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COMPARATIVE AND ONTOGENIC PHYSIOLOGY

Seasonal Fluctuations of Migratory Activity of Vertebrate Nuclear Hemocytes at Different Incubation Temperatures

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Abstract—Agarose migration test has shown that nuclear erythrocytes of *Cyprinus carpio*, *Rana ridibunda,* and *Gallus domesticus* are capable for spontaneous locomotions. The migration of red blood cells from frogs is associated with formations of long pseudopodia, whereas that from carps and hens—with short protrusions. It has been shown that migratory activity of nuclear erythrocytes and leukocytes from *Rana ridibunda* and *Gallus domesticus* under effect of temperature *in vitro* had seasonal nature, while that from *Cyprinus carpio* did not depend on the year season. In frogs and hens the circadian oscillations of blood cell migration area are coupled with the organism functional activity, whereas no such association is present in carps.

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Key words: migratory activity, nuclear hemocytes, vertebrates.

INTRODUCTION

According to the current general and evolutionary physiology, erythrocytes in the animal organism serve the breathing function, while leukocytes—the immunocompetent one [1–3]. Also, blood cells are known to have some duplicating functions, thus, enhancing guarantees of the life support and the integrity of the organism [4, 5]. Phagocytic cells realize their protective function due to their capability for migration [6]. There are quite a few literature data about mechanisms of locomotion activity of white blood cells in mammals and human [7, 8]. Peculiarities of leukocyte migration, both spontaneous and stimulated by various factors have been studied in functionally altered and pathologic states of the organism [9]. It is well-known that erythrocytes of lower vertebrate animals are capable for uptake of alien particles [10–12]. However, despite a wide range of studies on the problem of phagocytosis [13–15], some key questions about determination of phagocytic reactions by blood cells in lower vertebrates and birds remain to be elucidated. In these groups of animals, the migratory activity of leukocytes is studied insufficiently and there are no data on this issue in nuclear erythrocytes as well as on mechanisms of compensatory reactions of nuclear hemocytes under conditions of hypo- and hyperthermia. Very limited data are available in chronobiological studies dealing with the temperature-dependent seasonal fluctuations of migratory activity of the blood nuclear cells in representatives of the lower vertebrate animals and birds. This determined the goal of the present study—to investigate the circannual fluctuations of the migratory activity of nuclear hemocytes at different incubation temperatures.

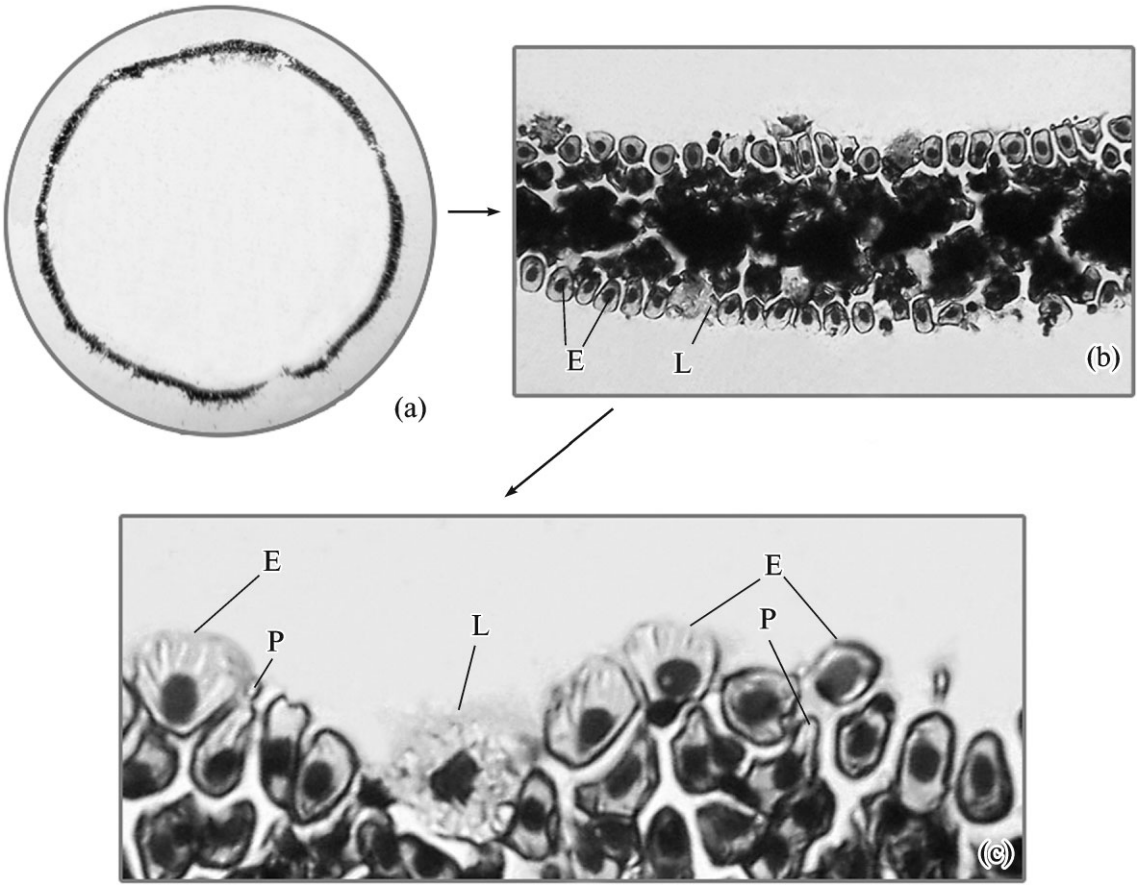

Fig. 1. Distribution of blood cells of the *Cyprinus carpio* in the well after 24 hr-incubation. (a) Small magnification (× 40), (b) larger magnification (× 400), (c) large magnification (× 2000). E—Erythrocytes, L—leukocytes, P—pseudopodia.

MATERIALS AND METHODS

In correspondence with the goal of our study, two sets of experiments were carried out. The first set was performed to elucidate the occurrence of physiological capability for spontaneous locomotion of nuclear erythrocytes of the common carp (*Cyprinus carpio*), the marsh frog (*Rana ridibunda*), and the hen (*Gallus domesticus*). In the second set, effect of temperature on migratory activity of nuclear erythrocytes and leukocytes of the common carp, the marsh frog, and the hen was evaluated under *in vitro* conditions with considering natural physiological activity of the animals during four seasons of the calendar year: in spring (April), summer (July), fall (October), and winter (January). In each of the seasons, before each set of the experiments the animals were adapted for 24 h to room temperature (20°C). Blood samples were drawn under light ether anesthesia: in the common carp—from the tail vein, in the frog—from the heart, and in the hen—by venipuncture. Heparin (10 U/ml) was used as an anticoagulant. The stabilized blood was centrifuged at 400 *g* for 4 min, the leukocyte layer and the lower layer of the plasma rich in leukocyte were collected. Erythrocytes and washed resuspended leukocytes were counted in Goryaev's chamber.

As a criterion of the spontaneous migration there was used the area of the spontaneous spread of erythrocytes and leukocytes, obtained in the agarose test. The classical method described in numerous papers [16–18] was used with some modifications [19, 20]. Three μl of the hemocyte suspension diluted with isotonic saline (0.8%, 0.6%, and 0.9% for the carp, frog, and hen, respectively) were placed into the wells cut in agarose placed on an object glass. This volume of the suspension contains approximately 1 mln blood cells of the carp and the hen and about 300 thou-

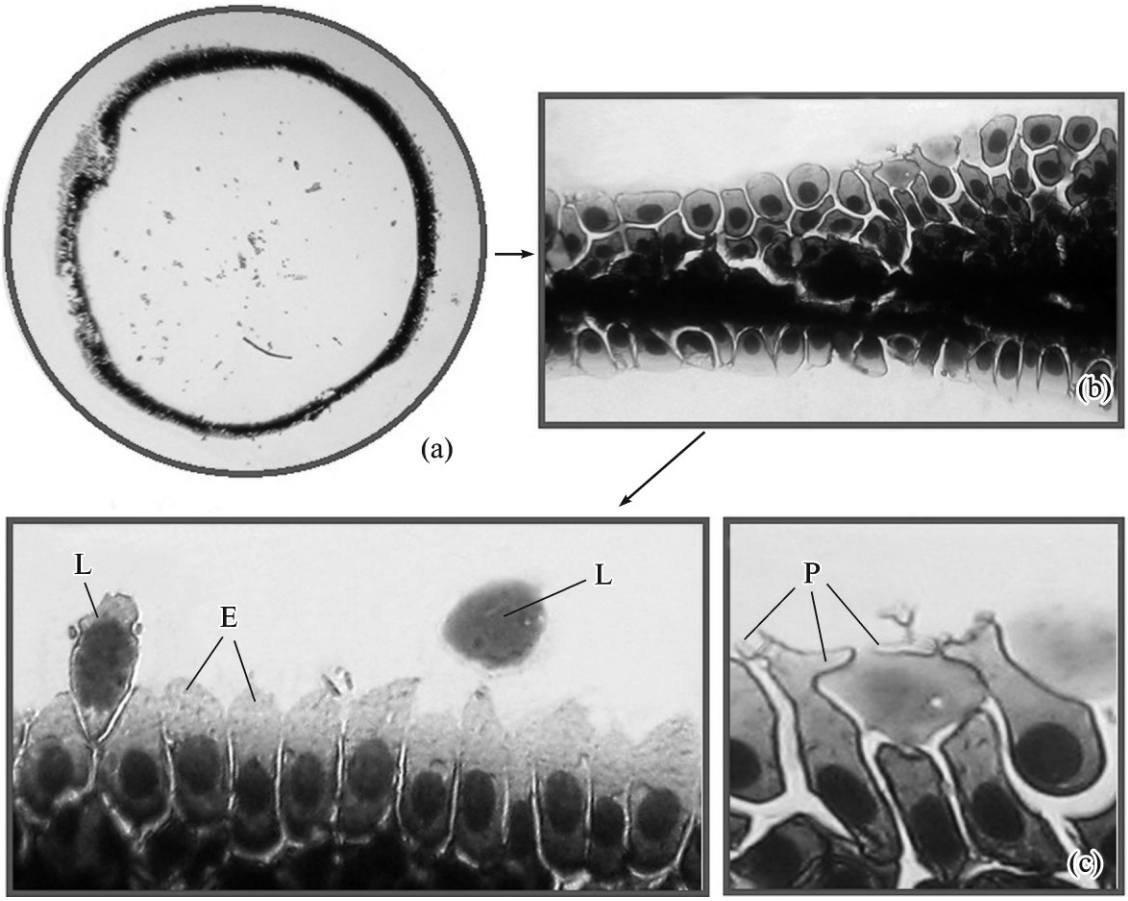

Fig. 2. Distribution of blood cells of the *Rana ridibunda* in the well after 24 hr-incubation. (a) Small magnification (× 40), (b) larger magnification (× 400), (c) large magnification (× 2000). E—Erythrocytes, L—leukocytes, P—pseudopodia.

sand hemocytes of the frog. Sections with blood of the carp and the frog were incubated for 24 h in the medium containing 5% CO_2 at three temperature regimes: at the room (20°C), lowered (5°C), and elevated (40°C) temperature. The hen blood cells were incubated under the same conditions with addition of the forth variant—at 45°C, with the temperatures of 5°C and 20°C being considered as lowered, 40°C—as optimal, and 45°C—as elevated. After the end of the incubation, the hemocytes were fixed for 1 h in glutaraldehyde and then stained with azure-eosin. Area of the spontaneous migration of individual cell pools was recorded and measured by using a VideoTest-Razmer 5.0 image analyzer (OOO Mikroskop-Servis, St. Petersburg, Russia).

The obtained results were processed by methods of variation statistics. Digital data presented as the mean (M) ± standard error of the mean (m) were introduced into a table only in the case of their normal distribution. The significance of differences was determined by Student's t-test ($p < 0.05$).

RESULTS AND DISCUSSION

Results of the first set of experiments conclusively demonstrate that the red blood cells of the common carp, the marsh frog, and the hen are capable for spontaneous locomotion. Figures 1–3 show that both leukocytes and erythrocytes of the experimental animals migrate outside the well after the 24-h-long incubation of the blood cells at room temperature.

It was established that the spontaneous locomotion of red blood cells of the frog is associated with formation on the leading edge of the long outgrowths—pseudopodia (lamellopodia) of varying shape typical of leukocytes (Fig. 2). In the com-

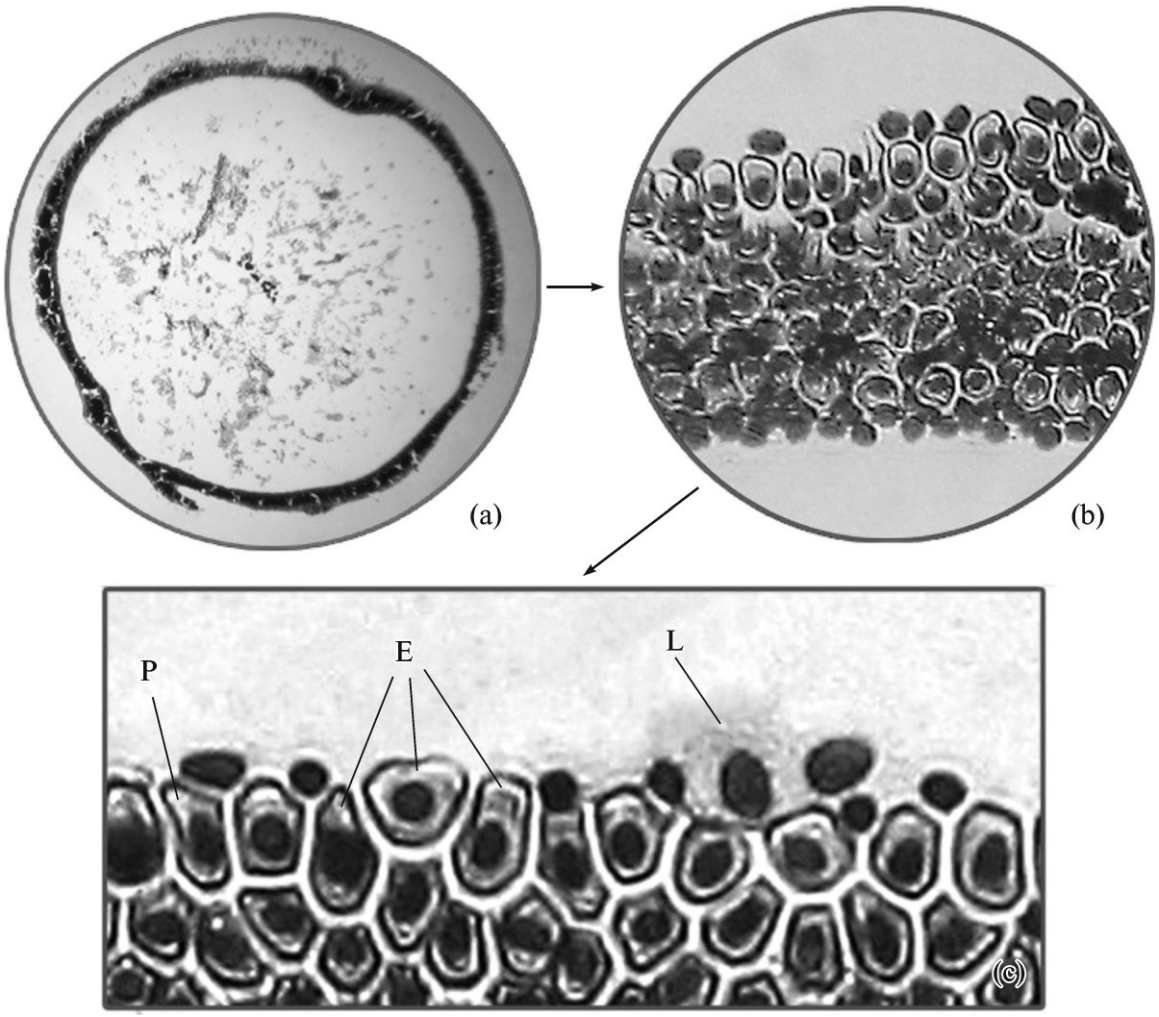

Fig. 3. Distribution of blood cells of the hen *Gallus domesticus* in the well after 24 hr-incubation. (a) Small magnification (× 40), (b) larger magnification (× 400), (c) large magnification (× 2000). E—Erythrocytes, L—leukocytes, P—pseudopodia.

mon carp (Fig. 1) and the hen (Fig. 3), migration of erythrocytes is based on formation of short protrusions. Some authors have shown [21] that nuclear erythrocytes of the *Rana ridibunda* exhibit higher plasmalemma folding than the similar cell pool of the *Cyprinus carpio* and the *Gallus domesticus* and, thus, have larger membrane reserve providing formation of long pseudopodia. Formation of long pseudopodia in nucleated erythrocytes of the frog and the shorter protrusions in the hen and the carp allows migration of the cells of this pool. The obtained data are in agreement with data of other investigators [22] that have showed that capability for locomotion of a cell is determined to the greater extent by the shape of the cell. The dynamics of the front edge of the cell makes the main contribution to the rate of migration [6].

The data obtained in the second set of experiments revealed species-specific pattern of migratory activity of nucleated erythrocytes and leukocytes of the *Cyprinus carpio*, *Rana ridibunda*, and *Gallus domesticus* in *in vitro* experiments depending on the temperature regimes.

The locomotion activity of blood cells of the common carp incubated at 5°C and 20°C was found to remain at the same level at any time of the year (Table 1). Given that in temperate latitudes the water temperature during the year ranges from 0 to 20°C [23], we can assume that just in this temperature range are realized innate mechanisms of cellular adaptation, determining the stability of migratory activity of hemocytes. Increasing the

Table 1. Areas of the spontaneous migration of hemocytes of the *Cyprinus carpio* (mm^2)

Incubation temperature, °C	Season							
	spring		summer		fall		winter	
	E	L	E	L	E	L	E	L
5	2.66 ± 0.12	2.77 ± 0.21	3.06 ± 0.13*	3.20 ± 0.24	3.15 ± 0.15	3.64 ± 0.60	3.08 ± 0.18	3.13 ± 0.12
20	2.80 ± 0.20	2.86 ± 0.22	3.39 ± 0.32	3.45 ± 0.38	3.27 ± 0.25	3.47 ± 0.25	3.12 ± 0.40	3.18 ± 0.50
40	2.44 ± 0.18*	2.24 ± 0.34*	2.48 ± 0.37*	2.58 ± 0.35*	2.97 ± 0.26*	3.31 ± 0.23	2.65 ± 0.16*	2.98 ± 0.43

Note: E—erythrocytes, L—leukocytes. *—significant difference in comparison with 20°C according to Student's *t*-criterion ($p < 0.05$).

Table 2. Areas of the spontaneous migration of hemocytes of the *Rana ridibunda* (mm^2)

Incubation temperature, °C	Season							
	spring		summer		fall		winter	
	E	L	E	L	E	L	E	L
5	3.24 ± 0.15*	3.13 ± 0.15*	3.16 ± 0.27*	3.05 ± 0.19*	2.95 ± 0.47	3.14 ± 0.47	3.13 ± 0.16	3.04 ± 0.22
20	2.82 ± 0.11	2.69 ± 0.13	2.78 ± 0.48	2.71 ± 0.23	3.16 ± 0.32	3.08 ± 0.30	3.07 ± 0.22	3.01 ± 0.22
40	2.79 ± 0.17	2.74 ± 0.19	2.72 ± 0.51	2.85 ± 0.36	2.60 ± 0.15*	2.84 ± 0.22*	2.58 ± 0.30*	2.52 ± 0.29*

Note: E—erythrocytes, L—leukocytes. *—significant difference in comparison with 20°C according to Student's *t*-criterion ($p < 0.05$).

incubation temperature to 40°C—the upper limit of survival of the carps [24], compared to 20°C causes a decrease in the area of locomotion of the *Cyprinus carpio* erythrocytes in all periods of the year, and leukocytes—in the spring and summer seasons. We believe that the high temperature of the blood cells incubation in the experiment in contrast to the optimal temperature range has negative impact on the physiological status of the fish. According to the literature, the temperature of 40°C is capable of causing not only the thermal stress in the fish, but even a thermal shock [24, 25].

In amphibians, a decrease of temperature of the cells incubation to 5°C increases the migratory activity of erythrocytes by 14.9 and 13.7% and that of white blood cells—by 16.4 and 12.6% in the spring and summer, respectively (Table 2). It can be assumed that the decline of temperature of functionally active animals is a factor in activation of protective functions, in particular, amplification of hemocytes locomotion. An increase of the incubation temperature (40°C) in the autumn and winter seasons caused a decrease in the area of the frog erythrocytes migration by 17.7 and 16.0% and of leukocytes—by 7.8 and 16.3%, respectively.

This result is consistent with the known data that amphibians have reduced body temperature and sharply limited locomotion activity during both the hibernation and the period between the breeding season and start of hibernation [26]. We believe that the decrease in leukocyte migratory activity in frogs in a state of anabiosis is due to two factors. It is possible that such a high temperature of incubation of blood cells appeared to be an additional physiological load or, alternatively, the 24 hr-incubation period was insufficient to activate the biochemical processes that stimulate cell function.

Before performing the experiments, we assumed that the incubation temperature of 40°C corresponding to the body temperature of the birds will be optimal for cell migration. However, results of this variant of the experiment showed that spontaneous migration of erythrocytes of the hen at incubation temperature of 20°C compared to 40°C during spring and summer increases by 11.9 and 19.3% and that of leukocytes—by 5.0 and 26.9%, respectively (Table 3). During fall, the migratory activity of erythrocytes and leukocytes of *Gallus domesticus* is increased not only at room temperature (20°C) (by 6.8 and 6.8%), but also at lower incubation temperature (5°C) (by 4.8 and 8.4%) as compared to 40°C. An increase of the locomotion activity of blood cells at the incubation tempera-

Table 3. Areas of the spontaneous migration of hemocytes of the *Gallus domesticus* (mm^2)

Incubation temperature, °C	Season							
	spring		summer		fall		winter	
	E	L	E	L	E	L	E	L
5	2.74 ± 0.20	2.84 ± 0.13	2.77 ± 0.35	2.90 ± 0.20*	3.26 ± 0.08*	3.21 ± 0.11*	3.02 ± 0.16*	3.06 ± 0.13*
20	3.00 ± 0.17*	2.94 ± 0.16*	3.03 ± 0.15*	3.02 ± 0.17*	3.32 ± 0.12*	3.16 ± 0.13*	3.06 ± 0.13*	3.04 ± 0.11*
40	2.68 ± 0.23	2.80 ± 0.18	2.54 ± 0.38	2.38 ± 0.27	3.11 ± 0.18	2.96 ± 0.10	3.17 ± 0.21	3.17 ± 0.16
45	2.63 ± 0.20	2.52 ± 0.33*	3.14 ± 0.32*	2.88 ± 0.21*	3.24 ± 0.15	2.92 ± 0.14	2.87 ± 0.17*	2.88 ± 0.15*

Note: E—erythrocytes, L—leukocytes. *—Significant difference in comparison with 40°C according to Student's *t*-criterion ($p < 0.05$).

tures of 5°C and 20°C seems to be due to the lower energy expenditure by the bird for morpho-physiological mechanisms of heat irradiation under the above-mentioned conditions [27]. A decrease of area of spontaneous locomotion of the hen's hemocytes in the winter variant of the experiment at low incubation temperature is likely to be related to the reduction of the level of the basal metabolism. A number of species of birds and mammals residing in the temperate zone are known to reduce slightly the basal metabolic rate in winter for a more economical energy balance at this period [27].

At increased incubation temperature (45°C), the migratory activity of hen erythrocytes and leukocytes is increased in the summer by 23.6 and 21.0% and decreased in winter by 9.4 and 9.1%, respectively, as compared with 40°C. These data suggest that enhancement of the spontaneous locomotion of the bird's blood cells *in vitro* during summer is a consequence of plasmalemma stimulation by the thermal factor. Indirect support of this suggestion is a results of the study [28], which demonstrated an increase of hemocyte activity not only in inflammation, but also after induction by agents of different nature. All the stimulants by anyway interact with the plasma membrane by altering its molecular topography. The membrane modification at the thermal load leads to formation of lipid domains and elastic deformations. In this case, the content and structure of protein macromolecules are not changed, which provides preservation and activation of membrane functions in connection with a reduction in the activation threshold [29, 30].

We believe that the differently directed dynamics of spontaneous migratory activity of blood cells in experimental animals in experiments *in vitro* is due to their habitat and, accordingly, to action at the cellular level of evolutionarily different mechanisms of temperature adaptation determined by various properties of the plasma membrane. The membrane properties in this case depend upon the phasic transitions of the lipid bilayer of the plasmalemma with different critical points in species of homoiothermal and poikilothermal animals [31]. Significance of the ambient temperature for cell membranes of both poikilothermal and homoiothermal organisms is due to the fact that it determines the so-called "weak" interactions between molecules regulating microviscosity of the lipid bilayer, the phasic distribution of lipids, the microsurrounding of proteins, the protein–lipid interactions, and other characteristics of the structural organization of membranes [32].

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