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Viral pyrogen affects thermoregulatory behavior of wintering *Planorbarius corneus* (L.) snails (Mollusca: Gastropoda)

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ABSTRACT

Exogenous pyrogens, isolated from viruses, bacteria and fungi, are widely used in research on fever in endotherms. The present study investigates the use of viral pyrogen poly (I:C) for inducing the symptoms of so-called behavioral fever in ectothermic animals *Planorbarius corneus* (Mollusca: Gastropoda). The observations of thermoregulatory behavior of the snails placed in the temperature gradient showed that the snails which were injected with 750 ng g^{-1} polyinosinic-polycytidylic acid-poly (I:C), after remaining latent for more than 24 h, moved to warmer areas (with the temperature around 30 °C) and stayed there until the end of the experiment. Transferred to the culture, they lived for a period similar to that of the control snails. Our observations indicate that *P. corneus* snails can exhibit a symptoms of fever response to poly (I:C) and could be used as model animals in the research on behavioral fever in invertebrates.

1. Introduction

Studies on the mechanism of fever are still an important part of the mainstream research on defensive processes in humans and animals (Bouchama et al., 2000; Kozak et al., 2000, Bicego and Branco, 2002; Kraybill et al., 2002; Leon, 2002; Cooper et al., 2010). Fever in endotherms, i.e. the adjustable rise in body temperature occurring as a result of an elevation of the thermoregulatory set-point (Glossary of Terms for Thermal Physiology, 1987), is generated in response to pathogenic factors such as microbial infections, parasites, surgeries etc. Viral, bacterial and fungal pyrogens are exogenous fever-producing agents, characterized in detail by Kozak (2009). The mode of action of exogenous pyrogens was described after the discovery of endogenous pyrogens, whose properties are shared by cytokines, intensively studied nowadays. However, the comprehensive explanation of this phenomenon was possible only after determining the function of prostaglandins, biochemical agents which connect pyrogens with the rise of the body temperature. Prostaglandins and cytokines were identified not only in endotherms but in ectotherms as well, both in vertebrates and invertebrates (Ruggeri and Thoroughgood, 1985). Although ectotherms do not have the ability to raise their body temperature endogenously, it has been frequently confirmed that bacterial infections and pyrogen injections generate their behavioral thermal responses (Marx et al., 1983; Ostberg et al., 2000; Basu and Srivastava, 2003; Blanford et al., 1998; Borges, 2000). Ectothermic vertebrates and invertebrates infected for the purpose of the experiment reacted by moving to warmer microenvironments to raise their body temperature (Seebacher and Craig, 2005; Grans et al., 2012; Casterlin and Reynolds, 1982). The elevated body temperature led to either the recovery from the infection or the inactivation of the injected pyrogen in the reaction referred to as behavioral fever (Louis et al., 1986; Richards-Zawacki, 2010).

Although thermoreceptory abilities have been identified in almost all animal groups (Nelson et al., 1984), thermoregulatory processes in invertebrates are studied less intensively compared to similar processes in vertebrates. Moreover, research on thermoregulatory behavior in invertebrates focuses mainly on arthropods, and insects in particular, due to their huge economic importance. In the pioneering study on thermoregulatory behavior of molluscs Casterlin and Reynolds (1980) pointed to non-random responses of *Nassarius trivittatus* snails placed in a temperature gradient and accentuated the fact that they were able to select and avoid particular microenvironments because of their specific temperature. Kavaliers et al. (1983), Kavaliers and Hirst, (1986) and Gutierrez (1992) also emphasized molluscs' ability to register and regulate the body temperature.

Molluscs were not included in the group of model animals for the research on behavioral fever after Cabanac (1990) published his study on the phylogeny of fever. The author maintains that the ability to exhibit fever response by animals appeared after the emergence of molluscs and before the emergence of arthropods, which means that older taxa including molluscs are not able to exhibit a fever response (Cabanac and Rossetti, 1987; Cabanac and Drolet, 1991). The loss of interest in behavioral fever in molluscs

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was sealed with the study of Lefcort and Bayne (1991), who observed a drop in body temperature in *Biomphalaria glabrata* in response to the invasion of *Schistosoma mansoni* larvae and therefore concluded that snails did not express a fever response to the parasites. The authors ignored the possibility that behavioral anapyrexia (lowering body temperature by cold-seeking behavior), similarly to behavioral fever, is a defensive mechanism of infected hosts (Żbikowska, 2004; 2005; 2011; Żbikowska and Cichy, 2012).

The results of our previous research on thermoregulatory behavior of *Lymnaea* sp. snails (Żbikowska, 2001) are consistent with the hypothesis of Cabanac and Rossetti (1987) that snails do not exhibit behavioral fever response. However, it was also established that *Planorbarius corneus*, observed during winter season, showed a fever response when infected with LPS or zymosan (Żbikowska et al., 2013). Hemolymph composition is one of the differences between Lymnaeidae and Planorbidae; the latter have hemoglobin, which, according to Zilletti et al. (1994) and Walentynowicz et al. (2006) can participate in a molecular mechanism of fever. The above facts inspired us to continue research with the use of pyrogens. In the present study we used a viral substance to determine its effect on the thermoregulatory behavior of *Planorbarius corneus*. It was discovered that viral poly (I:C) induced a fever response in rats through a mechanism dependent on interleukin – 1 (Fortier et al., 2004). Since different interleukins (including IL-1α and IL-1β) had been isolated from hemocytes of *Planorbarius corneus* (review: Coyne, 2011) we predicted that the potential positive effect of our research could be beneficial for the future use of snails as model animals in research on the mechanisms of fever.

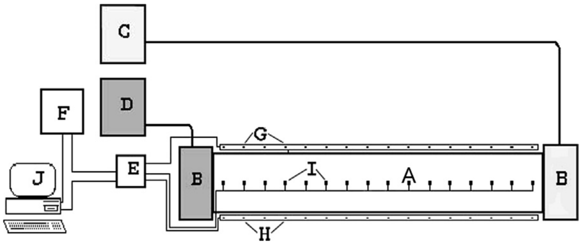

Fig. 1. Experimental setup for recording thermal behavior of snails: A – temperature gradient chamber (water level: 5 mm), B – fluid chambers, C – thermostat, D – cryostat, E – electronic switch to thermocouples, F – scanner, G – transmitters of infrared radiation, H – receivers of infrared radiation, I – thermocouples, and J – computer.

Table 1
The mean temperatures selected by the control and the experimental *Planorbarius corneus* snails.

Snails	*N*	MEAN 48 h [°C] (SD)	MEAN 1st day [°C] (SD)	MEAN 2nd day [°C] (SD)
Injected with 100 µl of 0.6% NaCl	43	19.0 (2.6)	19.1 (3.3)	18.9 (2.1)
Injected with 100 µl of 750 ng g^{-1} poly (I:C)	37	23.2* (2.1)	19.0 (0.0)	27.5* (3.1)

* Statistically different from control group, t-test (p«0.0001).

2. Materials and methods

2.1. Animals

Planorbarius corneus snails were collected in November 2011 from Lake Gopło in central Poland (water temperature +11.5 °C, pH 8.5, oxygen content 142%, conductivity 61.1 mS m^{-1}, total phosphorus content 0.214 mg l^{-1}, calcium content 71.9 mg l^{-1}). One hundred fifty snails with shell diameter 28–35 mm were placed in beakers with settled water in a refrigerator (temperature: 4–6 °C) for 12 weeks. Before the experiment in a temperature gradient the snails were subjected to a standard non-invasive method of diagnosing parasites. Only the snails which did not release trematode cercariae were qualified for further tests. In order to confirm the absence of parasites all the snails were dissected after death.

Before the experiment in the temperature gradient the snails were acclimated for three weeks at 19 °C in water changed weekly. This routine was aimed at stimulating the snails to move in the gradient and also enabled us to compare the results with those obtained for the active snails. During the acclimation, the experimental snails never laid eggs, which distinguished them from the active snails.

2.2. Treatment of snails with pyrogens

Polyinosinic-Polycytidylic acid (poly (I:C)) sodium salt (Sigma-Aldrich Chemicals) were dissolved in sterile water. Before injection, the stock solution of poly (I:C) (10 mg ml^{-1}) were diluted in a sterile 0.6% saline to the desired concentration. Two different doses of poly (I:C) (7.5 µg g^{-1}, 750 ng g^{-1}) were tested on snails. Both doses were calculated according to wet body mass of snails (without shell). The control group of snails were individuals injected with 0.6% sterile salt solution (CNaCl). Pyrogen was injected using sterile single-use syringe (1 ml). All snails were injected at noon without anesthesia. The snail foot was injected with 100 µl of liquid, and only animals that had not bled (95 injected individuals) were used in the experiment.

2.3. Observation in the temperature gradient

The snails were placed individually in an rectangular oblong thermal gradient (temperature range: +4 to +38 °C) (Fig.1). The temperature gradient was generated by circulating fluids (Petrygo Q and water) controlled by PolyScience ultrathermostats. The experiment was carried out in a room with the temperature of 19–20 °C. Each snail was used only once, and the number of repeats is shown in Table 1.

The results were automatically recorded and computed at 3-min intervals during 48 h using a custom data acquisition computer program GRAD. For the data analysis, excel plotting and presentation, the temperature recordings of the control and experimental snails' groups were pooled into 1-h, 24-h, and 48-h means. Longer than 48 h keeping snails without food in a thermal gradient caused the starvation response. Regardless of the treatment the starved animals moved at the cool end of the thermal gradient (Żbikowska, 2009).

2.4. Statistical analysis

Thermal preference data (24-h, and 48-h means) were analyzed with t-test. A repeated measures ANOVA followed by LSD Fisher test was used to analyze 1-h temperature means.

3. Results

The experiment involved 95 parasite free *P. corneus* individuals including 43 control snails, 15 snails inoculated with 7.5 µg g^{-1} poly (I:C) and 37 snails inoculated with 750 ng g^{-1} poly (I:C). During the 48 h observation the control snails tended to select temperatures ranging from 8 °C to 34.3 °C but the mean temperatures recorded for 48 h (MEAN 48 h) as well as the mean