Leeches *Baicalobdella* sp. feed on hemolymph but do not affect the cellular immune response of amphipod *Eulimnogammarus verrucosus* (Amphipoda, Crustacea) from Lake Baikal (#93789)

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Leeches *Baicalobdella* sp. feed on hemolymph but do not affect the cellular immune response of amphipod *Eulimnogammarus verrucosus* (Amphipoda, Crustacea) from Lake Baikal

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Lake Baikal is one of the largest and oldest freshwater reservoirs on the planet with a huge endemic diversity of amphipods (Amphipoda, Crustacea). These crustaceans have various symbiotic relationships, including the rarely described phenomenon of leech parasitism on amphipods. It is known that leeches feeding on the hemolymph of crustacean hosts can influence their physiological status, especially under stressful conditions. Here we show that leeches *Baicalobdella* sp. found on the gills of the amphipod *Eulimnogammarus verrucosus*, one of the most abundant amphipods in the Baikal littoral zone, indeed feed on the hemolymph of their host. However, the leech infection had no effect on such immune parameters as hemocyte concentration and phenoloxidase activity, as well as glycogen content. The intensity of hemocyte reaction to foreign bodies in a primary culture was identical between leech-free and leech-infected animals. Artificial infection with leeches also had almost no modulating effect on bacterial influence on the hemocyte concentration and composition in hemolymph of amphipods after the injection modeling the microbial outburst. Thus, our study shows that the influence of a few leeches on *E. verrucosus* is probably negligible, and leech-infected amphipods can be used at least for some types of ecophysiological experiments.

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Abstract

Lake Baikal is one of the largest and oldest freshwater reservoirs on the planet with a huge endemic diversity of amphipods (Amphipoda, Crustacea). These crustaceans have various symbiotic relationships, including the rarely described phenomenon of leech parasitism on amphipods. It is known that leeches feeding on the hemolymph of crustacean hosts can influence their physiological status, especially under stressful conditions. Here we show that leeches *Baicalobdella* sp. found on the gills of the amphipod *Eulimnogammarus verrucosus*, one of the most abundant amphipods in the Baikal littoral zone, indeed feed on the hemolymph of their host. However, the leech infection had no effect on such immune parameters as hemocyte concentration and phenoloxidase activity, as well as glycogen content. The intensity of hemocyte reaction to foreign bodies in a primary culture was identical between leech-free and leech-infected animals. Artificial infection with leeches also had almost no modulating effect on bacterial influence on the hemocyte concentration and composition in hemolymph of amphipods after the injection modeling the microbial outburst. Thus, our study shows that the influence of a few leeches on *E. verrucosus* is probably negligible, and leech-infected amphipods can be used at least for some types of ecophysiological experiments.

Introduction

Various parasites are now considered as a significant environmental factor influencing survival of aquatic animals under stressful conditions and sometimes acting synergistically with such factors as pollution [1,2]. In particular, some parasites have been shown to manipulate behavior, distort sex ratio, modify energy budget and compromise the immune defense in amphipods (Amphipoda, Crustacea), one of the most important groups of freshwater invertebrates [3]. Leeches are annelid worms (Hirudinea, Annelida), many species of which parasitize various animals and feed on the host blood or hemolymph. Importantly, saliva components of these parasites can have anticoagulant, anti-inflammatory and other roles, but such bioactive components and their effects are mostly studied in medically important species [4,5,6]. Leeches and crustaceans can exist in different types of ecological relationships. For example, leeches of the species *Myzobdella lugubris* Leidy, 1851 are parasites of crabs *Callinectes bocourti* Milne-Edwards, 1879 feeding on their hemolymph and laying eggs on the surface of the crab body [7]. The South African leech *Marsupiobdella africana* is a facultative ectoparasite of the amphibian *Xenopus laevis* and has a phoretic relationship (i.e. promoting spreading of the attached phoront) with the freshwater crab *Potamonautes perlatus* Milne-Edwards, 1837. The sex of the host crab has been shown to be important in leech infestation. In addition, the period of residence of the leeches on crabs corresponds to the development of leech eggs, which may indicate additional benefits of these relationships for leeches [8]. The crayfish *Orconectes rusticus* (Girard, 1852) has the cleaning leech-like symbiont *Cambarincola fallax* Hoffman, 1963 that removes fouling organisms and thus improves growth rates of the host [9,10,11]. The fish leech *Johanssonia arctica* (Johansson, 1898) is also an epibiont of the red king crab *Paralithodes camtschaticus* (Tilesius, 1815) [12,13].

Lake Baikal is among the largest and most ancient freshwater reservoirs on the planet and also the birthplace of outstanding endemic diversity of amphipods playing various roles in the lake ecosystem [14]. Over 350 morphological species and subspecies of amphipods have been described from Baikal, constituting about 19% of all known freshwater species and demonstrating tremendous morphological variety [15,16]. Yet, symbionts and parasites of Baikal amphipods and their potential influence on physiology of these crustaceans are understudied. It is known that the hemolymph of the amphipods can contain various bacteria [17] and DNA of microsporidians [18]. Baikal endemic amphipods are also known to be intermediate hosts for acanthocephalans, but the fraction of infected individuals is generally low [19].

However, the parasites that can be most easily found on amphipods in Lake Baikal are leeches. According to our observations, leeches are mostly attached to the gills of the largest morphological species in the Baikal littoral zone such as *Eulimnogammarus verrucosus* (Gerstfeldt, 1858) or *Pallasea cancellus* (Pallas, 1772) and much less often to a smaller *E. vittatus* (Dybowsky, 1874). The hypothesis that the parasites prefer larger species as hosts is also supported by observations of leeches on even larger deep-water Baikal amphipods [20]. Again, according to our previous observations in *E. verrucosus*, leeches can infect a substantial proportion of the population on the order of dozens of percents at least in some seasons. These parasites of *E. verrucosus* belong to the genus *Baicalobdella* containing at least two species, *B. cottidarum* Dogiel, 1957 and *B. torquata* (Grube, 1871) [21,22,23]. *E. verrucosus* is a widespread and abundant morphological species in the littoral zone of Lake Baikal [24] and yet influence of leeches on its physiology is fairly unstudied. Moreover, the whole phenomenon of leeches infecting amphipods seems to be very rare if not unique to Lake Baikal, which might be related to the larger size of many Baikal endemics in comparison to most freshwater amphipods. Literature search gave us no other examples of such a phenomenon, and a recent review categorizing parasites of amphipods do not mention leeches at all [25].

If leeches indeed feed on hemolymph of amphipods in Lake Baikal, the infection may directly impair their immune defense and indirectly lower the available energy resources besides the potential effects of leech saliva. The crustacean immune system relies on hemolymph components such as hemocytes (i.e. circulating cells) and the phenoloxidase system. Hemocytes perform phagocytosis and encapsulation of foreign bodies, while phenoloxidase is responsible for the melanization process, which is also a part of foreign body encapsulation and hemolymph clotting after injury [26]. So, in this study we mostly concentrated on testing the effects of leech infection on these immune factors in E. verrucosus from Lake Baikal.

Materials & Methods

Animal sampling and handling

All experimental procedures were conducted in accordance with the EU Directive 2010/63/EU for animal experiments and the Declaration of Helsinki; the protocol of the study was registered and approved before the start of the experiments by the Animal Subjects Research Committee of the Institute of Biology at Irkutsk State University (protocol #2022/11). Leech-free and leech-infected amphipods Eulimnogammarus verrucosus (Gerstfeldt, 1858) were collected by kick sampling with a hand net in Baikal littoral zone near the Listvyanka village (51°52'05.5"N 104°49'47.1"E) at depths 0-1.2m (the animals belong to the W barcoding species [27]). Amphipods were acclimated to the laboratory conditions in well aerated 3 L plastic aquaria at 6°C in MIR-254 incubators (Sanyo, Osaka, Japan) for at least 3 days prior to any experiments. Typically, leeches were attached to the gills of amphipods (Figure 1A,B).

Identification of leech species

After sampling~~s~~ in October 2022, February and April 2023 all clearly visible leeches were detached from amphipods ~~in~~ and fixed in 96% ethanol for further species identification. Morphological analysis of fixed specimens was performed on a stereo microscope SPM0880 (Altami, Russia) according to the standard keys (Bauer, 1987; Lukin, 1976). DNA extraction from the posterior sucker of leeches was performed using the S-sorb kit (Syntol, EX-516, Russia). PCR amplification of the cytochrome oxidase subunit I (COI) gene fragment was performed with a 5× Screen Mix (Evrogen, Russia), the Folmer primers (LCO1490/HCO2198 [28]) and the following program: 94°C for 1 min, 30 cycles of 94°C for 20 s, 43°C for 2 min, and 72°C for 1 min.

The sequencing reactions were performed in both directions using BigDye Terminator v3.1 Cycle Sequencing kit (Life Technologies, USA) and analyzed with a Nanophor-05 Sanger sequencer (Syntol, Russia). Sequencing reads were basecalled with the programs Mutation Surveyor v5.1 and Chromas v2.6.6. Consensus sequences were compiled with UGENE v41.0 [29] using the sequence from *Baicalobdella* sp. (NCBI Genbank accession MN854834) as the reference COI fragment. Sequences were aligned with the MAFFT algorithm [30] and trimmed to 559 bp in the UGENE program. The sequences were deposited to the NCBI GenBank database with accession numbers OR077511-OR077525. Similar sequences were searched with BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in the nt database. Phylogenetic tree was built with the IQ-Tree web server (http://www.iqtree.org/) using automatic model selection with Model Finder [31] and ultrafast bootstrap for assessment of the branch support values [32]. The resulting phylogeny was visualized with iTOL (https://itol.embl.de/) [33].

Injection of fluorescent latex beads into amphipods and further visualization

After sampling in June 2023, we analyzed the ability of leeches *Baicalobdella* sp. to consume amphipod hemolymph. For this, leeches were detached from amphipod gills with tweezers and kept in aquaria separately from hosts for ~24 h. Next, 10 non-infected individuals of E. verrucosus were injected with 1 µl of suspension containing about 3*106 latex microbeads (L3030, Sigma-Aldrich) using a IM-9B microinjector (Narishige, Tokyo, Japan). Right after the injection, the amphipods were placed in aquaria with free leeches, which attached to the new hosts during 30 min.

Four hours post injection we anesthetized the amphipods in clove oil suspension (50 µL of clove oil per 50 mL of Baikal water) and detached leeches and two pieces of gills from each individual for further observation under an inverted fluorescent microscope Celena S (Logos Biosystems, Republic of Korea). Prior to the visualization, the leeches were placed into sterile 1.5-mL microtubes and homogenized with 50 µL of phosphate buffered saline using a plastic pestle.

Hemolymph extraction of characterization of hemocytes

Before the hemolymph extraction, the dorsal side of the amphipod pereon surface was always sterilized with 70% ethanol. The central hemolymph vessel was punctured with a sterile needle, and hemolymph was collected with a sterile glass capillary. The amphipod hemolymph was mixed (1:1) with the isotonic anticoagulant solution (150 mM NaCl, 5 mM Na2HPO4, 30 mM sodium citrate, 10 mM EDTA, pH 8.0; filtered through a 0.45 µm syringe filter) on ice to avoid degranulation of granulocytes [17]. Amphipod hemolymph was always extracted before detachment of leeches. Hemocytes were visualized using the Celena S inverted microscope (Logos Biosystems, Republic of Korea) or the Mikmed-2 microscope (LOMO, Russia). Total hemocyte count (THC) and granulocyte percentage was estimated in disposable hemocytometers (Aptaca, Italy).

Characterization of hemocyte types was performed with a CytoFLEX flow cytometer (Beckman Coulter, USA, CA). Hemolymph of 8 non-infected amphipods E. verrucosus was extracted and measured for forward (allows for the discrimination of cells by size) and side scatter (gives the information about cell complexity).

Biochemical measurements of phenoloxidase activity and glycogen content

Along with estimation of THC, part of infected and non-infected animals collected in October 2022, February or April 2023 were used for measurements of phenoloxidase activity and glycogen content. For phenoloxidase measurements, hemolymph was collected between the 7th and 8th segments of mesosome as described above, mixed 1:1 with a buffer solution (150 mM NaCl, 10 mM $Na2HPO4$, 7 mg/mL phenylmethanesulfonyl fluoride, pH 8.0) and frozen at -80 °C. The samples were melted at 4°C and centrifuged for 10 min at 500 g and 4°C to precipitate the cellular pellets. 10 µl of hemolymph extract were mixed with 40 µl of buffer solution, 280 µL of distilled water, and 40 µl of 4 mg/ml 3,4-dihydroxy-L-phenylalanine. Measurements were performed with the CLARIOstar Plus microplate reader (BMG Labtech, Germany) at 490 nm (absorbance) for 40 min. Activity of phenoloxidase was assessed in arbitrary units as the slope of the reaction curve during the linear phase [17].

Glycogen extraction was performed as described previously [34] with modifications. Frozen amphipod tissues (after hemolymph extraction) were ground into a powder, mixed with the solution (0.5 mL per 100 mg of wet weight) containing 0.6 M $HClO4$, and further homogenized in a Potter-Elvehjem tissue grinder until no visible particles remained. Next, 20 µL of the homogenate was mixed with 75 µl of 1% amyloglucosidase (10115-5G-F, Sigma-Aldrich, Germany; 5250 U/µL) in a 0.2 M acetic acid buffer (acetic acid/sodium acetate; pH 4.8). The mix was incubated at 40°C for two hours and then 62.5 µl of 0.6 M $HClO4$ and 100 µl of 1 M $KHCO3$ were added. The supernatant was centrifuged at 13000 rpm for 15 minutes. Glycogen concentration was measured with the kit "Glucose-Vital" (Vital Development, Russia), 40 µl of experimental sample was added to 190 µl of "Glucose-Vital" monoreagent and incubated at 25 °C for 15 minutes. Light absorption was measured at 510 nm with a CLARIOstar plus microplate reader (BMG Labtech, Germany).

Assessing encapsulation of Sephadex beads by amphipod hemocytes in primary culture

Sephadex microbeads (G100120-50G, Sigma-Aldrich, United States) were washed with 5 mg/ml streptomycin and 5000 U/mL penicillin solution (1.3.18, Biolot, Russia). The bead suspension was pipetted into a sterile 96-well plate (GT204-0096DV, Minimed, Russia) in a laminar flow box. Then, 100 µL of complete medium 1X L-15 (Leibovitz medium with L-glutamine, L4386-10X1L, Sigma-Aldrich, United States) containing 15% fetal bovine serum (FBS-HI-11A, Capricorn Scientific, Germany) was added, and the beads were imaged under Mikmed-2 microscope (LOMO, Russia) with attached EOS 1200D camera (Canon, Taiwan) and counted using the Count Things application (CountThings.com). Since the amount of microbeads per well varied substantially, for further tests we used only the wells with approximately the same number of beads (on average 230±70 beads per well).

Hemolymph was extracted from 10 leech-free and 12 leech-infected amphipods (collected in April 2023) as described above and was pooled within each group. 10-μL aliquotes were collected from the pools to estimate the hemocyte concentrations. Then, each pool of hemolymph was divided to the selected wells with microbeads contolling for the equal amounts of hemocytes for leech-free (15 wells) and leech-infected (11 wells) groups (on average, $1 \pm 0{,}4 * 10^5$ cells per well).

After cell sedimentation to the well bottom, the upper layer of the suspension was collected, and 100 μL of fresh medium was added. The hemocyte response to the Sephadex microbeads was analyzed after 24 hours of incubation, and the number of microbeads with hemocyte aggregates was counted under the Celena S inverted fluorescent microscope (Logos Biosystems, Republic of Korea). We categorized 4 stages of the encapsulation reaction: no reaction, low reaction, medium reaction, the stage showing partially encapsulated beads, and the intense reaction showing fully covered beads (Figure 1C,D). The hemocyte nuclei were stained with 10 μg/mL 4′,6-diamidino-2-phenylindole (DAPI, A4099, AppliChem, Germany) to visually contrast the encapsulation reaction (Figure 1D). Cell viability was assessed by staining with 1 μg/mL propidium iodide (81845-100MG, Sigma-Adrich, Germany).

Artificial infection of amphipods with leeches and bacteria

In this research, we used the bacterial strain *Pseudomonas* sp. H5-2 (belongs to the *P. fluorescens* species group) that was previously extracted from the hemolymph of *E. verrucosus* collected in the same location [17]. For the cultivation, we used the tryptic soy broth (TSB) medium (casein peptone, dipotassium hydrogen phosphate, glucose, NaCl, soy peptone) as suggested previously [35,36]. For injection into amphipods, *Pseudomonas* sp. cells were washed by centrifugation and resuspended in physiological solution (150 mM NaCl, 10 mM Na_2HPO_4). 2.5 μL of physiological solution with 10^5 *Pseudomonas* sp. cells per 1 μL was injected into the central hemolymph vessel of amphipods between 5th and 6th segments with an IM-9B microinjector (Narishige, Tokyo, Japan).

After ~15-30 min, the amphipods with and without the bacterial injection were infected by leeches as described above with 1:1 parasite to host ratio. After 1.5 hours, 1 and 3 days hemolymph was extracted from amphipods and mixed 1:1 with anticoagulant solution (150 mM NaCl, 5 mM Na_2HPO_4, 30 mM sodium citrate, 10 mM EDTA, 50 mM EDTA-Na2, pH 8.0). This adjusted anticoagulant solution allows to fix hemocytes in the state when nulei and granules are visible more clearly [37] and was applied to later visually distinguish granulocytes among all hemocytes. THC and granulocyte proportion were estimated under the Mikmed-2 microscope (LOMO, Russia) in a glass hemocytometer.

All experimental groups for 1.5 h and 1 day timepoints included 10 animals per group and showed no mortality both with and without bacterial infection (since hemolymph samplings always failed for part of animals, the number of analyzed hemolymph samples could be reduced down to 7 for some groups). The first round for 3-day timepoint also included 10 animals per each experimental group but showed high mortality specifically for animals injected with bacteria (60% for leech-free and 50% for leech-infected) with no mortality for amphipods without injection. Since this high mortality could be an artifact of specific injection procedure, we performed the second round of the experiment with bacterial injection into 9 animals per experimental group. Both leech-free and leech-infected animals showed no mortality during 3 days post injection, and their hemolymph was used for the tests along with hemolymph of the animals from the first round.

Statistical analysis

Statistically significant differences between experimental groups were always estimated using Mann-Whitney U test with the Holm's correction for multiple comparisons. Mann-Whitney tests were performed in the program Past 4.03 [38], and Holm's corrections were applied in R v.4.3.1 [39]. The differences were considered statistically significant with $p < 0.05$.

Specifically for the experiment with artificial infections with bacteria and leeches we applied a generalized linear model (GLM) for analysis of factor effects in R v.4.3.1 [39]. The model was fitted using the glm() function with gaussian distribution to three independent factors (time as numeric variable, absence or presence of leech and injected bacteria) and all of their interactions. The assumptions for GLM were mostly met for the dataset: the outcome with time was acceptably linear (slightly violated specifically for THC), the residuals were always homoscedastic and the normality assumption was slightly violated only for THC.

Results

Infection rate and identification of leeches

We collected amphipods *E. verrucosus* infected with leeches at the same location in Lake Baikal but in different seasons. The infection rate was not estimated precisely, but it clearly varied greatly. In October 2022, ~80 % of individuals were infected, while in February and April 2023 the rate was substantially lower on the order of ~5 %. There were from one to nine leeches per animal with 2-4 parasites found on most individuals.

Morphological analyses showed us that all 42 leeches sampled in these seasons belong to one genus *Baicalobdella* with prevalence of morphospecies *B. torquata*. Several leeches were identified as potentially belonging to morphospecies *B. cottidarum* but recent data [40] indicate that *B. torquata* may have significant morphological variability, so identification of these specimens remained uncertain. In order to clarify the diversity of the leeches, we performed sequencing of the COI gene fragment in 15 specimens in total; all samples with ambiguous morphological identification were included in the analysis, and the rest identified as *B. torquata* were chosen randomly from all three samplings.

The phylogenetic tree clearly showed that all 15 leeches belong to the same species (Figure 2). They were found to be approximately 90% similar to the available sequence of *Baicalobdella sp.* (#MN854837.1). However, the sequence of *B. torquata* (#MN854834) belongs to a different group. Previously, it was found that morphological species *B. torquata* may in fact be a complex of cryptic species [41], and the leeches found in our study could belong to one of them but we have no means to identify the species more precisely. However, since *E. verrucosus* was found to be infected with only one species of *Baicalobdella* locally, we had the possibility to test the physiological influence of these leeches onto the amphipods.

Leeches *Baicalobdella* sp. consume amphipod hemolymph

The assumption that the leeches attached to gills of amphipods also feed on their hemolymph is obvious, but these ectosymbionts may simply be in phoretic relationships with specifically these hosts. In order to test this assumption, we injected fluorescent microbeads into the hemolymph of *E. verrucosus* and tracked their distribution. Five hours post injection the microbeads were easily observable in amphipod gills and also inside some ciliates that were found to be attached to gills (Figure 3A,B). The homogenate of 5 out of 10 tested leech bodies also contained these fluorescent microbeads (Figure 3C). Since the leech oral apparatus is not suitable for consumption of ciliates [22], our data unambiguously confirms that the leeches *Baicalobdella* sp. indeed can feed on hemolymph of amphipods *E. verrucosus*.

Characterization of amphipod hemocytes

Since hemocytes are an important component of the crustacean immune system, before further analysis we investigated their possible subdivision into populations. Flow cytometry clearly differentiated hemocytes of *E. verrucosus* into two main groups, one with smaller cell size and lower internal complexity and the other with larger cell size and higher internal complexity (Figure 4). The groups are usually called hyalinocytes and granulocytes respectively [42] and can also be observed with common phase contrast microscopy. In particular, larger size of granulocytes is evident right after the sample is placed under the microscope, while higher amount of vesicular structures in granulocytes is better visualized after attachment to the surface (Figure 4). Additionally, we observed hemocytes with intermediate internal complexity and size between granulocytes and hyalincytes, i.e. semi-granulocytes, but their proportion was only ~10%.

Influence of leeches on hemocyte concentration and other parameters of amphipods in different seasons

We used the amphipods collected in October 2022, February and April 2023 for discriminating the effects of leech infection on several physiological parameters of *E. verrucosus* in different seasons. The consumption of hemolymph by leeches may directly reduce the hemocyte concentration and phenoloxidase content in the hemolymph and indirectly reduce the available glycogen due to the compensation of the tissue loss.

Total hemocyte count (THC) varied with season, but leech-infected and non-infected amphipods never had a statistically significant difference in this parameter (Figure 5A). Moreover, median THC in February 2023 was even slightly higher in infected than in non-infected animals despite the fact the size of leeches was the largest (Figure 5B,C) and hemolymph consumption would be expected to be the highest. It could be hypothesized that hemolymph consumption, on the contrary, lowers specifically for the largest leeches that are ready to switch their host. Interestingly, there was indeed a statistically significant difference between infected amphipods in October and February (Figure 5A), which partially supports this hypothesis. Since most amphipods were infected with 2-4 leeches, we could not check the correlation between THC and leech size directly, but the dependence between THC and summarized leech width per host was absent or even indicated slightly higher THC in amphipods with higher biomass of leeches (Figure 5D). In general, these data show that influence of leeches on THC is either negligible or the hemocyte loss is compensated by the host.

Similarly, phenoloxidase activity of *E. verrucosus* hemolymph (analyzed only in April 2023) was not significantly different between infected and non-infected amphipods (Figure 5E).

Finally, glycogen content was also almost identical between the groups, which indicates no prominent energetic burden of the infection.

Overall, our data suggest that infection with leeches does not have substantial deleterious effects for amphipods *E. verrucosus* at least in the analyzed seasons.

Cellular immune response of infected and non-infected amphipods estimated *in vitro*

Despite the fact that leeches did not substantially affect the amounts of immune components in amphipod hemolymph, they might modulate intensity of the immune response through bioactive components in their saliva. For preliminary testing of this hypothesis, we chose the primary culture of amphipod hemocytes as a convenient model system and Sephadex microbeads (consisting of specifically processed dextran) as model foreign bodies. The primary hemocyte culture allows for observing the behavior of these immune cells and quantitative estimation of their reactions such as aggregation and further encapsulation of foreign bodies.

In particular, we measured the fraction of Sephadex beads encapsulated by hemocytes originally extracted from leech-infected and non-infected amphipods 24 h after contact with the beads. This time point was previously shown to be enough for development of strong immune reaction even to artificial non-microbial foreign bodies [43]. We found no difference in the intensity of the immune reaction between the experimental groups since the proportions of fully encapsulated (~10%) and partially encapsulated microbeads (~85%) were equal for hemocytes from infected and non-infected amphipods (Figure 6A). Some of the beads were not encapsulated at all, and there was a high mortality of hemocytes around Sephadex microbeads in contrast to free hemocytes, as indicated by propidium iodide staining (Figure 6B).

However, the humoral components of hemolymph were mostly removed during hemocyte transfer into culture medium (dilution was ~12x), which could alleviate the potential influence of leech infection during the experiment. Thus, here we could not fully exclude the possible minor effects of *Baicalobdella* sp. saliva on the intensity of cellular immune response in amphipods of Lake Baikal.

Changes in hemocyte concentration and composition after injection of bacteria and artificial leech infection

Finally, in order to evaluate the potential synergistic effects of leeches and other immunity-related factors we experimentally tested the influence of leeches on the ability of amphipods to deal with bacterial infection. In this study we performed (i) an artificial infection of leech-free *E. verrucosus* with the *Pseudomonas* sp. strain originally extracted from hemolymph of the same species and (ii) an artificial infection with leeches. The amount of injected bacterial cells was comparable to the number of circulating hemocytes in the animal hemolymph to model a significant microbial outburst. Some of the amphipods were then infected with one leech per animal, and such parameters as hemocyte concentration in the hemolymph and the fraction of granulocytes were evaluated for three days (Figure 7; Table 1).

The mortality during the three-day experiment was mostly low, and it never was higher for leech-infected animals than for leech-free ones. The generalized linear model indicated that infection with leeches itself and time after the infections had no statistically significant effects on both the concentration of hemocytes in amphipod hemolymph and the fraction of granulocytes among them, while the injection of bacteria clearly leads to a statistically significant decrease in hemocyte concentration by ~2800 cells per µl on average and an increase in granulocyte proportion by ~16% (Table 1). Interestingly, the interaction between bacterial injection and infection with leeches, oppositely, led to a statistically significant decrease in the fraction of granulocytes by 12% but caused no statistically significant changes in hemocyte concentration (Table 1). Other interactions between factors even being statistically significant in the case of granulocytes percentage did not exceed 1% by module in the estimated effect. However, pair-wise comparisons between leech-free and leech-infected animals gave no statistically significant differences between any experimental groups not only in hemocyte concentration but also in granulocytes fraction during the whole experiment (all adjusted $p > 0.05$; Figure 7). Thus, the effect of leech infection partially compensating the effect of bacterial infection specifically for hemocyte composition deserves further attention, but overall our results clearly demonstrated no synergistic interaction between these two factors.

Discussion

Our research group focuses on environmental physiology of the amphipods endemic to Lake Baikal, and almost exclusively the previously published experiments were made with amphipods without visible leech infection [44,45,46,47] since the infected individuals were considered as potentially weakened. Here we questioned this assumption.

Sequencing of leeches from *E. verrucosus* collected in three independent sampling campaigns clearly demonstrated that the parasites in the used sampling location belong to the same species, and thus their influence on these amphipods can be studied without preliminary species identification. Since the phylogeny and diversity of leeches in Lake Baikal are still being revised [48,20,49,50], we could not identify the species precisely but it belongs to the genus Baicalobdella (Figure 2). Our tests also showed that the leeches can consume amphipod hemolymph (Figure 3), and thus the effects of the infection on amphipod physiology are worth studying. However, within the 4-hour experiment, only a half of the artificially attached leeches consumed hemolymph, and according to our observations, in nature the leeches were mostly attached to gills with their posterior sucker, which indicates that these parasites do not consume hemolymph constantly.

The studies investigating the host-symbiont relationships of amphipods commonly use such techniques as histological analysis, spectrophotometry, metagenomics, PCR and microscopy, while such an important component of the immune system as hemocytes is rarely mentioned [25]. Rigaut and Moret studied phenoloxidase activity of *Gammarus pulex* (Linnaeus, 1758) and *G. roeselii* and found a correlation between infection by acanthocephalans and a decrease in the enzyme activity [51]. Another freshwater amphipod *G. fossarum* (Koch, 1835) was used as a test organism to investigate potential pollutant-parasite interactions for infection with larvae of the acanthocephalan species *Polymorphus minutus* (Zeder, 1800). Phenoloxidase activity, glycogen, and lipid concentrations showed a significant increase in infected gammarids [52]. In the other case acanthocephalan infection was associated with reduction of the phenoloxidase activity and the hemocyte concentration [53].

Here we were looking for any substantial effects of leech infection on hemocytes and other related parameters of amphipods. Most studies on crustacean hemocytes have been performed for decapods and revealed three main types of these immune cells with different morphology and functions: hyalinocytes (hyaline cell), semi-granulocytes (semi-granular cells) and granulocytes (granular cells) [42]. Such information for amphipods is less abundant. Using light and electron microscopy the following hemocyte types were found in the body of the amphipod *G. setosus* (Dementieva, 1931): granulocytes, adipohemocytes, plasmatocytes, and rare prohemocytes [54]. In the hemolymph of the amphipod *G. pulex* four types of circulating cells were identified with microscopy and histochemical staining: hyalocytes I (cells with a transparent cytoplasm), hyalocytes II (cells with a slightly basophilic cytoplasm), granulocytes, and adipohemocytes (with large nucleus surrounded by granules) [55]. In the case of *Parhyale hawaiensis* (Dana, 1853), it was shown that hemolymph contained tree typical type of hemocytes: granulocytes, semi-granulocytes and hyalinocytes with semi-granulocytes being rare [56]. Our research on *E. verrucosus* seems to be the first or among the first studies checking amphipod hemocyte diversity with flow cytometry, which demonstrated the prevalence of two types of hemocytes, granulocytes and hyalinocytes, while the intermediate semi-granulocytes were found to be relatively rare (Figure 4).

We used leech-free and leech-infected amphipods from the same samplings and of similar size in order to compare the concentration of hemocytes in hemolymph, phenoloxidase activity and glycogen content and found no influence of leeches on these parameters (Figure 5). Hemocyte concentration varied greatly with sampling campaigns and could be partially influenced by the reproduction season, which starts in autumn for *E. verrucosus*. Interestingly, the infection rates were also very different in different months (dropped from ~80% to ~5% from October to April), which indirectly indicates that the same individual of this species can be infected with different leeches multiple times during their lifespan of about five years. A previous transcriptomic study indicated that even *E. verrucosus* without visible leech infection sometimes can bear the parasites, so the mentioned values might be an underestimate [44]. However, the infection seems to substantially affect neither immune defense, nor energy budget of the animal. This discovery sheds some light into the host-symbiont relationships of *E. verrucosus* and *Baicalobdella* sp. showing that the infection with leeches is probably more natural for large amphipods of Lake Baikal than we would assume.

Next, we checked for potential influence of leech saliva on reaction intensity of hemocytes to artificial foreign bodies in the primary cell culture. Hemocytes from leech-free and leech-infected animals demonstrated the same results (Figure 6), but since humoral components of hemolymph were diluted for ~12 times during extraction into the primary culture, we can only exclude a very intense influence of the saliva components. Finally, we checked for potential synergistic interaction, of leeches with artificial bacterial infection and found no or even a slight antagonistic interaction, as indicated by the estimates of granulocyte fraction among all hemocytes (Figure 7; Table 1). Artificial infection with leech did not influence hemocyte concentration or granulocyte percentage in the amphipod hemolymph at all, while injection of bacteria clearly decreased the first and increased the second (Figure 7; Table 1). The decrease in THC was expected from a number of studies [57,58,59,60]. The increase in fraction of granulocytes among all hemocytes probably reflects high mortality of hyalinocytes during the immune response to bacteria but possible discharge of granulocytes from some tissues also cannot be excluded. The antagonistic interaction of leech infection with bacterial injection specifically in the case of the granulocyte fraction among all hemocytes might be speculatively explained by a potential decrease in the concentration of bacteria due to hemolymph consumption by the leech, but this effect clearly demands further exploration.

An unexpected finding of our research was the discovery of numerous parasitic ciliates on the gills of *E. verrucosus* that clearly consumed amphipod hemolymph (Figure 3B). It is known that ciliates of the genus *Lagenophryidae* can seat on gills of amphipods *E. verrucosus* [61]. However, their potential influence on the amphipods is a subject for a separate research.

Overall, our study indicated no substantial influence of leeches on the amphipods *E. verrucosus* from Lake Baikal. Therefore, the individuals infected with *Baicalobdella* sp. can or sometimes even should be included into ecophysiological experiments for performing them on the representative part of the population.

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Figure 1

Representative photos of the research objects.

(**a**) Photo of amphipod *Eulimnogammarus verrucosus* with a leech attached to its gills indicated by the black arrow. (**b**) Microscopic photo of a leech with amphipod gill after detachment. (**c,d**) Hemocytes of *E. verrucosus* and stages of their encapsulation reaction to Sephadex beads after DAPI staining. c, bright field channel; d, DAPI channel of the fluorescent microscope. Pink arrows, no response; green arrows, low response; dark blue arrows, intermediate response; red arrows, intense response.

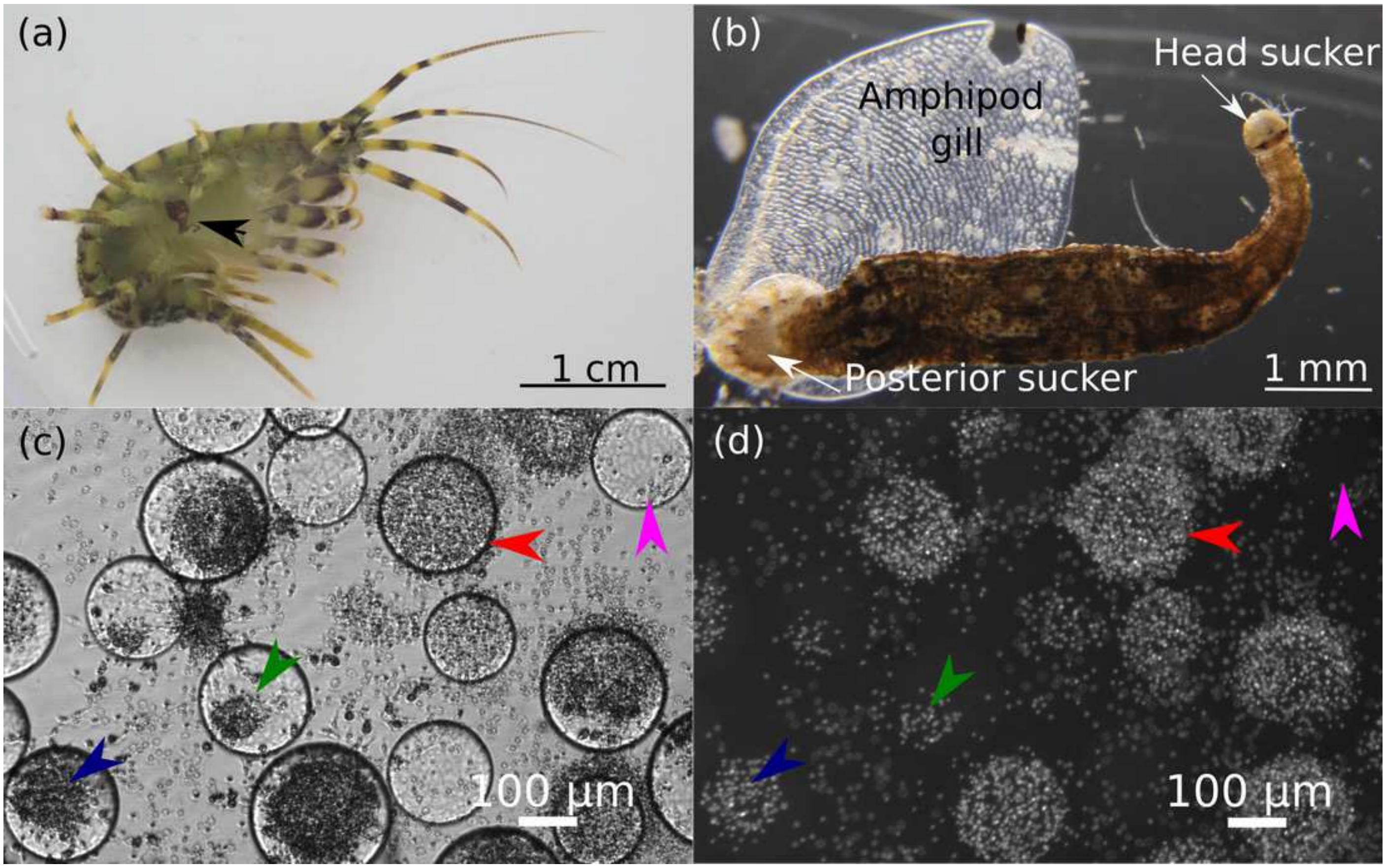

Figure 2

Phylogenetic tree of partial COI gene sequences of leech samples detached from amphipods *E. verrucosus* collected in Baikal littoral zone nearby Listvyanka village (highlighted in blue) and sequences of other closely related leeches.

The numbers next to the nodes mean percent of their ultrafast bootstrap support.

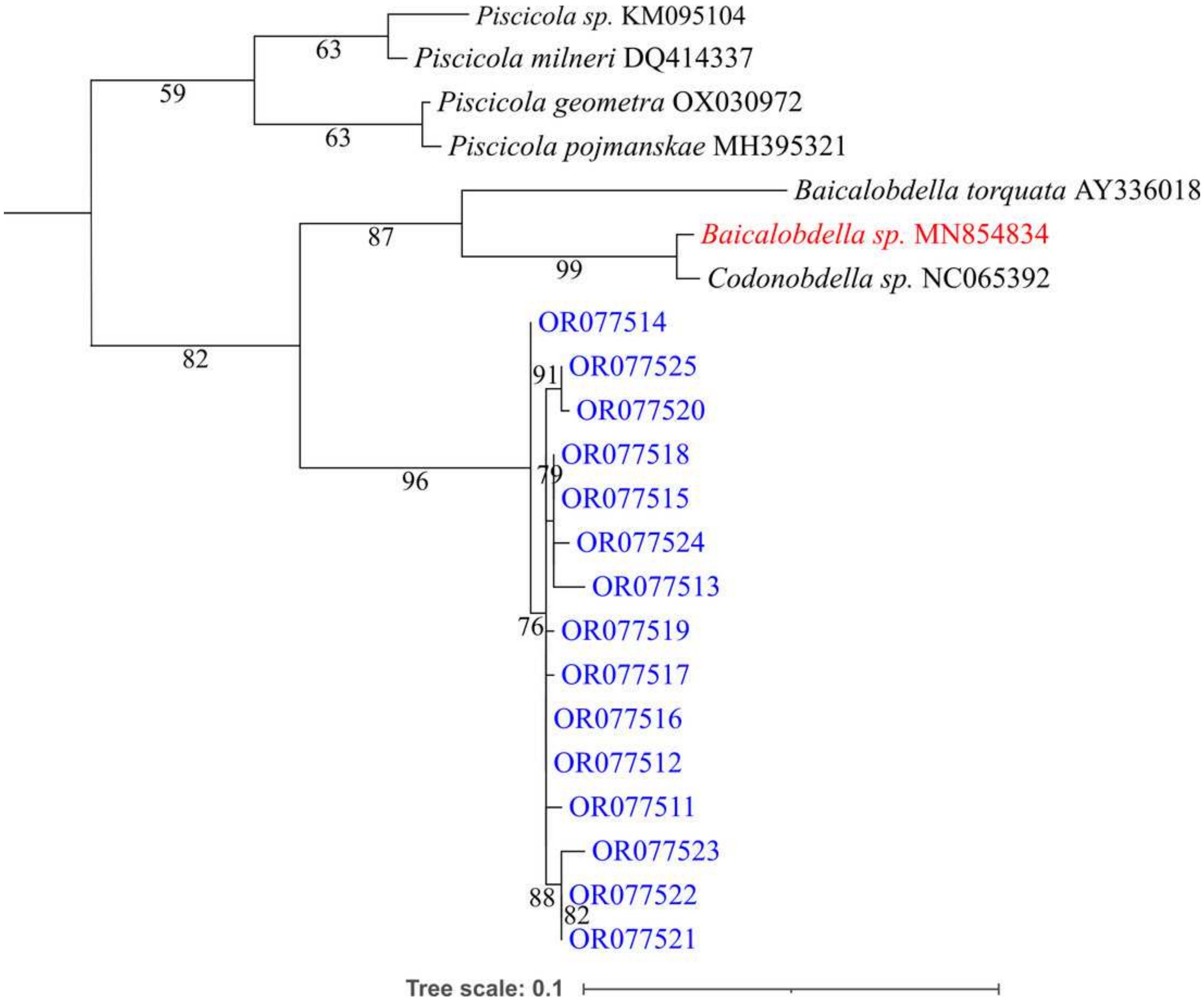

Figure 3

Distribution of latex microbeads 5 hours after injection into the central amphipod hemolymph vessel.

(**a**) Amphipod gill with latex microbeads and hemocytes. (**b**) Ciliate cells on the surface of gills with the microbeads inside them. (**c**) Content of leech body with latex microbeads. The pictures are merged photos obtained in brightfield and RFP channels with the same camera settings. Red arrows, latex microbeads; blue arrows, ciliates with microbeads inside.

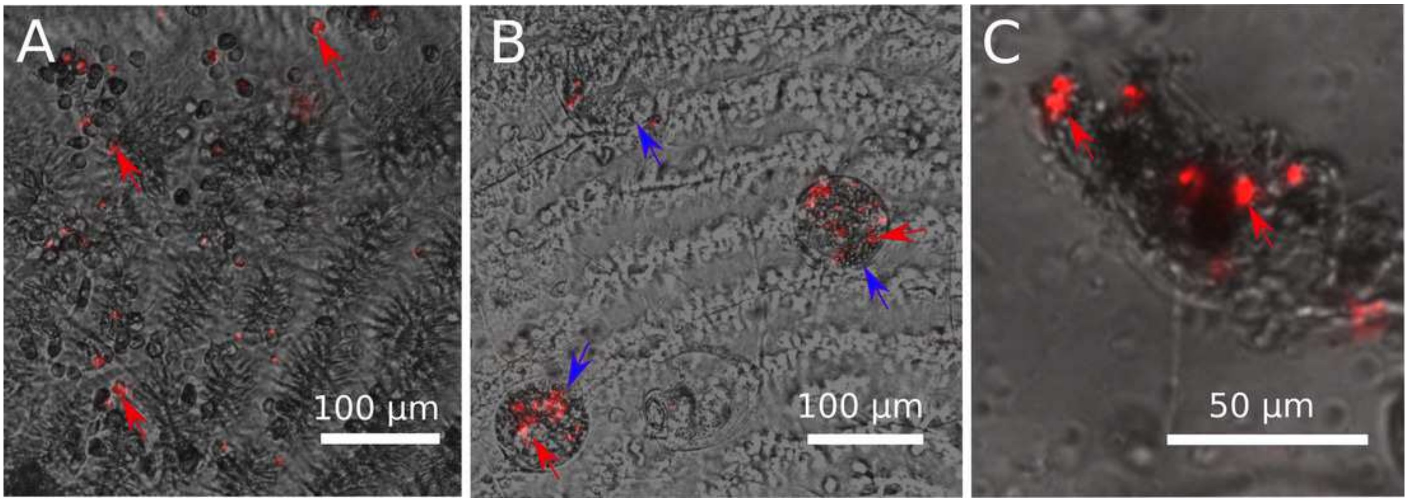

Figure 4

Characterization of *E.verrucosus* hemocytes using flow cytometry and microscopy.

Left panel shows the internal complexity (side scatter, SSC) against the cell size (forward scatter, FSC) of hemocyte populations (hyalinocytes and granulocytes), and other panels depict their respective phase contrast photos before (center) and after (right) attachment to a glass surface.

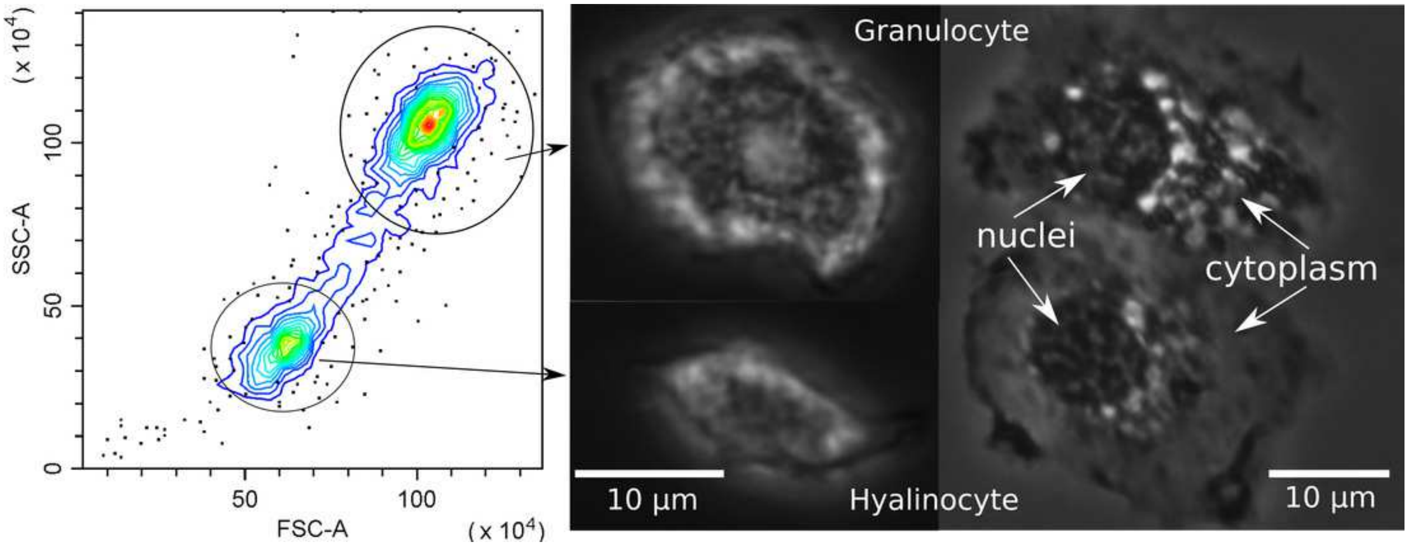

Figure 5

Different parameters of leech-infected non-infected amphipods *E.verrucosus* collected from natural environment and the leeches in different seasons.

(**a**) Total hemocyte count of infected and non-infected *E.verrucosus* collected in different seasons (n = 5-10). (**b**) Width of leeches in the middle of the body in different seasons. (**c**) Representative photos of leeches detached from *E.verrucosus* in different seasons. (**d**) Dependence of hemocyte count on summarized width of all leeches infecting the animal. The difference of the regression coefficient from zero is not statistically significant (p = 0.34). (**e**) Phenoloxidase activity in hemolymph of leech-free and leech-infected *E. verrucosus* collected in April 2023 (n = 10). Color legend is identical to panel (a). (**f**) Amount of glycogen in leech-free and leech-infected *E. verrucosus* collected in October 2022, February and April 2023 (n = 10-11). Color legend is identical to panel (a).

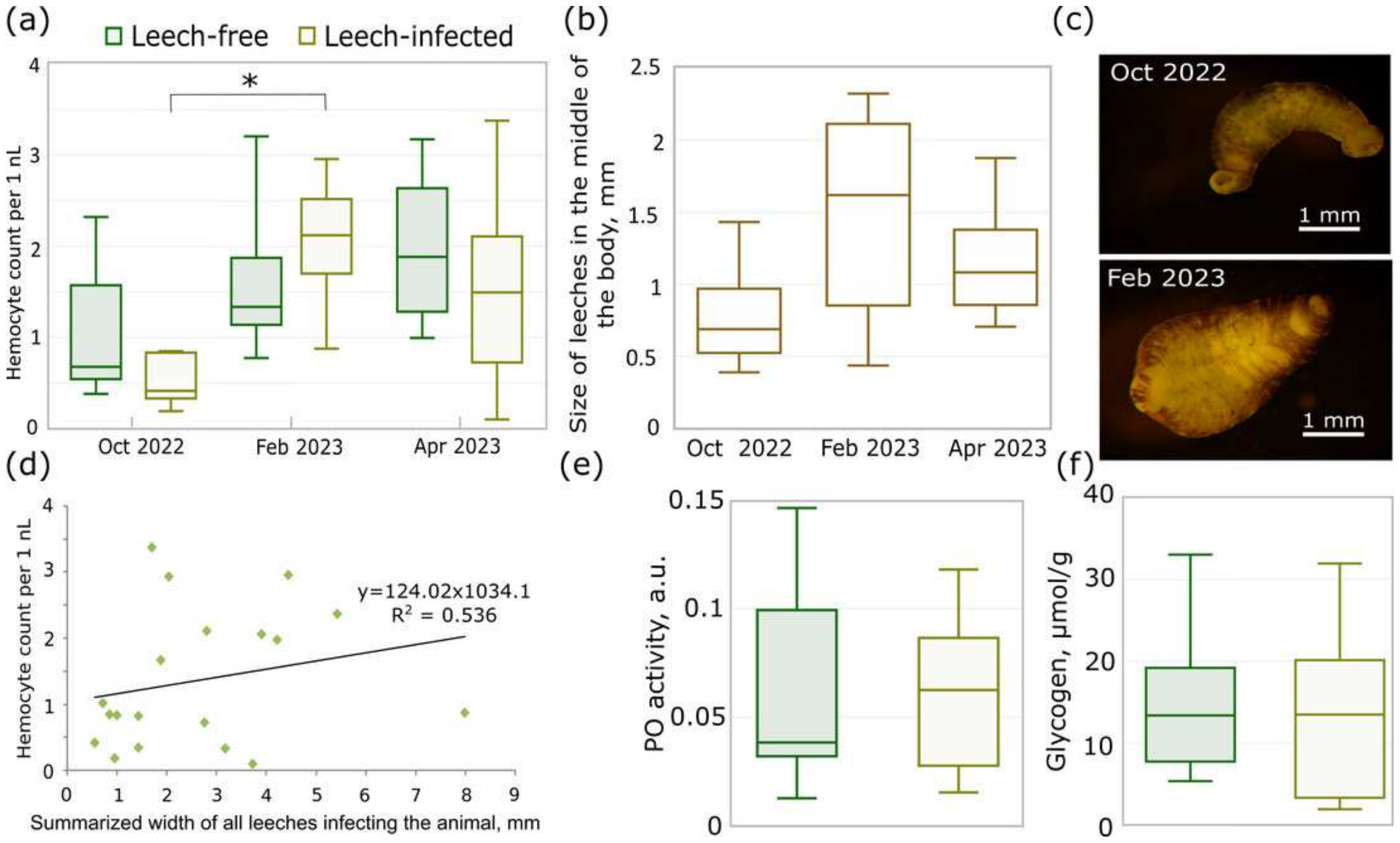

Figure 6

Intensity of the cellular immune response of hemocytes extracted in primary culture from leech-free and leech-infected amphipods *E. verrucosus*.

(**a**) Fractions of Sephadex microbeads partially and fully encapsulated by hemocytes after 24 hours of contact. (**b**) Example photos of microbeads' encapsulation in hemocyte primary culture, propidium iodide staining. Pink arrows — Sephadex microbeads, red arrows — aggregates of hemocytes. Photos in RFP channel were obtained at the same camera settings in different groups, but time of staining could be different.

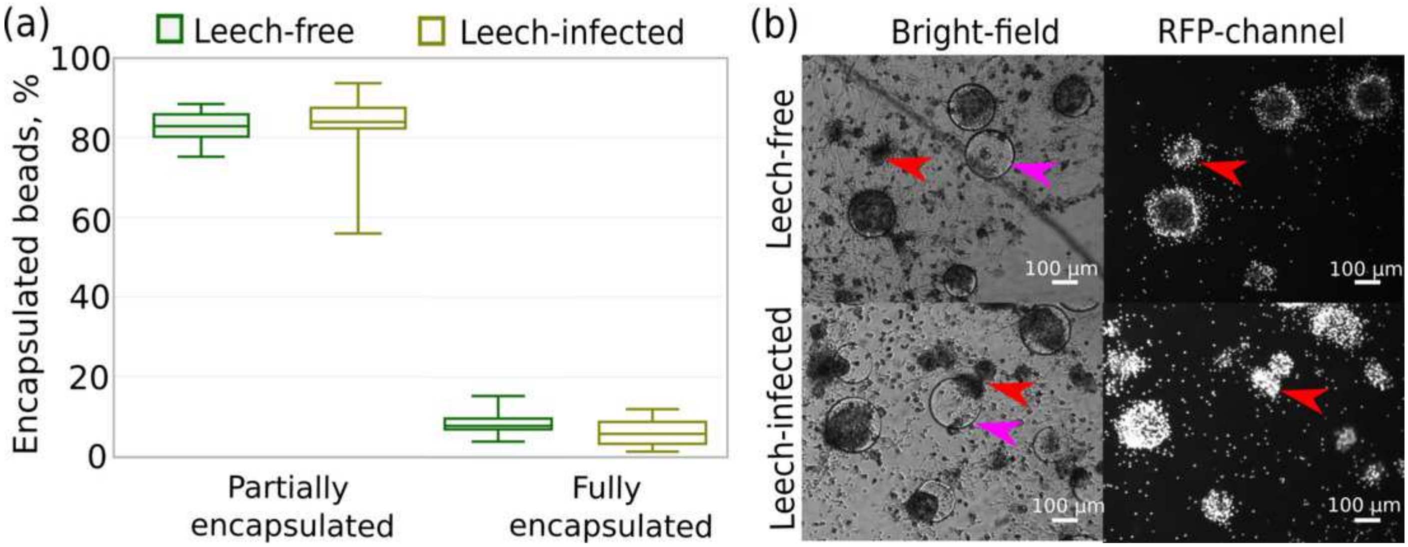

Figure 7

Immune cells in the hemolymph of leech-free and artificially leech-infected amphipods with and without bacterial injection.

(**a**) Total hemocyte count (n = 7–13). (**b**) Granulocyte fraction among all hemocytes (n = 7–13). The legend is identical for (a) and (b). Injection of bacteria to amphipod central hemolymph vessel was performed about 15 minutes before leech infection. Pair-wise comparisons of leech-free and leech-infected animals in each time point with Mann-Whitney U test with Holm’s correction for multiple comparisons gave no statistically significant differences both for amphipods with and without bacterial infection.

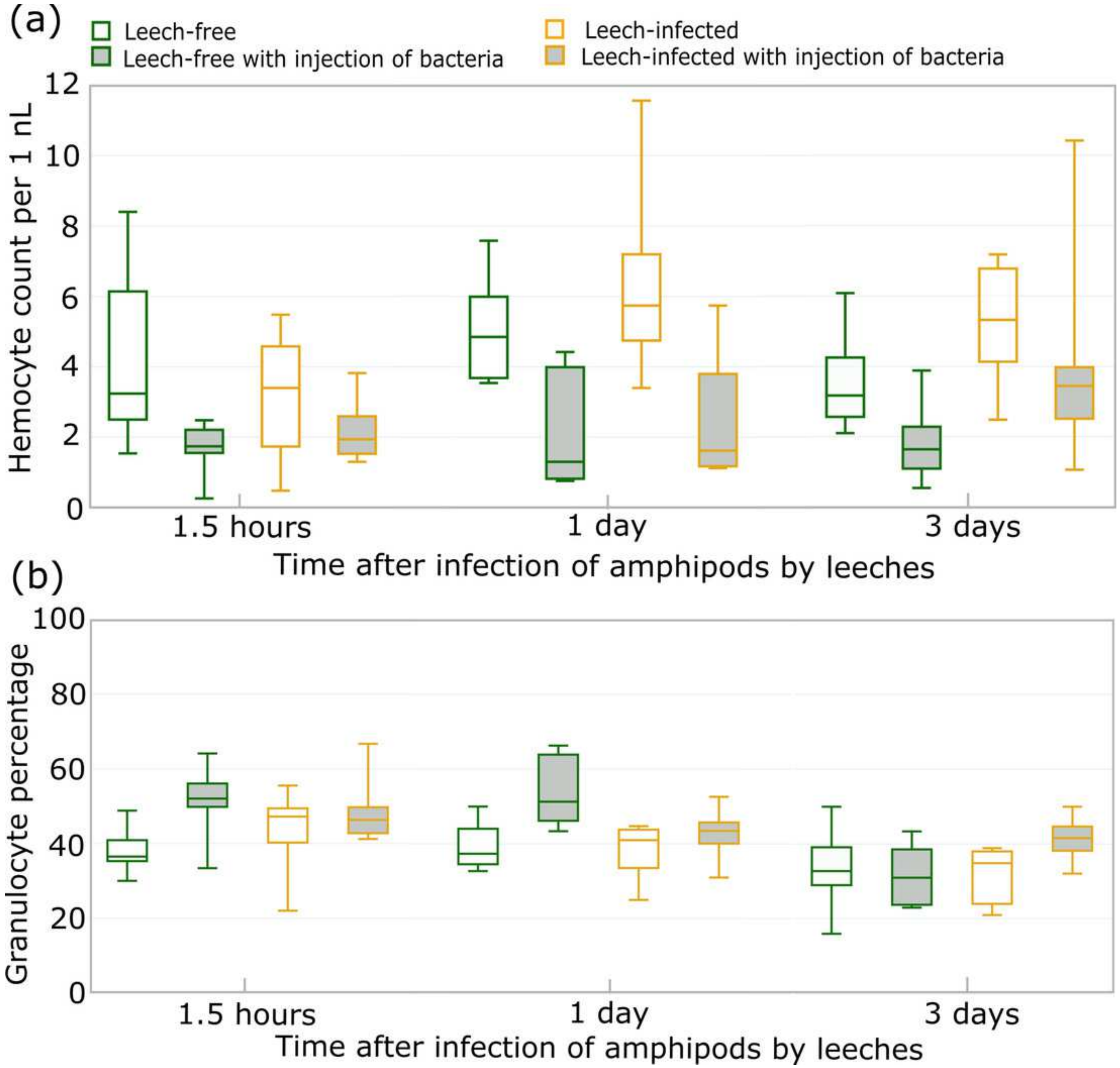
(a)
Leech-free
Leech-free with injection of bacteria
Leech-infected
Leech-infected with injection of bacteria
Hemocyte count per 1 nL
0
2
4
6
8
10
12
1.5 hours
1 day
3 days
Time after infection of amphipods by leeches
(b)
Granulocyte percentage
0
20
40
60
80
100
1.5 hours
1 day
3 days
Time after infection of amphipods by leeches

Table 1(on next page)

Output of generalized linear model with the Gaussian distribution fitted to total hemocyte count and granulocyte percentage in leech-free and artificially leech-infected amphipods with and without bacterial injection (see Figure 7).

All interactions between factors were allowed, but the results only for three independent factors and their statistically significant interactions with substantial effect estimates are depicted here.

Variable	Total hemocyte count		Granulocyte percentage	
	Estimate, cells/μl	P-value	Estimate, %	P-value
Time	-15.1	0.248	-0.061	0.284
Leech	-514.1	0.537	4.149	0.256
Bacteria	-2846.1	< 0.001 ***	16.457	< 0.001 ***
Leech:Bacteria	603.3	0.601	-12.223	0.019 *