 120 sec twice, 1200 cercariae for 625 sec, 1300 cercariae for 110, 120, and 130 sec, 1400 cercariae for 1225 sec, 1600 cercariae for 875 and 925 sec, and 3300 cercariae for 275 and 325 sec. Two fish died soon after the experimental infection. They were exposed to 1200 cercariae for 575 sec and to 1400 cercariae for 1175 sec. Thus the final number of Arctic charr included in the analysis was 19.

The strength of parasite challenge used in the present study was decided mainly on the basis of the experimental infection carried out by Betterton (1974). Betterton (1974) exposed 10 one-summer-old rainbow trout (TL 44 ± 8 mm, mean ± S.D.) to 400 *Diplostomum* spp. cercariae shed from the infected snails *L. stagnalis* and *R. balthica* for 4 min (*R. balthica* was formerly known as *Lymnaea peregra* and *Radix peregra*). The fish were not naïve, but had had eye fluke infection previous to the experiment at the fish farm. In the case of Betterton (1974), fish size (TL) did not correlate with the number of *Diplostomum*-parasites located in the fish eye lenses as a result of the experiment (Pearson's $r = 0.388$, $P = 0.268$, the coefficient was calculated

from the original data for the present study), but it strongly correlated with the number of parasites established themselves in the fish as a consequence of uncontrolled contact between the fish and parasites at the fish farm ($r = 0.806$, $P = 0.005$, calculated for the present study). Stronger parasite challenges than that used by Betterton (1974) were emphasized in the present experiment, as the fish infected by Betterton (1974) were younger and smaller.

Fish handling

After the experiment, the fish were maintained singly at 17-20 °C in 2 l flow-through tap water aquaria for 24 h. The migration rate of the parasite inside fish decreases after 20 h at 15 °C, which corresponds to the maximum life-span of *D. spathaceum* cercariae at 20 °C (Karvonen et al., 2003). The larvae that have not settled in the fish eye lens during that time are phagocytised (Ratanarat-Brockelman, 1974). Then the fish were anaesthetized with sodium bicarbonate ($NaHCO_3$) buffered tricaine methane sulfonate (Sigma Chemical, St. Louis, Missouri) and their FM and TL were measured. Finally, the fish were decapitated and their eyes were dissected and compressed between two microscope slides. The number of *Diplostomum* spp. located in the fish eye lenses was counted using a stereomicroscope. The experimental infection was carried out with permission from the committee of the University of Joensuu (renamed as University of Eastern Finland in 2010).

Statistical analyses

A linear regression model was fitted to the infection-exposure relationship. The number of parasites located in the fish eye lenses within 24 h after the exposure was used as a dependent variable in the model. The number of parasite to which the fish were exposed (dose) and the duration of parasite challenge (time) were used as independent variables. The analysis was performed with the IBM SPSS 19 for Windows.

Results and discussion

The best fit model in the infection-exposure relationship was:

$$Y = 0.035 \times D + 0.071 \times T - 1.300 \times TL - 9.374$$

Y: the number of parasites located in the fish eye lenses
D (dose): the number of parasites to which the fish were exposed
T (time): the duration of parasite challenge in seconds
TL: the total length of the fish in centimeters

The model explained 85% ($r^2 = 0.853$) of the original variation. Statistically the model was highly significant (ANOVA: $F_{2, 16} = 46.341$, $P < 0.001$). Dose had a slightly greater effect on the number of parasites located in the fish than time (standardized coefficient 0.844 and 0.761, respectively) (Figure 1). Standardized residuals were all $<|2|$ indicating no outliers (Figure 1B). Moreover, the standardized residuals were normally distributed (Kolmogorov-Smirnov test, $P = 0.679$) and they did not correlate to a statistically significant extent with the number of parasites located in the fish (Pearson's $r = 0.384$, $P = 0.105$). Consequently, the outcome of the experimental infection of Arctic charr with the cercariae of *Diplostomum* spp. appeared to be predictable, when experimental conditions were standardized and the parasite challenge was well-controlled. Adding fish length (TL) or mass (FM) as an explanatory variable in the model did not increase the model's explanatory

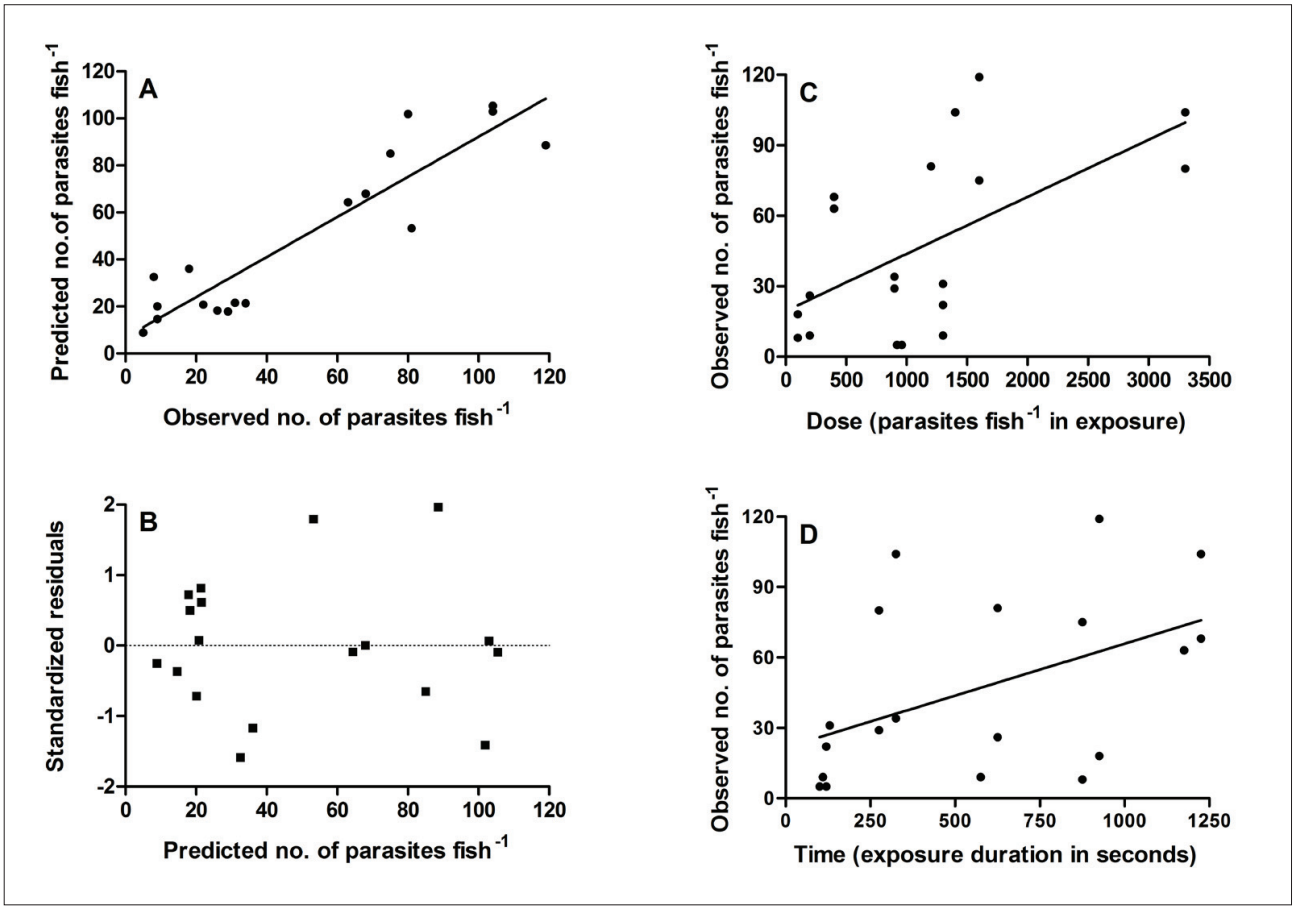

Figure 1. Fitting a linear model to the infection-exposure -relationship: A) the predicted number of parasites fish^{-1} plotted against the observed number of parasites fish^{-1} and B) standardized residuals plotted against the predicted number of parasites. The number of parasites located fish^{-1} correlated positively both with C) exposure dose and D) time. The line denotes linear relationship.

power in the present case, but TL was included to improve the use of the model in general. At the end of the experiment, there was an average of 47 (range 5-119) parasites fish^{-1} as a result of the experimental parasite challenge. Within the present range of parasite challenge from 100 to 3300 parasites fish^{-1} and exposure time from 110 to 1225 seconds, there was no evidence of "saturation". Rather, the number of parasites in the fish eye lenses increased linearly with the strength of parasite challenge (Figure 1). This was a slightly unexpected finding, as in three cases the total number of parasites per fish exceeded 100, which means that there is not enough space in the eye lens for the parasites to grow further (cf., Voutilainen et al., 2010b).

Experimental infections of hosts have to be well-controlled to ensure reliable results and valid conclusions drawn from the results. This requirement emphasizes testing the experimental procedure prior to carrying out more applied studies. The present results concerning the relationship between Arctic charr and *Diplostomum* spp. have a general meaning to researchers who are searching for the right strength of parasite challenge for their experimental infections of Arctic charr with *Diplostomum* spp. cercariae.

It is obvious and acknowledged that the appropriate strength depends on the fish and parasite species in question together with the prevailing conditions, but the present results can be considered as a starting point. This is in accordance with the ethical principles for animal experiments (e.g., ASAB, 2006), as the researchers can lower (minimize) the number of animals used in their experiments, if they are able to find adequate background information from the literature.

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