Journal of Invertebrate Pathology 166 (2019) 107220

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Influence of *Himasthla elongata* (Trematoda: Echinostomatidae) metacercariae on heart rate in blue mussels (*Mytilus edulis*)

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ARTICLE INFO

Keywords:
Himasthla elongata
Mytilus edulis
Heart rate
Monitoring
In situ

ABSTRACT

Whether metacercariae of the digenean *Himasthla elongata* (Himasthlidae) are harmful to their secondary intermediate hosts, the blue mussel, *Mytilus edulis*, is a disputable question. To shed light on this issue, we performed field monitoring of the heart rate (HR) in mussels infected with *H. elongata* over a period of 1.5 years. We observed a significant rise in HR in infected mussels, but only 1 year after infection and at temperatures of 15–17 °C. HR variance also grew sharply in infected mussels within the same temperature range. We also detected a decreased ability of infected mussels to compensate for the rise in the environment temperature. Finally, growth rate in the infected mussels was slower compared to the control group. We provide evidence for measurable pathogenic effects caused by *H. elongata* metacercariae in their secondary intermediate mussel hosts.

1. Introduction

Digenean metacercariae are among the most common parasites of bivalves (Lauckner, 1983) and, in many cases, they are harmful to their hosts. For example, Gymnophallidae metacercariae are motile, grow in the host, and have significant pathogenic effects (Bartoli, 1974; Lauckner, 1983; Pekkarinen, 1986; Ituarte et al., 2001; Cremonte and Ituarte, 2003). At high infection rates these pathogens can cause increased mortality in molluscs (Goater, 1993; Bowers et al., 1996; Addino et al., 2010). The metacercariae of Echinostomatidae, Renicolidae, Zoogonidae and Lepocreadiidae are quite different; they become encysted and are immobile, and are often considered to have little impact on the well-being of their molluscan hosts (Bower et al., 1994; Laruelle et al., 2002). However, several studies have discovered a pathogenic influence of Himasthlidae (formerly a subfamily within Echinostomatidae) metacercariae on bivalves. A few parameters were found to decline following the infection of a mollusc with these metacercariae: rate of byssal thread production (Lauckner, 1983), growth rate (Wegeberg and Jensen, 2003; de Montaudouin et al., 2012), condition index (Gam et al., 2009; de Montaudouin et al., 2012; O'Connell-Milne et al., 2016), and filtering activity (O'Connell-Milne et al., 2016). Moreover, infection with Himasthlidae metacercariae may lead to decreased tolerance of bivalve hosts to hypoxic conditions (Wegeberg and Jensen, 1999) and increased mortality among strongly infected individuals, especially of older individuals (de Montaudouin et al., 2000; Desclaux et al., 2004, 2006; Nikolaev et al., 2006; Gam et al., 2009).

Considering this background, it is surprising that no studies have looked at the influence of metacercariae on physiological parameters of bivalve hosts. Heart rate (HR) in marine invertebrates has been shown to be correlated with fluctuations in environmental factors (Marshall and McQuaid, 1994; Santini et al., 2000). HR is also known to be related to oxygen consumption in molluscs (Marshall and McQuaid 1992; Santini et al. 1999; Bakhmet, 2017), thus providing an estimate for a relative metabolic rate. Here, we determined *in situ* the impact of infection by *Himasthla elongata* Mehlis, 1831 (Echinostomatidae) metacercariae on the physiology of the blue mussel *Mytilus edulis* L., 1758, by investigating the HR of infected molluscs.

The trematode *H. elongata* (Echinostomatidae) is the most common macroparasite of intertidal mollusks in northern Europe(Galaktionov, 2001). Its first intermediate hosts are periwinkles *Littorina littorea* (L., 1758), in which parthenitae (asexual stages) develop and produce cercariae. Cercariae leave the mollusc and infect the second intermediate host, for example, mussels *M. edulis*. The final hosts, sea gulls, acquire infection when feeding on mussels infected with invasive metacercariae. Adult worms develop in gulls and produce eggs containing larvae, the miracidia. Eggs are dispersed in the faeces, and miracidia are the source of infection for the first intermediate hosts.

Our study site includes a number of unique features. First, White Sea temperatures are extremely low during the winter season (to −1.5 °C), and approximately from January to May the White Sea is covered with

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https://doi.org/10.1016/j.jip.2019.107220
Received 31 December 2018; Received in revised form 22 July 2019; Accepted 23 July 2019
Available online 06 August 2019

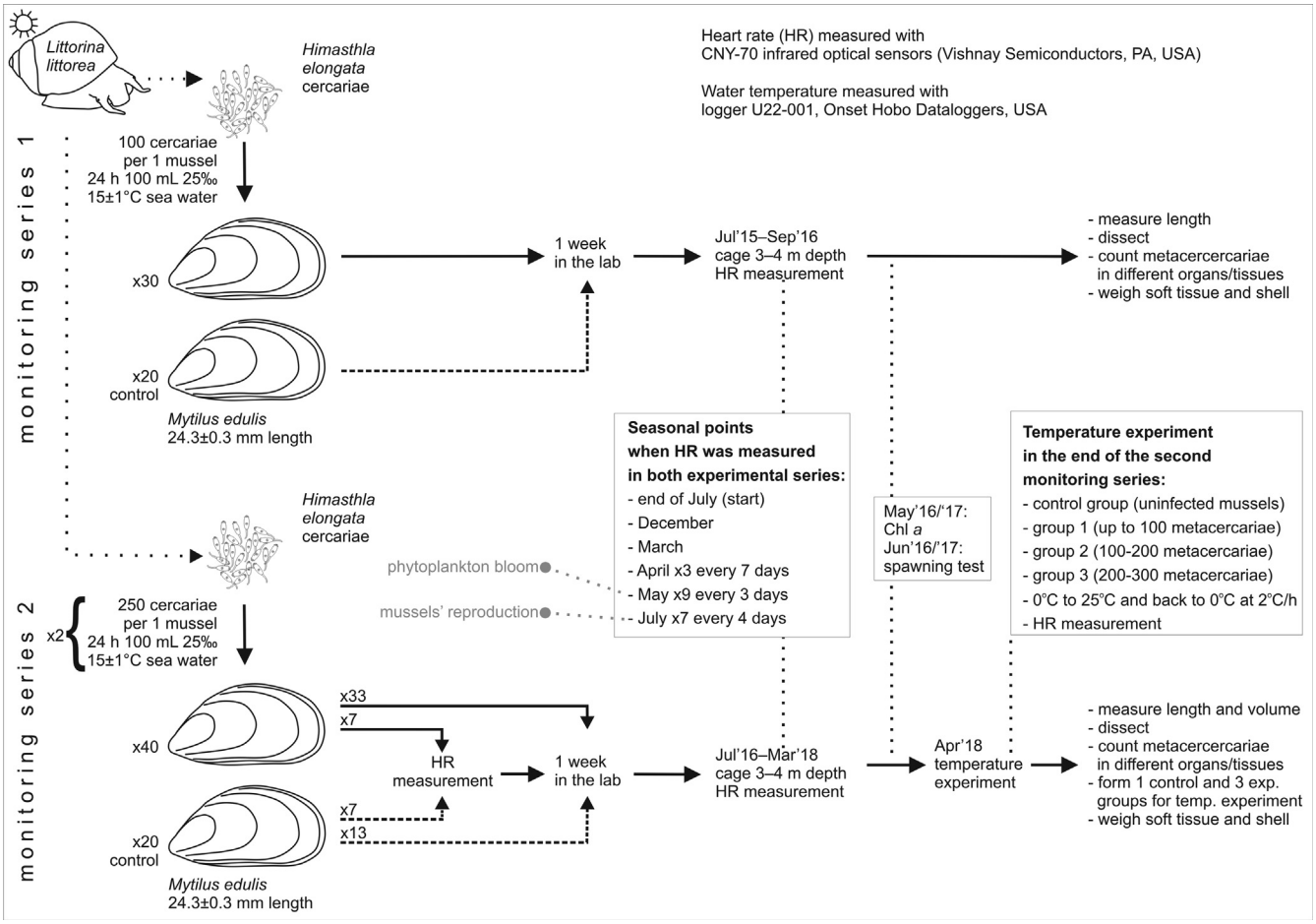

Fig. 1. Schematic showing the design of the two monitoring series and additional procedures.

30–40 cm layer of ice. The blue mussels have been shown to maintain a relatively high level of cardiac activity under these severe conditions (Bakhmet, 2017). Second, phytoplankton concentrations in the White Sea are generally low over the entire year (from 0 to 50–150 cells per mL) with two peaks in April and May (approximately 800 and 1000 cells per mL) (Bakhmet et al., 2018). Because of these conditions, blue mussels reach commercial size in 3–4 years, too slowly for mussel aquaculture to be profitable.

2. Materials and methods

The study was based at the White Sea Biological Station of the Zoological Institute RAS "Kartesh" (Chupa Inlet, Kandalaksha Bay, White Sea) and comprised two monitoring series. The first monitoring series, with low infection rates, was run from July 2015 to September 2016; the second series, with high infection rates, was run from July 2016 to March 2018 (detailed experimental design: Fig. 1).

Mussels were collected from artificial substrates on aquaculture rafts. Aquaculture facilities at the White Sea are placed at least 50 m away from shore, and mussels are not infected with *H. elongate* (Kulatchkova, 1985). We selected mussels of similar size (24.3 ± 0.3 mm) to exclude the influence of body mass on major physiological parameters and infection rate (Poulin, 2010).

We used a naturally infected intertidal gastropod. *L. littorea,* as a source of cercariae to infect mussels in the experiment. Individual snails were placed in 100-mL plastic containers with seawater and exposed to sunlight for 1 h. The containers were then screened under a stereomicroscope and molluscs shedding cercariae were selected. Cercariae, 2–3 h after emission, were used to infect mussels, as shown on Fig. 1.

HR was monitored following the non-invasive technique of Depledge and Andersen (1990), using optical sensors consisting of a phototransistor axially coupled with an infrared light-emitting diode. The sensor was attached to the shell of each individual mussel in experimental and control groups, with acrylic glue applied at the posterior end of the hinge region (the spot corresponding to pericardial cavity). Electric wires of the sensor were held above the water surface and accessed to record the HR signal at necessary time points. The signal was filtered, amplified, digitized (Adruino controller) and transferred to a computer through the USB-port. HR was measured in the field for the first time at the end of July 2015—one week after cages with mussels had been placed in natural conditions. We also measured HR at time points listed on Fig. 1, for months with multiple measurements; data collection was performed at even intervals. Throughout the monitoring period, temperature changes were registered (logger U22-001, Onset Hobo Dataloggers, USA). Data on Chl*a* was obtained from WSBS "Kartesh" long-term monitoring (for details see Usov et al., 2013) and converted into carbon using the carbon to chlorophyll ratio (C = 50 Chl*a*; Banse, 1977). In June 2016 and 2017 we dissected five subtidal mussels from the artificial aquaculture substrates every 4 days and determined gonadal maturity under a compound microscope in order to detect when mussels were ready for spawning. Every 3–4 months, cages were removed from water to clean them of algae and to measure mussels. Mussel growth rate (GR) (mm/day) was measured as [ln (final shell length) – ln (initial shell length)] / number of days (de Montaudouin et al., 2012).

At the termination of the second monitoring series (high infection) in April 2018 (ambient sea water temperature of 0 °C) we conducted an additional experiment to estimate the influence of the infection on the

adaptive potential of molluscs. Mussels from the second monitoring series were involved in this experiment. When the experiment ended and mussels were dissected, mussels were divided into one control and three experimental groups with different infection levels (Fig. 1) to analyse the experimental results. In this experiment HR was measured as temperature was increasing (Fig. 1). Critical temperatures were determined according to the Arrhenius method (Stillman and Somero, 1996). To estimate the differences between regression lines (HR vs temperature) ANCOVAs were applied: one for the descendent and one for the ascendant portion.

At the end of each monitoring series, the shell length of each mussel was measured by calliper to the nearest 0.1 mm. In the second series we also measured the shell volume using a graduated cylinder, after which mussels were dissected. To search for metacercariae of *H. elongata,* we pressed soft tissues of mussels between two glass slides and observed them under the stereomicroscope at 28×. Metacercariae were counted separately inside the foot, the mantle edge, the gills and the visceral mass (digestive gland, labial palps, gut and pericardium) of each mussel, then, the soft tissues and shell of mussels were weighed.

To compare HR in infected and uninfected molluscs we used Student's test (Sokal and Rohlf, 1995). When interrelation was found between (1) HR and water temperature, and (2) heart rate and number of metacercariae, we used Pearson's correlation analysis. Comparison of regression lines was performed with ANCOVA. To determine condition index we used the following formula: CI = weight of soft tissues in g × 1000/shell length in mm (Lundebye et al. 1997). The HR variances were calculated using standard formula. All data are given as arithmetic mean ± standard error. Analyses were performed in STATISTICA 10.0 software package.

3. Results

In the first monitoring series we recovered 19 mussels infected with *H. elongata* metacercariae, from 1 to 40 parasites each. Seventeen mussels had metacercariae in the foot, one in the mantle edge, and one in the gills. HR in mussels with metacercariae in the mantle edge and gills did not differ from mussels with the metacercariae in the foot. GR of control mussels was significantly higher than that of infected individuals from May to July 2016 (0.0023 and 0.0015 mm/day, respectively; $N = 17$; $t = 2.57$; $p = 0.02$). This parameter was 0.0008 mm/day in both groups from July to September 2016.

In the second monitoring series, HR of infected mussels dropped sharply during the first 2.5 h of the infection process, and then reached the level of the controls within 3 h. After that time point, HR of the infected and the control mussels did not differ significantly up to the next summer season. Infection levels varied from 1 to 302 metacercariae in 29 mussels tested. Parasites were mostly localized in the foot (up to 216) and mantle edge (up to 186). Metacercariae occurred in the visceral mass (from 1 to 25) of five mussels. The ratio of metacercariae numbers in the foot to abundance in the mantle edge varied from 0.5 to 89, with the mean of 14.4. No metacercariae were found in the gills. As in the first monitoring series, parasite localization did not affect mussel HR. However, unlike in the first series, we observed a significant relationship between HR on the 1st of August 2017 and the total number of metacercariae: HR fell linearly as the number of metacercariae grew to 200 ($HR = -0.06^*$ (number of metacercariae) + 29.68; $r = -0.67$; $p < 0.05$) (Fig. 2). As number of metacercariae continued to grow and reached 300, HR of the infected mussels also increased significantly (up to 27.7 ± 2.2 beats/min). The monitoring period was longer for the second series and we found a larger number of infected molluscs and higher infection rates. This allowed estimation of the relationship of changes in GR of infected and uninfected molluscs, and infection rates and season. Significant differences in GR of infected and uninfected molluscs were only detected from March to July 2017 ($N = 17$; $t = 2.17$; $p = 0.04$). A significant relationship between GR and infection rate was observed ($GR = -0.000002 \times$ (number of metacercariae) + 0.00138; $N = 40$; $r = -0.42$; $p < 0.001$). From July to September 2017 and from September 2017 to March 2018, no such relationship was found, but GR was significantly higher in the uninfected mussels than in the infected individuals. We observed gradual slowing of volume growth rate as infection rate increased (from 0.0017 to 0.0012 mL/day). In mussels with the highest infection rates (200–300 metacercariae) the rate of shell volume growth increased and reached 0.0014 mL/day. This pattern was similar to the pattern of HR in highly infected mussels.

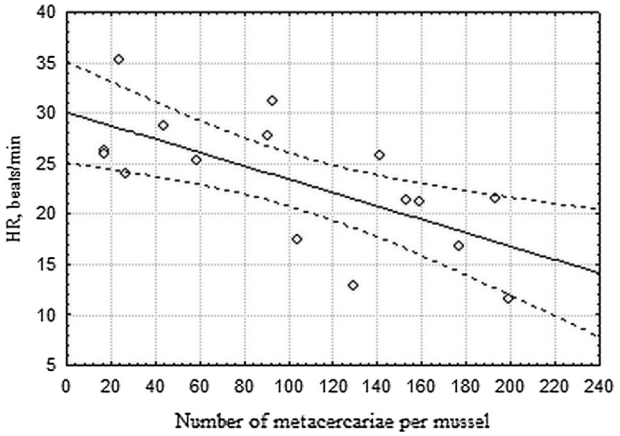

Fig. 2. Relationship of heart rate in infected mussels and number of metacercariae.

Water salinity was quite stable (24–26) during both HR monitoring series. On the contrary, temperature varied from −1.2 °C in March to +18.2 °C in July. Monitoring showed that HR in infected molluscs did not differ from HR in uninfected molluscs in the first monitoring series for the entire experimental period (Fig. 3). In the second series, HR in uninfected mussels was higher than in the infected individuals ($N = 18$, $p = 0.006$) during the summer but did not differ during other seasons (Fig. 4). The relationship of HR and temperature is described by an exponential equation with coefficients that are similar in both groups of experimental mussels (Table 1). In May, when temperature remained constant, HR in infected molluscs also increased in both series. It did not differ significantly from HR growth in the control groups observed in the same period (Figs. 3, 4). This coincided with the period of sharp rise in phytoplankton concentration (from 0.01 to 0.16 μg carbon per ml), followed by a decrease to 0.04 μg carbon per ml in the end of May.

From the end of May to early July, as temperatures rose sharply (9.2 to 16.8 °C), we did not note any significant changes in mussel HR. At the end of June spawning occurred. In the first week of July, HR of mussels increased significantly without noteworthy temperature fluctuations (Figs. 3, 4). By September HR was falling in accordance with temperature changes (Figs. 3, 4).

Variance in HR of the infected and uninfected mussels in winter and summer was similar, while in summer and autumn it rose sharply in infected mussels and was 2–4 times higher than in uninfected mussels (Figs. 5, 6).

Condition index (CI) of mussels decreased with the increase in infection rate as follows: 41.85 ± 3.46 (control group), 31.19 ± 2.32 (1–100 metacercariae per mussel), 26.31 ± 2.32 (100–200) and 26.34 ± 3.48 (200–300). Only CI of the control group was significantly higher ($N = 28$; $t = 2.05$; $p = 0.02$).

Changes in mussel HR during the temperature experiment differed in every group (Fig. 7). In the control group the initial increase of HR stopped at 20.7 °C (first critical temperature). Then, at 25 °C, the increase resumed until the gradual decrease started at 19.5 °C. Variation in HR in the first group of the infected molluscs (up to 100 metacercariae per mollusc) followed a pattern similar to that in the control group. However, the first critical temperature was 22.4 °C, at which HR

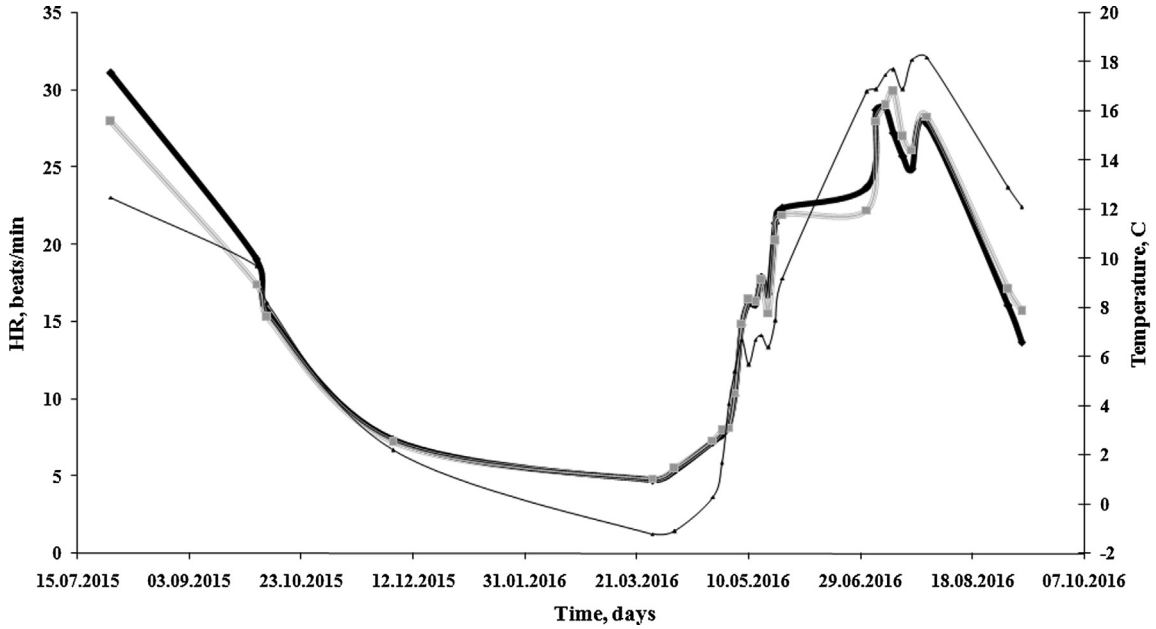

Fig. 3. Changes in heart rate of infected (gray line) and uninfected (thick black line) mussels in the first monitoring series (thin black line = temperature).

would decrease for the first time. A definitive decrease in HR also commenced at 19.5 °C. HR in the first group fluctuated strongly during the temperature rise (Fig. 7). In the second group of the infected mussels (100 to 200 metacercariae per mussel) there was only one critical temperature, and HR increased linearly followed by decrease at 23.3 °C. In this group HR reached the highest mean value, 39.1 beats per minute (bpm), while in the control and the first group it never exceeded 30.5 bpm. In the third group of the infected mussels (200–300 metacercariae per mussel) HR changes first coincided with data from the control and the first group, but the second HR rise occurred at 28.0 bpm (while it was at 25.4 and 20.9 bpm in the control and the first group, respectively). Comparing the regression lines (HR vs temperature) during temperature increase, only the second group differed significantly from others (ANCOVA: $F = 23.8$, $p < 0.001$; $F = 45.4$,

Table 1
Indices of equations – HR = axt + b – blue musselheart rate HR and temperature (t) correlation over the year.

		a	b	r	p <	R^2	N
1st group	Uninfected	1.16	7.11	0.90	0.001	0.81	25
	Infected	1.18	6.81	0.93	0.001	0.86	25
2nd group	Uninfected	1.60	4.78	0.93	0.001	0.90	24
	Infected	1.49	4.77	0.95	0.001	0.87	24

- r – correlation coefficient; p – probability; R^2 – determination coefficient, N – number of animals.

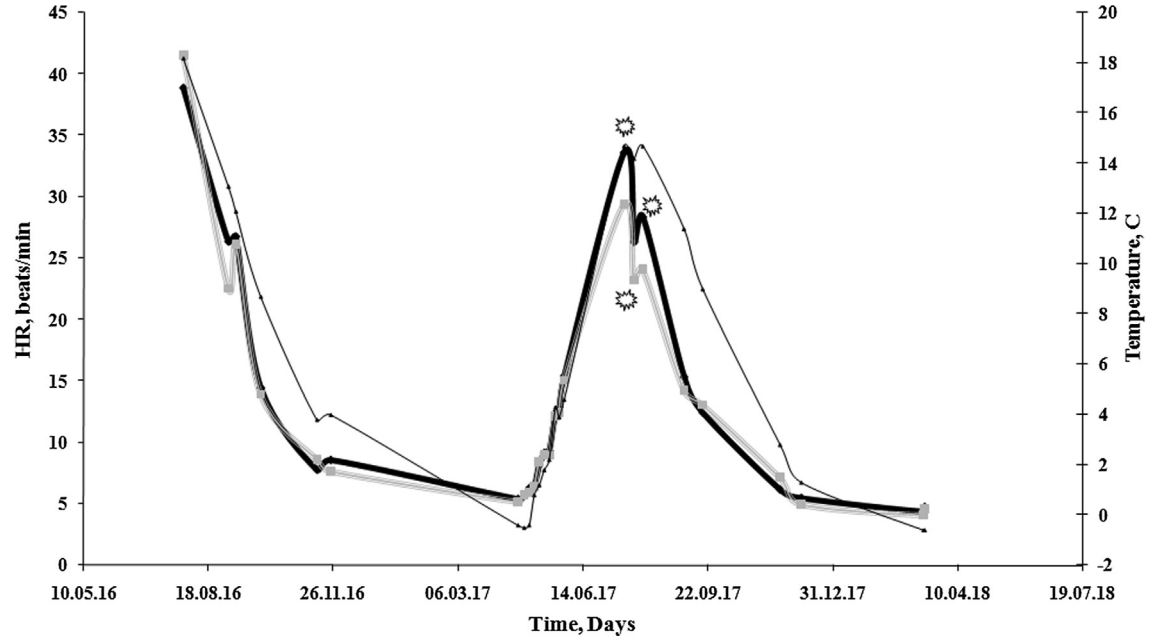

Fig. 4. Changes in heart rate of infected (gray line) and uninfected (thick black line) mussels in the second monitoring series (thin black line = temperature) (asterisks = significant differences).

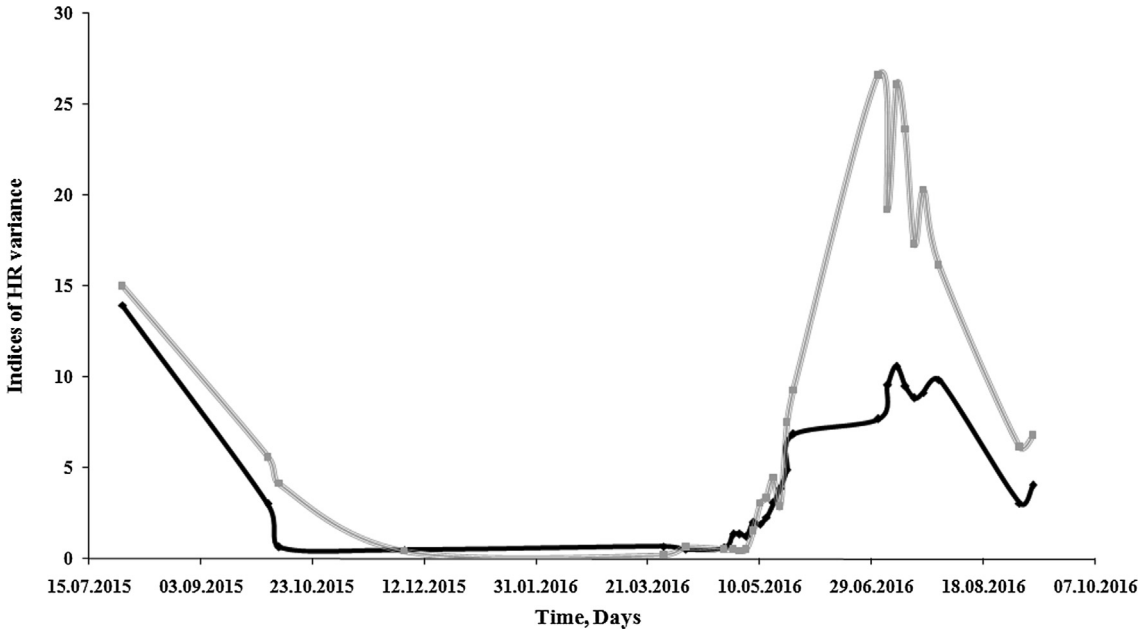

Fig. 5. Changes in heart rate variance for infected (gray line) and uninfected (thick black line) mussels in the first monitoring series.

$p < 0.001$; $F = 33.8$, $p < 0.001$). On the contrary, during temperature decrease, a significant difference for the third group was shown (ANCOVA: $F = 10.8$, $p < 0.001$; $F = 12.8$, $p < 0.001$; $F = 11.9$, $p < 0.001$).

4. Discussion

Lack of significant differences in mean HR of infected and uninfected musselsin the first series may indicate lack of explicit impact of *H. elongata* metacercariae on the host individual. On the other hand, infected mussels had first shown a decrease in GR, and then a sharp increase in the variance on HR values. Similar data on GR were obtained for *Cerastoderma edule* (L., 1758) infected with a closely related digenean species *Himasthla interrupta* Loos-Frank, 1967 (de Montaudouin et al., 2012). HR variance values in the infected molluscs are of special interest. Increase in intra-individual variation of different parameters is known to be treated as an indicator of stress (Zakharov, 1989; Graham et al., 1993; Leung and Forbes, 1996). We consider the increase in HR variance in the molluscs infected with *H. elongata* metacercariae as a sign of stress and impact of the parasite on the host individual. Variation also could be due to the inter-individual variability in the infection. Moreover, HR variance may explain why our results do not correspond to those published previously (Bakhmet et al., 2017). In the previous study mussels with decreased HR apparently were analyzed, while more material was available in this study and we observed molluscs with both increased and decreased HR, thus high variance values. Subsequently mean HR values of the infected mussels did not differ from those of the uninfected mussels. For example, in the month of July, HR of the infected molluscs ranged from 21 to 38 bpm, while in the uninfected it ranged from 25 to 35 bpm.

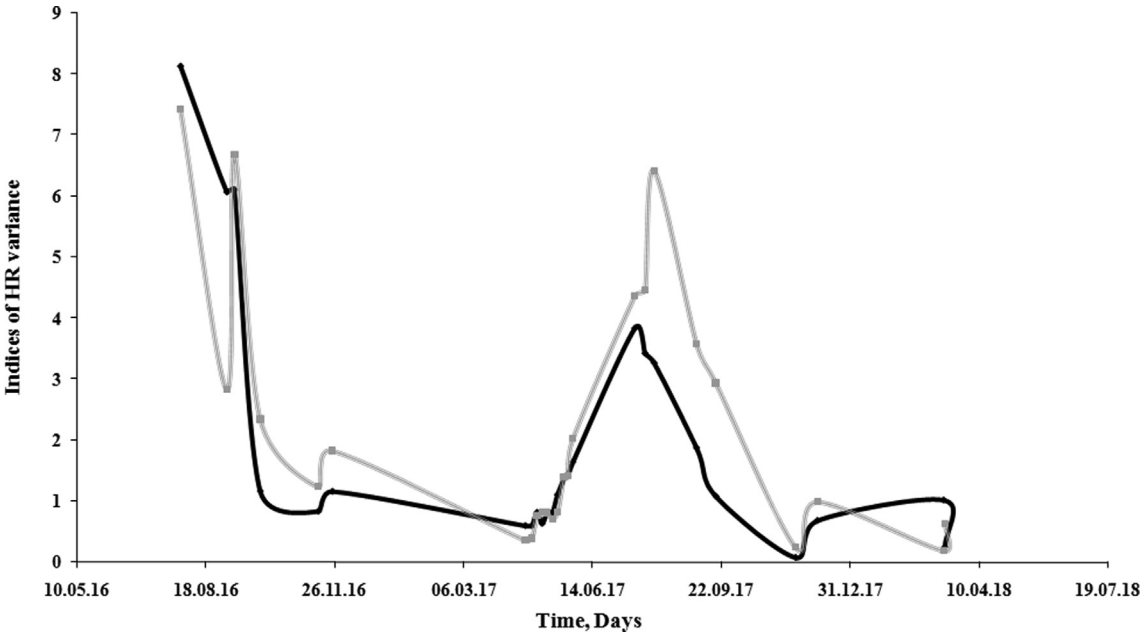

Fig. 6. Changes in heart rate variance for infected (gray line) and uninfected (thick black line) mussels in the second monitoring series.

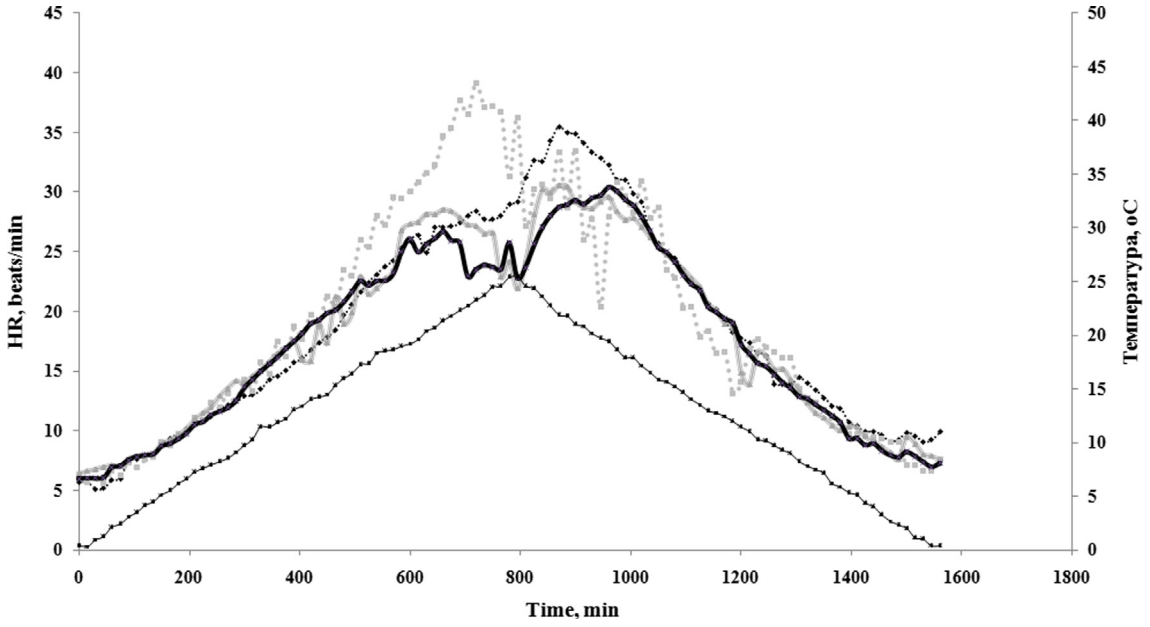

Fig. 7. Changes in heart rate of infected and uninfected mussels in the temperature experiment (thick black line = the control; thick gray line = infection rates of up to 100 metacercariae per mussel; gray dotted line = infection rates of 100–200 metacercariae per mussel; black dotted line = infection rates of 200–300 metacercariae per mussel; black thin line = temperature).

In the second monitoring series we more clearly established that metacercarial infection affected the mollusc host. There was conspicuous HR variance among the infected molluscs in summer (Fig. 6), and HR also was lower in infected mussels during the summer (Fig. 4). There was a linear relationship between mussel HR and metacercariae numbers (up to 200). Condition index decreased as infection intensity increased, a pattern also observed for mussels infected with *Renicola roscovitus* Stunkard, 1932 cercariae (Stier et al., 2015) and reflecting the decrease in relative soft tissue weight in infected molluscs. Finally, mussel growth rate also decreased upon infection.

Additional evidence for impacts on the host was obtained in the temperature experiment. Minimal differences in HR in the control and the first experimental group indicated the minimal impact of infection by less than 100 metacercariae. However, we observed prominent cyclic changes in the mussels' cardiac activity in the first experimental group that may point to the effect of metacercarial presence. Sharp difference in HR between the second trial infected group and the control shows that infection rates of 100–200 metacercariae per mussel affect host metabolism much more. The response of the third group may be explained by the initial high HR of infected mussels, which, as mentioned above (see Fig. 2), is probably due to the more pronounced inflammatory process. Notably, upper critical temperatures were higher in the infected mussels than in the control group. This may mean that infected mussels have decreased ability for temperature compensation (Jankowsky, 1973).

The observed differences in HR of infected mussels were prominent initially during infection, but only for the first 2–3 h. Decreased HR in mussels that are infected is probably due to the damage of their host tissues by cercariae. At this moment (start of infection) mussels close their shell valves, with an associated decrease in HR. Then, impact of metacercariae on molluscan host individuals was only observed in summer and, most importantly, only one year after infection. Apparently, pathogenic effect of metacercariae is limited at first and then grows with time, as the negative effects accumulate. This may explain why many authors did not detect influence of Echinostomatidae cercariae on the organism of the second intermediate host (Bower et al., 1994; Laruelle et al., 2002). The mechanism of *H. elongata* metacercarial influence on mussel individuals is not yet clear. Moreover, it is difficult to explain the sharp increase in HR and thus metabolism in highly infected mussels. Perhaps the pathogenic effects of metacercariae cause molluscs have to employ additional defence mechanisms (protein synthesis, increased haematopoiesis (Gorbushin and Iakovleva, 2008)), which require additional energy consumption. To test this idea further experiments will be necessary.

Acknowledgements

We are deeply grateful to the staff of the White Sea Biological Station of the Zoological Institute of the RAS. Also we thank the dean of Pskov State University Prof. V.V. Prokofiev and the student D.A. Egorov.

Financial support

This work was supported by a Russian Foundation for Basic Research (project no. 16-04-00820 A) and by federal funding for government-ordered project themes Nos. 0221-2017-0045, AAAA-A19-119020690109-2, and AAAA-A19-119022690122-5.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2019.107220.

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