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COMPARATIVE AND ONTOGENIC  
PHYSIOLOGY

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## Acid-Base Balance Modulates Respiratory and Alimentary Behavior of the Mollusc *Lymnaea stagnalis*

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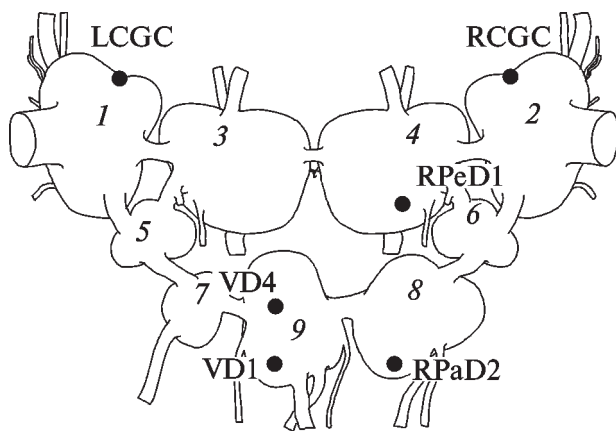
**Abstract**—An active reaction (change of pH) in hemolymph of the freshwater pulmonate mollusc *Lymnaea stagnalis* is studied in various physiological states: hunger, satiety, and alimentary excitation. In satiated animals the hemolymph pH is shifted to the acid area ( $7.6 \pm 0.003$ ) as compared with hungry animals ( $7.9 \pm 0.05$ ). The satiated individuals are characterized by an increased respiratory activity and decreased food consumption as compared with hungry animals. Acidification of the medium leads to an increase of excitability of the respiratory network interneurons (RPeD1, VD4, VD1/RPD2) and to inhibition of activity of the alimentary network interneurons (R/L CGC), whereas alkalization, to opposite effects. It is suggested that pH is one of factors coordinating activity of functional systems of *Lymnaea stagnalis* due to effect on neuronal correlates of the respiratory and alimentary behavior.

### INTRODUCTION

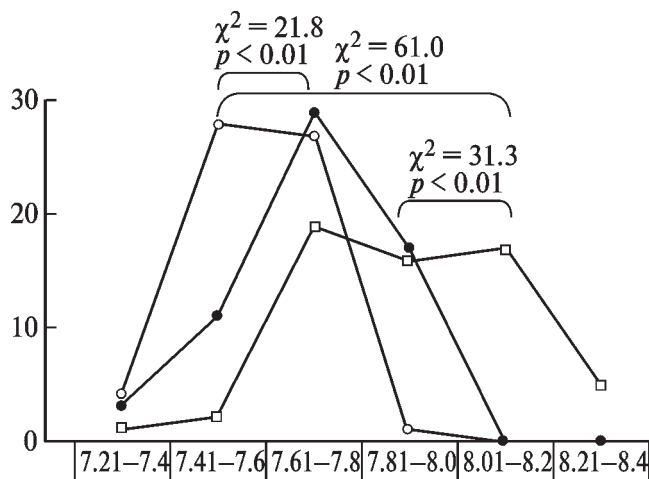
Changes of the acid-base balance of the organism internal medium immediately affect conditions of the occurrence of several metabolic and physiological reactions. Molluscs, like other invertebrates as well as several vertebrates (fish, amphibians), are characterized by significant pH fluctuations in the internal medium—hemolymph or blood. Analysis of literature data has brought attention to the fact that when performing electrophysiological studies on CNS of one of the classic objects of neurobiology, the pond snail *Lymnaea stagnalis*, different groups of researchers use hemolymph-substituting Ringer's solutions with pH values from 7.3 to 8.2 [1–3]. Experiments on cultivation of *Lymnaea* neurons *in vitro* have shown the pH fluctuations within the 0.3 unit (in the range of 7.6–7.9) to produce no effect on cell survival [4]. A preliminary performed pH-metric analysis of *Lymnaea* hemolymph samples has re-

vealed a significant dispersion of data. The assembly of *Lymnaea* individuals demonstrates different behavioral forms, the most significant of them being alimentary, respiratory, defensive, and sexual behavior. The lack of possibility of realization of at least one of them leads to death of the individual or of its possible offspring.

We have suggested the existence of an association between the hemolymph pH and the organism physiological state realized as certain forms of behavior. Disturbances of the acid-base balance are known to be of two kinds, metabolic and respiratory [5]. In this connection, our attention was focused upon analysis of changes of the internal medium pH in the process of respiration (at various stages of the pulmonary cycle) and nutrition (at different degrees of activation of the food-procuring system—the states of satiety, hunger, and alimentary excitation). We also studied reactions of interneurons of respiratory and alimentary networks of *Lymnaea stagnalis* due to a change of pH.



**Fig. 1.** Scheme of CNS of *Lymnaea stagnalis*. Location of the studied neurons is presented. The view is from the dorsal side. Buccal ganglia are not shown. The cerebral commissure is sectioned. Ganglia: (1) and (2) left and right cerebral, (3) and (4) left and right pedal, (5) and (6) left and right pleural, (7) and (8) left and right parietal, (9) visceral. Latin designations of neurons are in the text.



**Fig. 2.** Distribution of samples of the *Lymnaea stagnalis* hemolymph by pH values. Open circles and squares—satiated and hungry animals, respectively; black circle—animals in the state of alimentary excitation. Values of  $\chi^2$  and the statistical significance level ( $p$ ) are indicated. Abscissa: pH of hemolymph, ordinate: frequency of occurrence (units).

## MATERIALS AND METHODS

The work was performed on a representative of freshwater pulmonate molluscs, the pond snail *Lymnaea stagnalis*. The animals were kept in the

laboratory at the water temperature of 24–26°C and free access to food (leaves of lettuce and dandelion).

The molluscs were placed into 5-l vessels (10 individuals in each vessel) filled with sediment tap water. The food was present on the bottom of the vessel. The state of hunger was achieved by depriving the animals of the food for 24 h. The state of alimentary excitation was induced by providing hungry animals with access to food.

To study the respiratory behavior, the animals were transferred to 0.5-l vessels (one individual in each vessel) half-filled with water. The number of respiratory acts (opening-closing of the pneumostome) for 1 h of observation and duration of the respiration act were recorded. To analyze the alimentary behavior, the amount of the consumed food was determined, as described in the work [6].

The whole-body withdrawal response accompanied by release of a significant part of hemolymph [7] was produced by a strong tactile stimulation of the foot sole. The pH value of the obtained sample, 1–2 ml in volume, was determined using a PerpHecT pH-meter, Model 310 (ATI Orion, USA), at 25°C. The hemolymph was collected in satiated ( $n = 30$ ) and hungry ( $n = 30$ ) molluscs as well as in animals in the state of alimentary excitation ( $n = 30$ ) in 1 h after the access to food. One half of them were the samples taken directly before the beginning of pulmonary respiration, the other half, the samples obtained from the molluscs that completed the respiratory act and left the water surface.

Semi-intact preparations ( $n = 15$ ) were obtained by dissecting the body wall of the mollusc freed from shell from the oral orifice to the pulmonary cavity. Edges of the cut were moved apart with preparation needles to isolate the peripharyngeal ring of ganglia and the buccal mass. Esophagus and salivary gland ducts were preserved intact. Using an MBS-10 binocular ( $\times 14$ ), movements of the buccal mass and pneumostome were observed after application onto the CNS surface of the Ringer's solution for *Lymnaea* of the following composition (mM): 44.0 NaCl, 1.7 KCl, 4.0 CaCl<sub>2</sub>, 1.5 MgCl<sub>2</sub> · 6H<sub>2</sub>O, 10.0 HEPES at pH 6.8, 7.3, 7.8, 8.3, and 8.8.

Electrophysiological experiments were performed on preparations of the isolated nervous system ( $n = 11$ ) at 25°C, using the procedure described

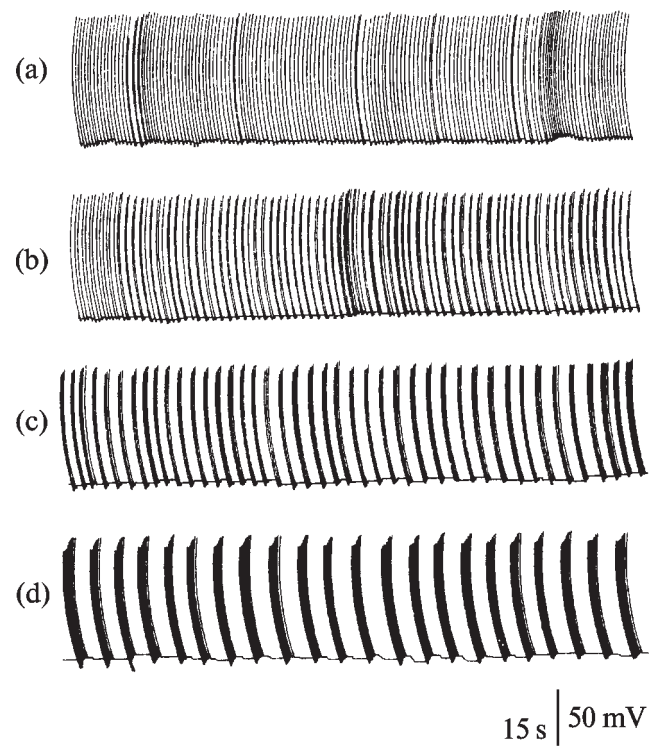
earlier [8]. The pH values of the Ringer's solution were the same as those indicated above. The perfusion system provided a fast change of solution with the pre-assigned pH in the whole volume of the experimental chamber (200 ml). There were studied reactions of the respiratory (RPeD1, VD4, VD1/RPD2) and alimentary (R/L CGCs) networks (Fig. 1) identified according to [2, 3, 9].

## RESULTS AND DISCUSSION

The hungry molluscs were characterized by an elevated hemolymph pH ( $7.9 \pm 0.05$ ) as compared with that in the satiated animals ( $7.6 \pm 0.03$ ). Distribution of pH values obtained for the molluscs in the state of alimentary excitation differed highly statistically significantly from that obtained for the satiated and hungry animals (Fig. 2) that also differed from each other. No differences in the hemolymph pH were recorded at various stages of the pulmonary respiration cycle in all experimental groups.

Study of respiratory behavior has shown a significant fall of the number of respiratory acts in hungry molluscs ( $2.1 \pm 0.27$  units/h,  $n = 10$ ), as compared with satiated animals ( $10.8 \pm 0.58$  units/h,  $n = 20$ ). Their duration was longer in satiated animals and amounted to  $149.2 \pm 10.30$  s ( $n = 20$ ). In hungry animals this parameter was equal to  $87.2 \pm 6.44$  s ( $n = 13$ ). As compared with this organism physiological state, the state of alimentary excitation was characterized by an enhancement of pulmonary ventilation in the form of an increase of the number ( $4.0 \pm 0.42$  units/h,  $n = 10$ ) and length ( $177.2 \pm 9.94$  s,  $n = 20$ ) of the respiratory acts.

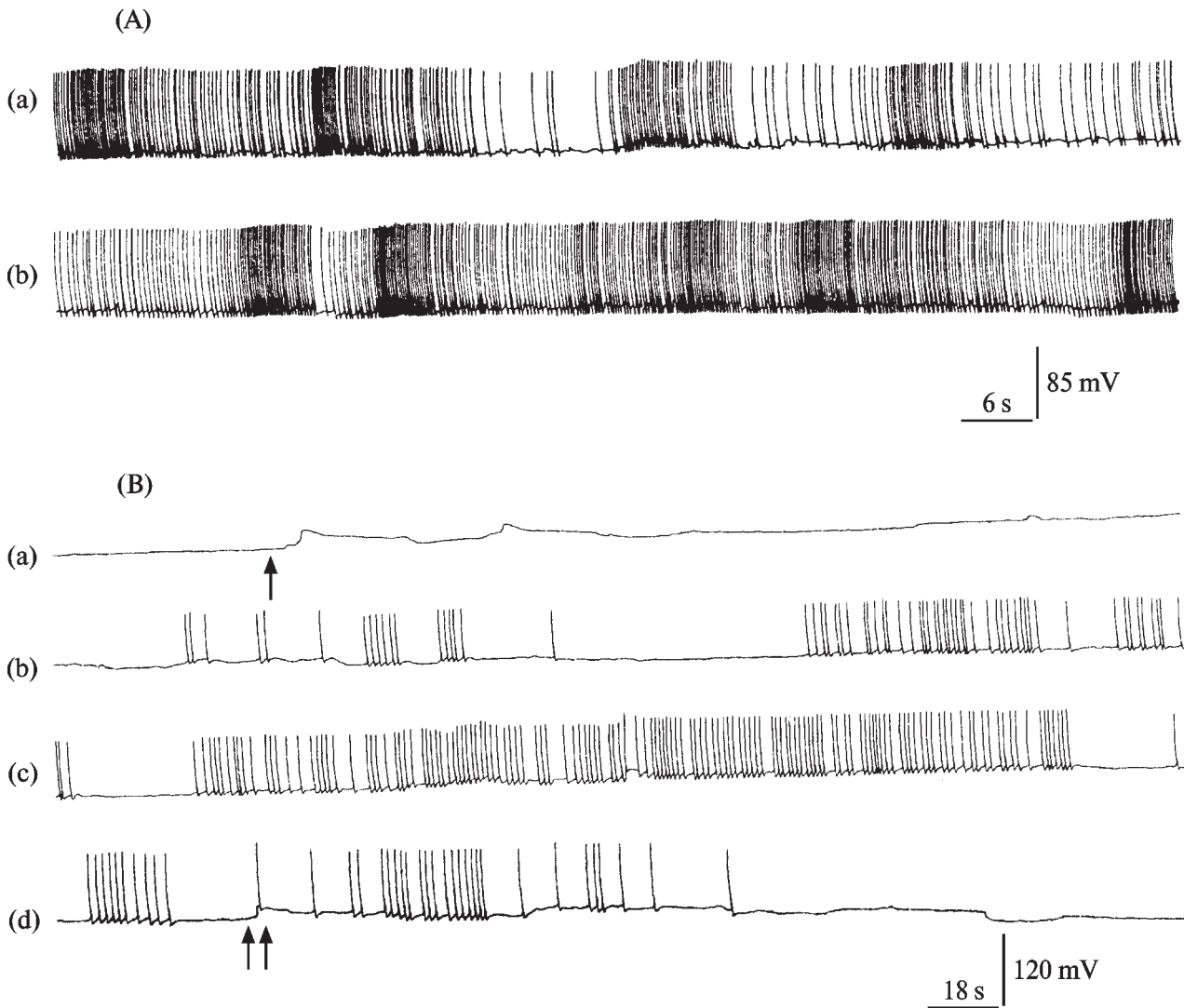
Hungry animals consumed 3 times more food than satiated individuals. Values of this parameter per one mollusc amounted to  $49.3 \pm 5.0$  mg/2 h ( $n = 15$ ) in the satiated and  $130.0 \pm 15.7$  mg/2 h ( $n = 15$ ) in the hungry molluscs. In 60% of semi-intact preparations obtained from hungry molluscs, we observed pronounced spontaneous contractions of the buccal mass. In 40% of preparations such activity was practically absent. On the contrary, in 80% of preparations obtained from satiated animals the buccal rhythm was not observed. Spontaneous pneumostome movements were not pronounced in both experimental groups. Application of Ringer's solution with pH 7.3 (or 6.8) onto the CNS



**Fig. 3.** Electrical activity of a neuron of the alimentary network of *Lymnaea stagnalis* (L CGC) at various pH. (a) 7.3, (b) 7.8, (c) 8.3, (d) after application of SNAP ( $10^{-4}$  M).

surface for 1 min produced the complete cessation of movements or a significant (3-fold) decrease of their frequency. The subsequent washing out of the preparation with the solution with pH 7.8 somewhat increased frequency of the buccal mass contractions, although this frequency remained lower than the initially observed one. Application of the solution with pH 8.3 (or 8.8) onto the surface of the preparation did not produce any significant changes of the buccal rhythm.

Ringer's solution with pH 7.3 (or 6.8) induced movements of pneumostome. Its valve was elevated to open entrance into the pulmonary cavity. In several preparations the complete opening of the respiratory orifice was observed. These responses occurred in 1–2 min after the change of the solution washing the CNS. The subsequent change of the solution (pH 7.8) produced an immediate cessation of all pneumostome movements and its closing. Reaction to the solutions with pH 8.3 (or 8.8) was similar.



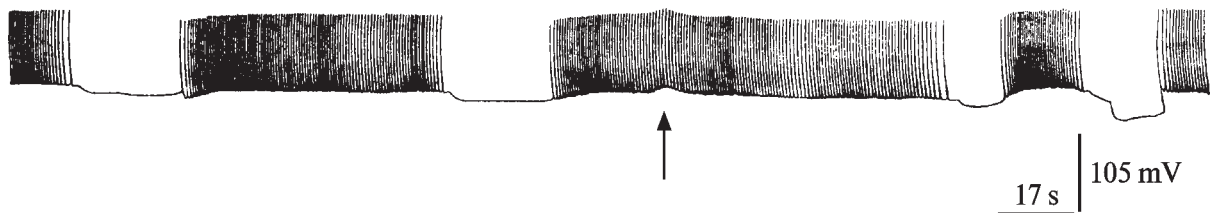
**Fig. 4.** Electrical activity of a neuron of the respiratory network of *Lymnaea stagnalis* (RPeD1) at changes of pH. (A) On the background of spontaneous activity: (a) pH 7.8, (b) 7.3; (B) in the absence of initial spontaneous activity: (a)–(d) (continuous recording); *one arrow*—the beginning of change of solution (pH from 7.8 to 7.3), *two arrows*—subsequent washing-out of the preparation (pH from 7.3 to 7.8).

Alkalization (pH 8.3 or 8.8) reduced frequency of generation of action potential (AP) by electrically coupled neurons R/L CGC shifting to the fire regime of the spontaneous activity. On the contrary, Ringer's solution with pH 7.3 (or 6.8) eliminated these effects and initiated the tonic activity regime of these neurons. Under the above actions, the resting potential did not change statistically significantly. If these cells initially were in the silent state, it was impossible to produce their AP generation by changing pH of the perfusion solution. It

is to be noted that such physical regime of activity of the neurons R/L CGC could be obtained by adding to the perfusion system of donors of nitrogen monoxide (NO), S-nitroso-N-acetylpenicillamine (SNAP) or sodium nitroprusside, at a concentration of  $10^{-4}$  M (Fig. 3).

Acidification (pH 7.3 or 6.8) depolarized neurons RPeD1 by  $10.3 \pm 1.2$  mV ( $n = 8$ ) and increased frequency of impulsion 1.7 times (Fig. 4A). Alkalization (pH 8.3 or 8.8) hyperpolarized (by  $5.2 \pm 0.64$  mV,  $n = 8$ ) such neuron and decreased its AP





**Fig. 5.** Spontaneous electrical activity of a neuron of the respiratory network of *Lymnaea stagnalis* (RPaD2) at changes of pH. Arrow—the beginning of changes of solution (pH from 7.8 to 7.3).

generation frequency. In the cases when the neuron RPeD1 was not spontaneously active, acidification of the medium brought about the appearance of AP (Fig. 4B).

Neuron VD4 was hyperpolarized by  $5.4 \pm 0.45$  mV after acidification and depolarized by a similar value ( $4.6 \pm 0.54$  mV) after alkalization of the medium. This cell is constantly in the silent state ( $n = 6$ ). Its AP never was observed at any pH values.

The pair of electrically coupled neurons VD1/RPaD2 was hyperpolarized (by  $7.5 \pm 0.68$  mV,  $n = 6$ ), and its spike frequency decreased (1.5 times) at pH 7.3 (or 6.8). In several preparations, the AP generation stopped completely. Alkalization somewhat increased (1.2–1.3 times) spontaneous impulsion and depolarized both VD1 (by  $5.1 \pm 0.44$  mV,  $n = 6$ ) and RPaD2 (by  $4.8 \pm 0.42$  mV,  $n = 6$ ).

The acid-sensitive ion channels (ASIS) in the mammalian nervous system are revealed in sensory neurons and central nociceptive pathways [10]. Responses of neuronal structures of the mammalian brainstem surface to changes of  $H^+$  concentration are well known [11]. Chemoreception of  $CO_2$  and maintenance of pH homeostasis in this CNS part are connected with pH sensitivity of  $K^+$  channels [12]. It can be suggested that the proton-gated channels appeared at earlier steps of biological evolution. Thus, study of non-identified neurons of *Lymnaea stagnalis* has revealed the presence of their structures differing by several properties from ASIS [13]. Changes of pH concentration are well known to produce a pronounced effect on  $Na^+$  permeability and, as a result, on the process of AP generation [14], biophysical properties of  $K^+$  channels [15], and functioning of electrical [16] and chemical [17] synapses. Fluctuations of pH inevitably occur in the case of cell damage or a marked change of metabolism.

Such deviations affect the ability of neuronal structures to perform their functions; hence, the pH value is one of factors regulating activities of the nervous system [18]. Various organism functional states, such as hunger, satiety, an increased motor activity, etc., are characterized by different degree of energy expenditure. This is also accompanied by changes of the acid-base balance in the organism, including various areas of the nervous tissue. Change of pH of the internal medium could be a consequence of both internal and external processes [19]. In turn, neuronal activity leads to pH fluctuations that are sufficient to affect proteins and functions of ion channels [20].

The serotonergic cerebral giant cells (CGCs) were revealed in representatives of such molluscs, as Pulmonata (*Lymnaea*, *Helisoma*, *Helix*) and Opisthobranchia (*Aplysia*, *Pleurobranchia*) [21]. Electric stimulation of CGCs, as well as application of serotonin, intensifies the buccal mass movement in *Lymnaea*, which is due to effect of CGCs on neurons of buccal ganglia [9]. Nitric oxide applied on the activated CNS preparation activated the food-procuring activity in *Lymnaea* [22]. The similarity of patterns of the CGS activity revealed in this work at elevated pH and under conditions of activity of NO donors can indicate that changes of the acid-base balance are able to modulate activity of the *Lymnaea* food-procuring apparatus.

Interneurons RPeD1 and VD4 are a part of the central rhythm generator (CRG) of respiration. VD4 is connected with closing of pneumostome, while RPeD1, with activation of activity of the whole neuronal assembly due to an increase of its own impulsion [2]. A similar role seems to be played by the pair VD1/RPaD2, whose pronounced hyperpolarization is observed at a decrease of  $pO_2$ , which leads to initiation of the respiratory

generator due to disinhibition [23]. These neurons have a pronounced pH-dependence of parameters of the electrical activity. Frequency of AP generation in RPeD1 rises under conditions of free access of molluscs to food, when the hemolymph pH is shifted to the acid side. In parallel with this, there occurs an increase of the number and duration of respiratory acts. Marked hyperpolarization (Fig. 5) and a decrease of frequency of impulsation in VD1/RPaD2 also facilitate this process. This is accompanied by hyperpolarization of VD4, which is necessary for its activation and, hence, for activity of the whole respiration CRG. A low probability of the appearance of AP in this cell is due to the initially low resting potential.

We failed to reveal changes of the acid-base balance in the *Lymnaea* hemolymph at various stages of the pulmonary cycle. The respiration in *Lymnaea*, like in most aquatic animals, seems to be regulated by the level of O<sub>2</sub>, rather than of CO<sub>2</sub>, which is due to a much higher water solubility of carbonic acid as compared with that of oxygen. It is possible that the pH value that changes essentially in the course of and due to changes of metabolism plays role of a “switch” of various behavioral programs by working in parallel with processes of coordination of functions based on direct anatomical connections. Sensitivity to factors producing “generalized” effect on CNS (temperature, pH, NO) already appeared at earlier stages of evolutionary development [24].

The pH effect at the cellular level can be realized due to changes of Ca<sup>2+</sup> concentration in hemolymph. It has been established that the Ca<sup>2+</sup> concentration in the acid medium rises, while alkalization leads to opposite effects [25]. At the same time, a decrease of the Ca<sup>2+</sup> amount evokes H<sup>+</sup>-mediated currents in *Lymnaea* neurons [13].

Thus, concentration of H<sup>+</sup> in hemolymph of the mollusc *Lymnaea stagnalis* plays an important role in realization of programs of alimentary and respiratory behavior. It is based on different pH dependence of the electrical activity of neurons controlling processes of pulmonary respiration and nutrition.

#### ACKNOWLEDGMENTS

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