

# Influence of rusty-spotted disease on river crayfish in aquaculture

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**Abstract.** In intensive culture of river crayfish the animals are suffered from lower immune status and high risk of infection with not only pathogenic, but also opportunistic fungi and bacteria. It is important to assess the possibility of contagious disease and adaptive capacity of these hydrobionts. This paper shows the results of studying influence of rusty-spotted disease on the physiological state of freshwater river crayfish *Astacus astacus* and *Astacus leptodactylus*. It was revealed that infection was caused by fungus *Saprolegnia parasitica*. The following indicators were investigated: active reaction of hemolymph, total number of hemocytes, hemocytic formula, cytochemical indicator of lysosomal cationic protein of hemocytes. An amount of 30% of *A. astacus* and 25% of *A. leptodactylus* were immune to parasitic fungus. In disease-resistant animals of both species being in contact with *S. parasitica* the content of lysosomal cationic protein in hemocytes decreased. Probably this protein is spent in the process of immune antifungal defense. The other studied biochemical indices in infected and healthy specimens significantly differed as well.

**Key Words:** fungus *Saprolegnia parasitica*, hemocytes, hemolymph, river crayfish, rusty-spotted disease.

**Introduction.** The crustaceans are popular items of contemporary aquaculture. The breeding and rearing of crustaceans could be followed by infectious diseases and mortality so permanent monitoring of health and immune status of water animals is a way to success (Lafferty et al 2015).

High density leads to a decrease in level of oxygen and increase in concentration of organic substances and number of pathogens (Bosma & Verdegem 2011; Li et al 2019). In crustaceans in conditions of high density the phenomenon of cannibalism is observed (Borisov et al 2007) and the risk of infection by rusty-spotted disease increases (Alexandrova & Tarasov 2020).

The river crayfish (family Astacidae) are characterized by open circulatory system with hemolymph instead of blood. The indices of hemolymph reflect the state of health because the content of this fluid reacts on different physiological and pathological processes in the tissues of the animal. The features of hemolymph limit the possibilities of analysis: high degree of agglutination, destruction of hemocytes *in vitro*, the differences from blood content of vertebrates, specific enzyme systems and mechanisms of activation as consequence of open circulatory system. The hemocytes are involved in the cell response through processes such as phagocytosis, encapsulation, melanization and in humoral response through the activation of molecules known as antimicrobial peptides (AMP) (Söderhäll & Junkunlo 2019). For these reasons, studies controlling the immune system of crustaceans, including river crayfish, are based on the evaluation of immune molecules, as well as on total and differential counting of hemocytes (Söderhäll 2016; Hernández-Pérez et al 2020).

In higher crustaceans (Decapoda) classification of hemocyte types is based mainly on presence of cytoplasmic granules in hyaline cells, semigranular cells, and granular cells. Three main types of hemocytes are determined: agranulocytes or hyalinocytes (hemocyte I), semigranulocytes (hemocyte II) and granulocytes (hemocyte III). Each cell type is active in different defence reactions, for example, the hyaline cells are mainly

involved in phagocytosis, the semigranular ones participate in encapsulation, while the role of granular cells includes storage and release of the prophenoloxidase system and cytotoxicity (Jeyachandran et al 2020; Johansson et al 2020).

In our research on river crayfish *Astacus astacus* and *Astacus leptodactylus* the supplementary type of cells was identified. This type of cells (hemocyte IV) distinguished from the three mentioned above types. In process of natural destruction *in vitro*, a large oval nucleus is microscopically determined on the glass. The ratio of the nucleus-cytoplasm and the ability to phagocytosis (by the presence of pseudopodia and the detection of cationic lysosomal protein) suggested that these cells are juvenile forms of hemocyte (Pronina & Koryagina 2015). The assumption that agranular cells (hemocyte I) with capacity to proliferation could be cells-precursors was not confirmed (Martynova et al 2008).

The level of cytoplasmic cationic proteins is well-spread and highly informative indicator used in medicine (Salazar et al 2014; Carter & Lazar 2018). Cationic proteins provide high bactericidal activity in synergistic interaction with the myeloperoxidase system – hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). In human the values of this indicator change during disease. So, in patients with bacterial angina, a decrease in the content of lysosomal cationic protein is noted. The changes of index depend on intensity of disease. Therapy leads to relief of clinical symptoms and normalization of the indicator (Nagoev et al 2005). Lysosomal cationic protein is found in all types of hemocytes with higher levels in agranulocytes and semigranulocytes (Pronina & Koryagina 2015), demonstrating their potential phagocytic ability.

Disease-resistance of river crayfish depends on inherent properties. The highly virulent agent of crayfish plague (*Aphanomyces astaci*) causes epizootics and high mortality in populations of European species of river crayfish (*A. astacus*, *A. leptodactylus* etc.) in the whole areal. However in most American species like *Pacifasticus leniusculus*, *Procambarus clarkii* etc. this fungus does not cause distinct damage (Oidtmann et al 2002; Gruber et al 2014).

In the presence of a weakly virulent pathogen in water (for example, fungus *Saprolegnia parasitica*), not all individuals of the same species are infected. Therefore, the question arises: "What properties of river crayfish allow them to maintain immunity and health in the presence of a pathogen?".

The aim of this work is the study of physiological and immunological state of specimens of river crayfish affected by rusty-spotted disease and physiological features of non-infected individuals.

**Material and Method.** The experiments with wild specimens of broad-fingered crayfish *A. astacus* and narrow-clawed crayfish *A. leptodactylus* were carried out in 2010-2011 in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, ETS N°123, Strasbourg, 1986. The experimental protocol was approved by the Ethics Committee of the Moscow Timiryazev Agricultural Academy (RSAU – MTA named after K.A. Timiryazev).

The crayfish *A. astacus* (n = 40) at age between 3+ and 4+ were caught by special traps in Yasskoye Lake of Pskov region (Russian Federation) during 10 days in September 2010. The average weight of animals was 29.0±3.9 g. The crayfish were transported in two paperboard boxes (20 specimens in each one) on ice without water to laboratory. The duration of transportation was 10 hours. The period of slow hydration of animals was equal to 4 hours. After that, crayfish were stocked in aquaria with conditioned water.

The individuals of *A. leptodactylus* (n = 40) of same age as *A. astacus* were caught in August 2011 by same traps in Chepolshevskoye Lake in Tver region of Russian Federation. The average weight of animals was 64.0±6.9 g. The methods of transportation and stocking were the same ones as in previous case.

The experiments were carried out in aquaria with aeration, the volume of one aquarium was 160 L. The animals of both species (*A. astacus* and *A. leptodactylus*) were divided on two groups. Each group was subdivided on two aquaria with 10 specimens each.

The aquarium water contained opportunistic microorganisms *Saprolegnia parasitica*, capable to induce rusty-spotted disease at high stocking density.

To prevent cannibalism, aquaria were equipped with ceramic shelters for crayfish (Figure 1). The pH index varied from 6.9 to 7.3, the water temperature was maintained at the level of 16-17°C. The crayfish were fed once a day by larvae of buzzer midge *Chironomus plumosus* and charophyte green algae *Chara fragilis* ad libitum.



Figure 1. The specimen of river crayfish *Astacus astacus* near shelter on the bottom of aquarium.

Two aquaria were used for experiments with each species of crayfish (*A. astacus* and *A. leptodactylus*). The hydrochemical regime in aquaria is presented in Table 1.

Table 1

The values of hydrochemical indices during experiments

Indices	<i>Astacus astacus</i>		<i>Astacus leptodactylus</i>	
	Aquarium 1	Aquarium 2	Aquarium 3	Aquarium 4
NO <sub>3</sub> (mg L <sup>-1</sup> )	8±0.3	10±0.4	9±0.5	9±0.5
NO <sub>2</sub> (mg L <sup>-1</sup> )	1.8±0.2	1.8±0.2	2.3±0.2	2.3±0.3
PO <sub>4</sub> (mg L <sup>-1</sup> )	0.1	0.1	0.2	0.2
Ca (mg L <sup>-1</sup> )	107±4.0	107±3.0	98±4.0	98±4.0
Mg (mg L <sup>-1</sup> )	30±2.0	30±2.0	25±1.0	26±1.0
Fe (mg L <sup>-1</sup> )	0.01	0.01	0.01	0.01
pH	8.0±0.3	8.0±0.3	7.4±0.3	7.5±0.2
NH <sub>3</sub> /NH <sub>4</sub>	0.1	0.1	0	0

The water samples in all experimental aquaria contained the hyphae of fungus *S. parasitica*. Thus, the animals were held in presence of opportunistic microflora and in contact with each other.

We carried out comparative physiological and immunological characterization of healthy and infected by rusty-spot disease animals. The samples of hemolymph were taken *in vivo* by puncture of ventral sinus (Figure 2).

The content of non-enzyme cationic protein in the cytoplasm of phagocytes of hemolymph of river crayfish was determined by cytochemical method with bromphenol blue. The mean cytochemical coefficient (CCS) was estimated by Astaldi method (Astaldi & Verga 1957) of differentiated count of cells with different degree of activity (from 0 to 3 points) measured on base of intensity of a specific color (Figure 3).



Figure 2. *In vivo* selection of the hemolymph of the broad-fingered crayfish *Astacus leptodactylus* from the ventral sinus.

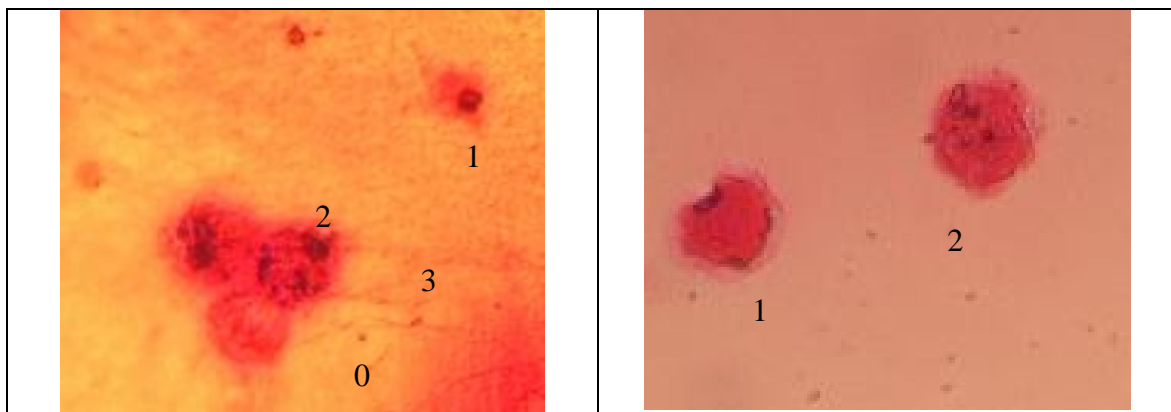


Figure 3. Hemocytes of broad-fingered crayfish *Astacus astacus* with cationic protein granules of different degree of cell activity, the numbers indicate the activity from 0 to 3 points. Magnification 400×.

Cationic protein was present in hemocytes of the following types: agranulocytes, semigranulocytes and transparent cells. This fact indicates that these hemolymph cells have phagocytic activity, especially with their digesting ability. Sometimes the cationic protein was present in small quantities in granulocytes as well.

The mean cytochemical coefficient (MCC) was calculated using the formula:

$$MCC = \frac{0 \times H_0 + 1 \times H_1 + 2 \times H_2 + 3 \times H_3}{100}$$

where:  $H_0$ ,  $H_1$ ,  $H_2$ ,  $H_3$  - number of hemocytes of river crayfish with 0, 1, 2 and 3 degrees of activity;  $H_0 + H_1 + H_2 + H_3 = 100$ .

The treatment of data was carried out by methods of variational statistics using standard Student test, differences were considered as reliable at  $p < 0.05$ .

## Results and Discussion

**The broad-fingered crayfish *Astacus astacus*.** The duration of experiment was 6 months. During this period the molting occurred in 8 animals. One animal affected by rusty-spotted disease died during molt.

The first signs of rusty-spotted disease were noted after 3 months of holding in artificial conditions in 3 individuals from aquarium 1 and 2 specimens from aquarium 2. These individuals had dark melanin spots on the carapace. In the end of the experiment we counted 14 affected individuals from 20, 6 of which died. So the number of survived

specimens in group of disease-receptive animals was 8. Only 6 specimens had no signs of rust-spotted disease and were included in group of disease-resistant individuals.

**The narrow-fingered crayfish *Astacus leptodactylus*.** In this experiment only 5 specimens were healthy in 6 months. Three disease-resistant individuals were found in aquarium 3 and another 2 in aquarium 4. The other 15 specimens had signs of rusty-spotted disease of different degree. Seven infected animals died, 8 survived. Two infected animals died during molt. Other authors also noted the mortality of crayfish infected by rusty-spotted disease (Perry & Jones 2018).

**Fungi lesion.** We studied the nature of rusty-spotted disease by methods of light microscopy using special preparations of invaded tissue exposed to 5% HCl solution during 5 minutes.

Microscopic studies of preparations showed the presence of gifs, cysts and hemes of *S. parasitica* (Figures 4 and 5). The viability of cysts was confirmed in tests with their sowing on Czapek's media.

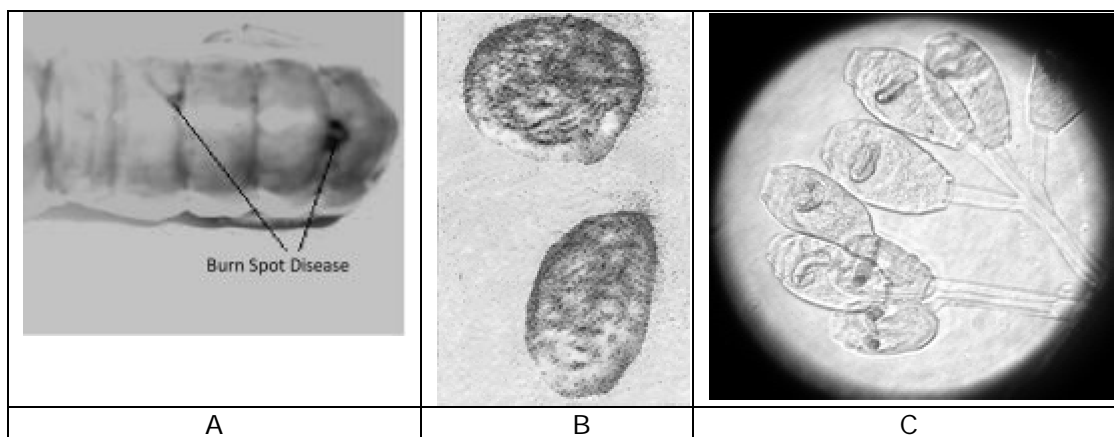


Figure 4. Melanin spot and its content in *Astacus astacus* crayfish. A - melanin spots on dorsal side of abdomen (macroscopic picture). Microscopic pictures: B - cysts of *Saprolegnia parasitica* from infected area of abdomen of the same specimen (magnification 400x). C - hemes of *S. parasitica*, from melanin spot (magnification 800x).

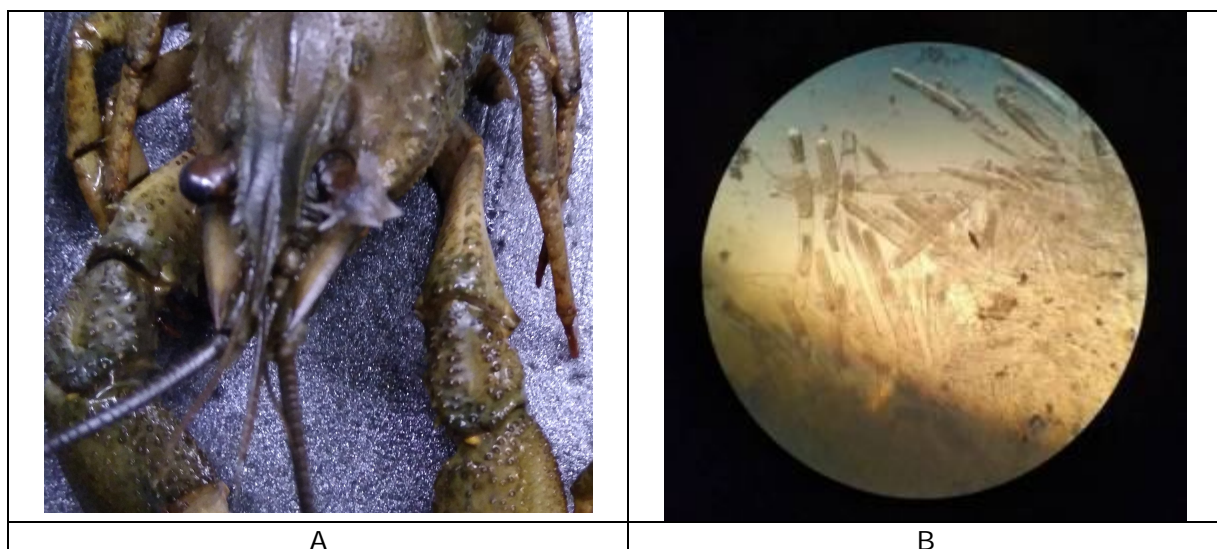


Figure 5. A – lesion of left eye in *Astacus leptodactylus* crayfish; B – gifs of *Saprolegnia parasitica* from affected area.

Studies have shown that even in case with low level of infection, molting does not remove *S. parasitica*. The same spots appeared on new cover of shell, but less pronounced because the cysts of fungus actively proliferate on new and soft tissues.

Observations on affected animals suggested that low level of lesion by *S. parasitica* at first time did not disrupt the processes of vital activity such as nutrition, breathing, molting. At the next stage of disease the destruction of the articular cuticles of the walking limbs reduced the moving activity of animals. At third stage the disruption of the third pair of maxilleped joints was followed by cessation of nutrition. A significant part of such crayfish died.

The contamination of aquatic environment with metabolic nitrogen substances during the period of food activity, especially at high stocking density, and other negative factors increased the possibility of infection of animals by fungus. Other investigators also noted higher probability of infection of crayfish (family Cambaridae) by pathogens of viral, bacterial and fungi nature when exposed to stress factors (Longshaw 2011; Fisher et al 2012).

Our studies confirm the data of other authors that inductors of rusty spot disease could be not only fungi of the genus *Fusarium* (according to the Russian classification - *Septocylindrium*), but also widespread in the Russian waters *S. parasitica*, which causes diseases in different species of water animals and affects fish eggs during incubation (Alexandrova & Tarasov 2020; Davies et al 2020).

In crayfish of both species affected by red-spotted disease the indices of cation protein (TCH) was significantly higher than in healthy ones (Tables 2 and 3).

Table 2  
Hematological and cytochemical parameters of broad-fingered crayfish *Astacus astacus*

<i>Parameters</i>	<i>Healthy animals</i>	<i>The infected by spots rusty disease animals</i>
pH hemolymph, units	4.8±0.4	5.5±0.1
THC, cells×10 <sup>6</sup> L <sup>-1</sup>	363±302	453±238
<i>Hemocyte formula, %</i>		
Hemocyte I	30.7±1.2	27.2±8.5
Hemocyte II	30.3±19.8	34.3±8.1
Hemocyte III	29.3±11.3	35.3±5.7
Hemocyte IV	9.1±8.5	3.2±1.4
<i>Hemocyte lysosomal cation test</i>		
MCC, units	0.87±0.12*	1.62±0.12*

Note: \* p < 0.05.

Table 3  
Hematological and cytochemical parameters of narrow-fingered crayfish *Astacus leptodactylus*

<i>Parameters</i>	<i>Healthy animals</i>	<i>The infected by spots rusty disease animals</i>
pH hemolymph, units	5.8±0.5	6.3±0.1
THC, cells×10 <sup>6</sup> L <sup>-1</sup>	401±49	453±238
<i>Hemocyte formula, %</i>		
Hemocyte I	41.8±1.3	29.7±8.5
Hemocyte II	28.6±1.4	34.3±8.1
Hemocyte III	25.8±2.6	32.8±5.7
Hemocyte IV	3.8±0.5	3.2±1.4
<i>Hemocyte lysosomal cation test</i>		
MCC, units	1.03±0.07*	1.84±0.05*

Note: \* p < 0.05.

In our research on river crayfish *A. astacus* and *A. leptodactylus* the supplementary type of cells was identified. This type of cells (hemocyte IV) was distinguished from the three mentioned above types. In process of natural destruction *in vitro*, a large oval nucleus was microscopically determined on the glass. The ratio of the nucleus-cytoplasm and the ability to phagocytosis (by the presence of pseudopodia and the detection of cationic

lysosomal protein) suggested that these cells were juvenile forms of hemocyte (Pronina & Koryagina 2015). The assumption that agranular cells (hemocyte I) with capacity to proliferation could be cells-precursors was not confirmed (Martynova et al 2008).

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The authors suggested that presence of exogenic factor (the inductor of rusty-spotted disease) turns on immune system of crayfish totally and immediately. Most of the cells circulating in hemolymph: agranulocytes and semigranulocytes (their content exceeds 60%) begin to phagocytize. It means that in river crayfish, the phagocytic system, as evolutionarily the most ancient, is quickly activated by pathogen invasion.

**Conclusions.** Our results suggested that total number of hemocytes, hemocytic formula, pH of hemolymph in affected and healthy individuals did not significantly differ. So it is better to use in this case the cytochemical indicator characterizing the phagocytic activity of cells. The results of research confirmed the presence of three types of hemocytes in the hemolymph of river crayfish: agranulocytes (hyaline cells) - hemocytes I, semigranulocytes - hemocytes II, granulocytes - hemocytes III. Besides a fourth type of hemocytes was identified and characterized – hemocytes IV, presumably being precursor.

The infection of crayfish by inductor of rusty-spotted disease, which was identified as the fungus *Saprolegnia parasitica*, caused a decrease in the content of lysosomal cationic protein in hemocytes. This is evidence of its involvement in the process of immune antifungal protection.

**Conflict of interest.** The authors declare that there is no conflict of interest.

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