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EXPERIMENTAL PAPERS

Glycogen as an Energy Storage Substrate in Fish Nucleated Erythrocytes

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Abstract—We studied the glycogen content in erythrocytes of two Black Sea cartilaginous fishes (*Raja clavata, Dasyatis pastinaca*) and four teleost species (*Trachurus mediterraneus ponticus, Spicara flexuosa, Diplodus annularis, Scorpaena porcus*). Glycogen accumulation in erythrocytes of teleost fish was about twice as high as in the ray fishes. Due to the unique ability of thornback ray *R. clavata* erythrocytes to well preserve their cell integrity in vitro in the cold (4°C), the dynamics of glycogen expenditure in erythrocytes was tracked over 11 days of storage until cell disintegration. Erythrocytes spent glycogen economically: during the entire storage period, only 52% of glycogen were spent. In the black scorpionfish *S. porcus*, erythrocytes were far less tolerant to being stored in the cold. The stability period of *S. porcus* erythrocytes suspended in physiological saline did not exceed two days. During this period, no significant changes in the glycogen concentration were observed.

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Keywords: glycogen, erythrocytes, cartilaginous fish, teleost fish, Black Sea

INTRODUCTION

Fish nucleated erythrocytes refer to unreduced cells having the nucleus, mitochondria and predominantly aerobic metabolism. This means that in the absence of oxygen fish erythrocytes actively consume it in the mitochondrial complex to compensate their energy demands [1]. Besides, fish erythrocytes have a complete set of glycolytic enzymes for anaerobic metabolism of glucose and a sufficient set of enzymes for glycogen synthesis [2]. The issue of which of the carbohydrate or non-carbohydrate substrates is principal in the fish erythrocyte energetics is being discussed in the literature. It might be supposed that, like in mammals, a major energy storage substrate in the fish blood plasma is glycose. However, this may be true, in part, only for those fish the plasma of which is characterized by a steadily high glucose level. In fish with a high plasma glucose level, the glucose concentration may vary across species from 2.0 to 11 mmol/L of blood plasma [3]. In the plasma of some fish, the glucose level may be an order of magnitude lower, fluctuating between 0.1–0.2 mmol/L blood plasma [4]. Such a level of glycemia in these fish can in no way be sufficient to cover all energy demands of their erythrocytes. In the literature, there are suggestions that such

erythrocytes can utilize other substrates to resynthesize ATP, including fats (oleates), amino acids (alanine), and derivatives of carbohydrate metabolism (pyruvate and lactate) [5]. Even less studied is the issue of the role and significance of intracellular glycogen in fish erythrocytes. Is glycogen a major energy storage carbohydrate in erythrocytes, as it has been proved, e.g., for fish muscles and liver, or it should be considered as a minor component of the substrate assortment? Since the glycogen level in erythrocyte of cartilaginous and teleost fish still remains unestablished, this work aimed to determine the intracellular glycogen concentration in erythrocytes of some cartilaginous and teleost fish inhabiting the Black Sea. To understand the involvement of glycogen in maintaining the cell energetics in vitro, this study also aimed at tracking the dynamics of glycogen utilization in fish erythrocytes when stored in the cold at 4°C.

MATERIALS AND METHODS

Experiments were carried out on erythrocytes of 2 cartilaginous and 4 teleost fish species caught at the eastern coast of the Crimean peninsula. The cartilaginous fishes, rays: thronback ray *Raja clavata* Linnaeus and stingray *Dasyatis pastinaca* Linnaeus; the teleost fishes: horse mackerel *Trachurus mediterraneus ponticus* Aleev, blotched pickarel *Spicara flexuosa* Rafinesque, annular sea bream *Diplodus annularis* Linnaeus, and black scorpionfish *Scorpaena porcus* Linnaeus. The chosen fish species had different positions on the evolutionary tree and different ecological specializations.

Rays refer to ancient cartilaginous fish. *R. clavata* and *D. pastinaca* have different temperature preferences. *R. clavata* is a psychrophilic species which inhabits at depths of 30–70 m and badly tolerates temperatures above 20°C. *D. pastinaca* is a thermophilic species, well tolerates a temperature of 25°C and above, can swim close to the shore and stay at a depth of 0.5 m in the summertime.

The above teleost fish differ in their speed and migratory characteristics. *T. mediterraneus ponticus* is the fastest pelagic swimmer which migrates permanently. *S. flexuosa* and *D. annulari* are neritic species, almost non-migratory though maneuverable, inhabiting underwater thickets of seaweeds. *S. porcus* is a neritic sedentary ambush predator hiding among bottom stones.

All the objects were caught near the eastern coast of the Crimean peninsula in the fall/wintertime. Before the experiments, the captured rays were kept for 5 days in a stagnant well-aerated basin (25 m^3) on the basis of 2 m^3 per individual. Teleost fishes were kept in two aerated basins, 1 m^3 each, on the basis of 100 L per individual. Water temperature was 14–16°C. Before the experiments, fish were deprived of any food.

Experimental protocols met all applicable international, national and institutional principles of handling and using experimental animals for scientific purposes.

Ray blood was sampled by cardiocentesis using a heparinized needle (from each individual per experiment) while in teleosts by paracentesis of the caudal vein using a specially sharpened glass pipette (from 5–7 individuals per experiment in *T. mediterraneus ponticus* and 2–3 individuals per sample in *S. porcus*, *S. flexuosa*, and *D. annularis*). The blood thus obtained was introduced into a tube containing ice-cold heparinized physiological saline (0.5 IU/mL, 1:10) for cartilaginous and teleost fishes. Erythrocytes were then double washed from plasma by saline and sedimented at 1500–2000 rev/min in a centrifuge K-23 (Germany). Supernatant was sucked off, and erythrocytes were diluted by saline to hematocrit values equal to 0.25–0.35. Bathing and incubation salines for the rays had the following composition (mM): 220 NaCl + 300 urea + 50 Tris-HCl (pH 7.4). For teleosts, both salines contained no urea and comprised (mM) 180 NaCl + 10 Tris-HCl (pH 7.4).

The glycogen level was assayed by a conventional method based on the color reaction of anthrone with polysaccharides [6] adapted by A.L. Morozova for fish [6]. To determine the amount of the carbohydrate substrate being studied, 0.1 mL of erythrocyte suspension with a precisely determined hematocrit (Ht = 0.25–0.35) was sampled for all the species studied, and this Ht value was subsequently taken into account in all calculations. The selected sample of erythrocyte suspension (0.1 mL) was fixed in 30% KOH

Table 1. Glycogen concentration (mg%) in erythrocytes of some Black Sea fishes

Fish class	Fish species	Glycogen concentration in erythrocytes, mg% of suspension wet weight	*n*
Cartilaginous fish	*Raja clavata* L.	142.8 ± 15.0*	8
	Dasyatis pastinaca L.	139.2 ± 10.0*	8
Teleost fish	*Trachurus mediterraneus ponticus* Aleev	372.3 ± 39.1	15
	Diplodus annularis L.	326.8 ± 22.5	7
	Spicara flexuosa R.	242.2 ± 14.0	12
	Scorpaena porcus L.	305.5 ± 35.5	11

n—the number of repeated experiments; *—significant differences ($p < 0.05$) between erythrocyte glycogen concentration values in a pairwise comparison of cartilaginous and teleost fish.

and heated in a boiling water bath for 20 min to hydrolyze proteins. Samples were then cooled, added with 0.5 mL of the basic solution (25 g $ZnCl_2$ and 5 g NaCl per 100 mL of water), and the hydrolysate was precipitated by 4.5 mL of ethanol. After precipitation, samples were centrifuged at 3000 rev/min for 15 min, the precipitate was separated, air dried and dissolved in 1 mL of 4% HCl. The resulting eluate was filtered and used for glycogen re-precipitation in 8 mL of ethanol per sample. After that, samples were re-centriguged at 5000 rev/min for 15 min, and the precipitate was dissolved in hot water (by 5 mL). 1 mL of such a solution was used for the anthrone color reaction (+ 2 mL of 0.2% anthrone dissolved in concentrated H_2SO_4). Concentration of the colored extract was determined using the Specol-11 spectrophotometer (Germany).

Calculations were made by the following equation:

$$X = \frac{E_{\text{sample}} \times 80\gamma \times 100000 \times 5}{E_{\text{stand}} \times 1000 \times d}$$

where E_{sample}—experimental sample extinction; E_{stand} —standard sample extinction; d—sample weight (g); 100000, 1000, 5—recalculation coefficients (mg%).

To evaluate the dynamics of glycogen utilization by erythrocytes, *R. clavata* and *S. porcus* erythrocytes were stored in a fridge at 4°C for 1, 3, 5, 8 and 11 days while performing a daily visual control of the hemolysis level in erythrocyte suspension and a determination of the glycogen level in erythrocytes by the above method.

The data obtained were expressed in mg% of cell wet weight. The data were statistically treated and presented as the arithmetic mean ± standard deviation. The results were obtained in 7–15 independent experiments for each of the fish species studied. Statistical significance of differences was evaluated using the Student's *t*-test; the differences were considered significant at $p < 0.05$.

RESULTS

Data on the glycogen level (mg%) in erythrocytes of cartilaginous and teleost fishes studied in our experiments are shown in Table 1.

As seen from Table 1, erythrocytes in all fish species accumulated glycogen in large amounts that were comparable with the accumulation of this substrate in fish muscles [7]. For instance, in fish erythrocytes its accumulation ranged from 242.2 to 372.3 mg%. It is necessary to point out that glycogen accumulation in erythrocytes of teleosts was on average twice as high as in cartilaginous fishes. At the same time, ray erythrocytes exhibited almost identical values of intracellular glycogen accumulation (Table 1). Among the teleosts, noteworthy is a correlation between the intracellular glycogen concentration and natural mobility of the species studied. For instance, erythrocytes in the most agile *T. mediterraneus ponticus* revealed a highest level of intracellular glycogen. In the highly maneuverable *D. annularis* characterized by a medium mobility, erythrocytes also demonstrate a high glycogen level. In the *S. flexuosa* and low-mobile *S. porcus*, the glycogen concentration proved to be lower by 18–35%

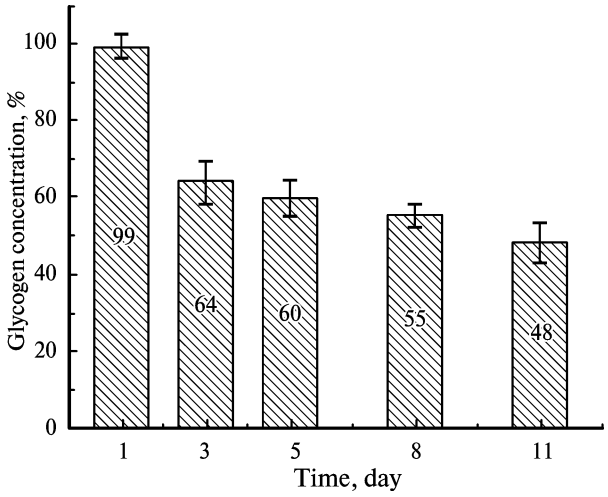

Fig. 1. Dynamics of glycogen concentration in erythrocytes of *Raja clavata* kept in the cold (4°C).

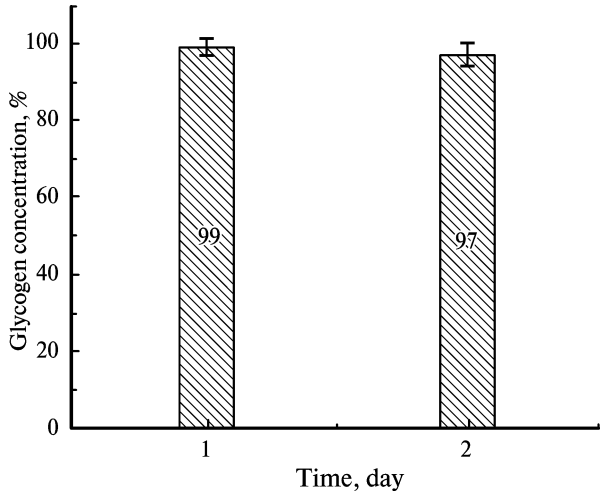

Fig. 2. Dynamics of glycogen concentration in erythrocytes of *Scorpaena porcus* kept in the cold (4°C).

(Table 1), indicative of quite a large spread in this parameter in erythrocytes of fish with a low level of motor activity.

In the *R. clavata*, erythrocytes are unique in having in vitro a high cold tolerance of their suspensions able to retain their integrity without any signs of hemolysis up to 11 days at 4°C. Because of this, we managed to track the dynamics of intracellular glycogen concentration in thornback ray erythrocytes throughout the above-mentioned storage period. The data obtained were expressed as a percentage of the physiological state taken for 100% (Fig. 1).

Figure 1 shows that *R. clavata* erythrocytes stored at 4°C were losing their glycogen quite slowly. During the first day, there were almost no glycogen losses. On the 3rd–5th days, the decrement was 36–40%. During the subsequent storage period, glycogen was depleted even slower, and its loss was 45% by the 8th day and 52% by the 11th day (Fig. 1).

In *S. porcus*, erythrocytes were less storage tolerant compared to thornback ray erythrocytes. The tolerance of *S. porcus* cell suspensions to storage in the cold did not exceed two days (Fig. 2). As seen in Fig. 2, *S. porcus* erythrocytes lost almost no glycogen over the 2-day period of their storage, and its level remained stable until the onset of hemolytic processes in the cell suspension, which commenced after 48 h since isolation (Fig. 2).

DISCUSSION

Fish erythrocytes refer to cells with aerobic metabolism, as follows from the literature [8] and our studies of oxygen consumption rates in erythrocyte mitochondria [9]. Due to the presence of mitochondria and the Krebs cycle, fish erythrocytes are potentially able to utilize in vitro different energy substrates. For instance, it was shown that in the rainbow trout *Oncorhynchus mykiss* and American eel *Anguilla rostrata* erythrocytes can oxidize glucose, lactate and alanine [5, 10]. Erythrocytes in the common carp *Cyprinus carpio*, in addition to these substrates, can utilize pyruvate and glutamate [11]. Using different substrates in fish erythrocytes could be considered as an important advantage of nucleated erythrocytes, especially under conditions of hypothermia, starvation, hypoxia and acidosis. However, the issue of using other substrates in the fish erythrocyte energetics under stress conditions remains debatable, particularly considering that their metabolic involvement requires oxygen which in these cases is exactly a limiting factor.

It is well known that mammalian erythrocytes have no nucleus and under physiological conditions utilize only glucose as an energy source. The lack of the cytochrome system minimizes oxygen demands of anucleate erythrocytes, and their energy-generating system functions on the basis of anaerobic glycolysis. Glucose penetrates into

erythrocytes from the blood flow through a specific membrane-bound transporter; it is not insulin-dependent and does not represent a limiting factor. Erythrocytes isolated from the human body and deprived of glucose quickly die because of a sharp decline in the intracellular ATP concentration, which causes a drop in the Na^+ and K^+ concentration gradients across the membrane, as well as in the accumulation of methemoglobin and oxidized glutathione [2]. This suggests that mammalian and human erythrocytes do not need to store glucose as glycogen. Nonetheless, active glycogen metabolism was demonstrated both in normal human erythrocytes [12] and in glycogen-rich erythrocytes characterizing the glycogen storage disease type III and IV [13]. Under normal conditions, the glycogen level in anucleate erythrocytes is low. In human erythrocytes, by different estimates, its level ranges from 57.3 to 69.5 mg/g Hb [14, 15].

In fish erythrocytes, like in other tissues, glycogen is an energy storage carbohydrate. However, unlike muscle or hepatic glycogen which in these fish tissues refers to a rapidly mobilizable energy substrate, the role of erythrocyte glycogen is obscure. For instance, it is not clear when and under what conditions this polysaccharide accumulates in erythrocytes. Also unclear is when and under what conditions it mobilizes, and even more unclear is its role as a substrate in the fish erythrocyte energetics. Sparse data on glycogen accumulation in fish erythrocytes indicate that its intracellular concentration varies within a broad range. For instance, in the Atlantic cod *Gadus morhua* its concentration in erythrocytes is 0.35 μmol of glucose units per g^{-1} of cells, while in the short-horn sculpin *Myoxocephalus scorpius* it is 2.9 μmol of glucose units per g^{-1} of cells [4]. The magnitude of glycogen accumulation in short-horn sculpin erythrocytes approximates its accumulation in erythrocytes of the sea raven *Hemitripterus americanus* (3.9 μmol of glucose units per g^{-1} of cells), while the level of its accumulation in Atlantic cod erythrocytes is comparable with its intracellular concentration in rainbow trout erythrocytes [16].

Our results on the glycogen content in erythrocytes of Black Sea cartilaginous and teleost fish show (Table 1) that in erythrocytes of the rays the accumulation of this energy substrate is twice as low as in the teleosts. A low glycogen accumulation may indicate a lower level of energy metabolism in erythrocytes of cartilaginous versus teleost fishes. This assumption is supported by our studies on the respiratory activity of the mitochondrial complex in erythrocytes of cartilaginous and teleost fish [9]. We demonstrated that erythrocytes of teleost fishes were characterized by higher oxygen consumption rates, as compared to erythrocytes of rays, in response to stimulation of the mitochondrial respiratory activity by energy substrates—glutamate, malate and succinate. A high glycogen level in erythrocytes of teleost fish may also indicate a stronger activation of their enzymes responsible for glycogen synthesis. Without question, an important factor for glycogenesis is the plasma glucose concentration. For this reason, plasma glucose and intracellular glycogen of erythrocytes can be considered as two interrelated components of the common pool of carbohydrate substrate supply in the plasma-erythrocyte system. Sparse data indicate that the plasma glucose level in stingrays is quite low. For instance, in the freshwater Atlantic stingray *Hypanus sabinus* it is 0.71 ± 0.1 mmol/L [17]. In the southern stingray *Dasyatis Americana*, it is also low (1.69 mmol/L) [18]. Apparently, a low plasma glucose level in the Black Sea rays is a significant prerequisite for a low glycogen level in erythrocytes of these fish. However, a low plasma glucose level in rays is not a common character of cartilaginous fishes. In the nearest relatives, more mobile sharks characterized by a high level of metabolism, the plasma glucose level is at least one order of magnitude higher. For instance, in the Caribbean reef shark *Carcharhinus perezi* the plasma glucose level is 10.4 mmol/L, and in the nurse shark *Ginglymostoma cirratum* it is 20.0 mmol/L [19].

According to the literature, the plasma glucose concentration in teleost fish is also highly variable, with the substrate level being below 0.1 mmol/L in some species and exceeding 10 mol/L in the others [20]. In marine fish species, such as the short-horn sculpin *Myoxocephalus scorpius*, blue perch *Tautogolabrus adsperdus*, Atlantic salmon *Salmo salar* and Atlantic cod *Gadus morhua*, plasma glucose concentration is, respectively, 0.73, 1.95, 4.1, and 4.0 mmol/L [4]. In our laboratory, we also deter-

mined the plasma glucose concentration in some marine teleost fish [7]. For instance, in *T. mediterraneus ponticus, S. flexuosa,* and *S. porcus*, it was, respectively, 7.7, 10.3 and 1.8 mmol/L. Based on these data, we established no correlation between the plasma glucose concentration and the glycogen level in erythrocytes of the Black Sea fish species (Table 1).

The conditions under which it occurs and glycogenesis *per se* in fish erythrocytes are practically unstudied. We failed to find any sources in the available literature devoted to this issue. Even more unclear is under what conditions fish erythrocytes utilize accumulated glycogen in vivo and in vitro. *R. clavata* erythrocytes refer to unique cells in terms of their ability to retain in vitro their integrity for a long time. Cells isolated and suspended in physiological saline were well preserved in the cold (4°C) for up to 11 days without any signs of hemolysis. This allowed tracking the dynamics of glycogen utilization in fish erythrocytes during long-term storage. As seen in Fig. 1, during storage, glycogen was gradually depleted in thornback ray erythrocytes to maintain their energy requirements. By the end of the storage period, its concentration was 48%, indicating the involvement of this polysaccharide in cellular metabolism in the absence of other energy substrates in physiological saline, where erythrocytes were washed and suspended for subsequent storage. The lack of glucose in physiological saline makes the process of intracellular glycogen utilization in erythrocytes an unavoidable and, most likely, unique way to maintain their metabolism. Quite a smooth fall in the intracellular glycogen concentration values can be explained by the ability of cells to block their energy costs in stress situations by 80% and thus to spend intracellular resources economically [8]. Cell isolating and suspending in physiological saline can exactly be referred to a strong stress exposure for fish erythrocytes. Due to instability of *S. porcus* erythrocytes stored at 4°C and their hemolysis after a 2-day storage (Fig. 2), we failed to track the dynamics of the intracellular glycogen concentration in these cells. Erythrocytes in the other teleost fish studied were even less stable and began to spontaneously disintegrate 17–24 h after isolation (unpublished data).

The very fact that fish erythrocytes utilize glycogen during storage indicates the importance of this substrate for these cells in which the value of its accumulation is comparable to the level of this polysaccharide in fish white muscles [7]. This emphasizes the significance of glycogen for erythrocytes in those situations when the use of other substrates is complicated or infeasible. The possibility of utilizing glycogen as an endogenous energy source was pointed out in the studies by Pesquero et al. [21]. It appears that a fall in the plasma glucose concentration is critical for the involvement of glycogen in metabolism of erythrocytes. Similar situations frequently occur in the fish life, being associated, e.g., with stress-related muscle loads, long-term starvation periods [22]. A special place is occupied by hypoxic conditions which also occur in fish quite frequently. As is well known, carbohydrates refer to substrates the utilization of which may occur in an anaerobic regime with no oxygen needed. In erythrocytes, metabolism proceeds via the classical Embden–Meyerhof pathway and, although the output of energy equivalents of cytoplasmic ATP is 16 times inferior to the aerobic breakdown, such a pathway of glucose utilization is demanded in fish. As shown in a number of studies [5, 11, 16, 21], plasma glucose in fish is a major energy-producing substrate. Even under normal oxygen tension, plasma glucose is utilized by erythrocytes in an anaerobic way, and at least 30% of this substrate is converted into lactate [5]. It appears that glucose and glycogen utilization by fish erythrocytes as an anaerobic source of energy allows saving oxygen carried by these cells. It should be emphasized that a low glycogen content in erythrocytes is inherent to psychrophilic fish species, such as the cod and rainbow trout. In these species, it makes no sense to save oxygen because there is no oxygen deficit in their cold-water habitats. In the thermophilic mobile species of teleost fish studied in our experiments (*T. mediterraneus ponticus, S. flexuosa, D. annularis*), the intracellular glycogen concentration was high. A high glycogen level was also noted in the neritic sedentary fish (European sculpin and sea raven [16], as well as *S. porcus* studied in our experiments) which had far larger reserves of this polysaccharide than the psychrophilic cod and rainbow trout. Neritic fish species experience

hypoxic conditions more frequently, and the utilization of glycogen and glucose in these situations allows saving oxygen to provide energy demands of other vitally important tissues and organs. The same oxygen-saving demands are inherent to thermophilic mobile fish in which the partial pressure of oxygen in the environment falls as the environmental temperature rises, while high energy metabolism requires erythrocytes to carry larger volumes of oxygen. As we suppose, the importance of the utilization of carbohydrates in specialized cells, such as erythrocytes, was "picked up" by natural selection and brought to "perfection" in mammalian anucleate erythrocytes. This enabled mammals to maximize the coefficient of performance of the gas transportation system and thereby to occupy a leading position in the animal kingdom.

In some fish, erythrocytes refer to cells which have a maximum tolerance to hypoxia/anoxia and other stress factors and can rapidly reprogram their metabolism towards the involvement of glycogen in the energy-related process to correct ATP production and consumption [23]. This allows preventing disastrous consequences of sharp fluctuations in the intracellular ATP level and energy status of cells in general. Such a "regrouping" of the cellular energetics under conditions of oxygen deficit towards a preferable utilization of glycogen is also typical of other tissues of stress-tolerant fish species [24–26] and appears to underlie their tolerance. As reported by Ferguson et al. [8], in the absence of oxygen, fish erythrocytes retain as little as 20–30% of energy metabolism, which coincides with the contribution of carbohydrates to basal metabolism in these cells [5]. Such a coincidence suggests that these 30% of energy costs represent a necessary and sufficient basis for providing current demands of erythrocytes in maintaining their functional status. The remaining 70% of the erythrocyte energetics seem to be consumed to provide biosynthetic and regenerative molecular processes, which promotes a longer maintenance of their gas transportation function and a longer presence of erythrocytes in the fish blood flow. As is well known, the lifespan of fish erythrocytes is about twice as long as in mammals (more than 200 days [27]).

The present data on the glycogen content in fish erythrocytes expand our understanding of the importance of the involvement of this carbohydrate substrate in the erythrocyte energetics and open new avenues for further investigations. These investigations should be aimed at identifying the factors and mechanisms that cause activation/deactivation of the glycogen involvement in energy metabolism of fish erythrocytes, which is important for understanding of adaptive abilities of these cells and hydrobionts in general.

AUTHORS' CONTRIBUTION

Yu.A. Silkin and E.N. Silkina were responsible for designing the experiments, data collection, writing and editing the manuscript. M.Yu. Silkin provided technical support during experiments, participated in data processing and discussing, as well as in graphic representation.

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COMPLIANCE WITH ETHICAL STANDARDS

Experimental protocols met all applicable international, national and institutional principles of handling and using experimental animals for scientific purposes.

This study did not involve human subjects as research objects.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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