Сибирский экологический журнал, 2 (2016) 228–236

УДК 577.15: 57.042: 57.017.32

DOI 10.15372/SEJ20160207

Seasonal variability of some biochemical parameters in the whitefish (*Coregonus muksun* and *Coregonus lavaretus*)

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Статья поступила 02.06.2015

Принята к печати 23.06.2015

ABSTRACT

The base levels and seasonal variability of ethoxyresorufin-O-deethylase (EROD) activity, glutathione-S-transferase (GST) activity, reduced glutathione concentration (GSH) and phospholipids concentration (PhL) of *Coregonus lavaretus* (Linnaeus, 1758) and *C. muksun* (Pallas, 1814) were investigated. The relationships between this biochemical markers, hepatosomatic index and seasonal growth were also examined.

Key words: *Coregonus*, EROD, GST, GSH, phospholipids, season.

The survival of living organisms depends on their ability to adapt to the habitat alterations. Biochemical adaptation means modification of the functioning and the content of certain macromolecules to maintain homeostasis of the internal environment under the changing conditions. Molecules involved in biochemical adaptation vary in accordance with current metabolic demands and as biomarkers may indicate the general state of the organism.

In this context, a number of biochemical parameters (biomarkers) have been investigated to assess disturbances of biological functions in aquatic organisms during biomonitoring studies [Sheehan, Power, 1999; Adams, 2002; Van der Oost et al., 2003; Martínez-Álvarez et al., 2005; Lehtonen et al., 2006]. It was established that most of the biochemical responses are not specific and susceptible to environmental factors fluctuations, such as diet, salinity, dis-

solved oxygen, etc. The metabolic rate of ectotermic organisms is also directly related to water temperature. These confounding factors may mask the effect of contaminant-induced stress signals and make the interpretation of the results quite challenging [Pavlović et al., 2010; Fonseca et al., 2011; Oliva et al., 2012; Koenig, Solé, 2012]. It was reported, that sometimes in aquatic ecosystems environmental variables have even greater influence on biomarkers than xenobiotic substances [Pavlović et al., 2004]. Thus, natural vulnerability of biomarkers must always be considered when designing an experiment and analyzing the obtained results.

Xenobiotic biotransformation enzymes and components of antioxidant defense system have been extensively used as valuable biomarkers of adverse effects of pollutants on hydrobionts [Martínez-Álvarez et al., 2005; Koenig, Solé, 2012]. However, little is known regarding seasonal differences in xenobiotic metabolism and antioxidant defense of aquatic organisms. Information is needed not only on the effects of anthropogenic factors, but also on the physiological adaptations of hydrobionts to a habitat [Lehtonen et al., 2006].

In this paper, biomarkers of xenobiotic metabolism such as enzymes ethoxyresorufin-O-deethylase (EROD) and glutathione-S-transferases (GST) as well as antioxidant defenses components, such as concentration of reduced glutathione (GSH) were studied. One of the important sources of cellular reactive molecules inducting the antioxidant defense system is lipids with their easily oxidized double bonds in the carbon chains, such as phospholipids (PhL). In this work, the relation between the studied biomarkers and the level of PhLs, which are the major structural molecules of biomembranes, was also evaluated.

The species selected were whitefishes *Coregonus lavaretus* (Linnaeus, 1758) and *C. muksun* (Pallas, 1814). As other Salmonids, the whitefishes generally prefer cold, clean, well-oxygenated water. Both species mostly feed on bottom-dwelling invertebrates or zooplankton and breed in autumn between September and November. *C. lavaretus* has big morphological diversity with a number of ecological forms. The fish length varies from 10–15 cm to 30–60 cm; the maximum age is 15–20 years. *C. muksun* is semi-anadromous Siberian endemic fish, most of the year it feeds in the area with low salinity. Its maximum age is 20–25 years, the average length of the fish is 40–60 cm [Berg, 1962; Reshetnikov, 1988; Evolution..., 2004; Kottelat, Freyhof, 2007]. These species are of commercial interest in fisheries and aquaculture in the northlands and can be chosen as an experimental model for biomonitoring studies in this region.

The main objective was to characterize baseline levels and the natural variability of selected biomarkers in two northern freshwater species *Coregonus lavaretus* and *C. muksun*. The investigation covered three different periods in the whitefish lifecycle: the first is low metabolic rate season before the ice break-up, the second is the beginning of the active feeding and growth period in spring, and the third is the most active feeding and growth period at the end of summer. The relationship between biomarker activities, hepatosomatic index and season related growth rate were also investigated. The current study forms part of a wider project investigating the biology of these two closely related whitefish species.

MATERIALS AND METHODS

Fish sampling. Juvenile fish (1+) of both species were obtained from a local fish farm (Ladoga lake, 61°60′ N, 30°9′ E). Samplings were done in March, May and August of 2013. The fish were visually healthy, without signs of infection and other considerable pathologies. Nine individuals of the same size were killed by severing the spinal cord, weighed, and liver and pieces of white muscle were excised. The tissues were weighed and divided into two parts. The first part was fixed in 96 % ethanol for lipid analysis. For an enzymatic assay the tissues were frozen in liquid nitrogen until further analysis.

Environmental parameters. Environmental parameters (average month temperature and oxygen concentration in the water) and feeding regime values were kindly provided by the fish farm administration (Table 1). Farmed fish were fed to satiation in accordance with the commercial protocol. Feeding frequency was

Table 1

Water temperature, dissolved oxygen values and feeding frequency during the study

	Water temperature, °C	Dissolved oxygen, mg · L^{-1}	Feeding frequency, times per week
March	0.2	9.3	2
May	3.9	11.5	7
August	15.8	7.6	14

minimal in the cold period followed by increasing feed application rate with the maximum in the end of summer.

Growth-related parameters. Specific Growth Rate (SGR) was calculated according to the following equation: $100 \cdot [\ln W_1 - \ln W_0] \cdot (\text{days})^{-1}$, where W is the weight of the sampled fish in grams, W_0 and W_1 are the initial and the final mean weights of the fish in grams. Hepatosomatic index (I_H, %) was determined as: liver weight · 100/fish weight.

Biochemical assay. The biochemical parameters were measured using the facilities of the Equipment Sharing Centre of the Institute of Biology, KarRC of RAS.

The EROD activity was measured fluorometrically in the liver tissue, according to the method described by M. D. Burke and R. T. Mayer [1974], after certain modifications. Samples were homogenized in an ice-cold buffer solution (EDTA 0.05 mM, *tris*-HCl 0.05 M, pH 7.5) using the Potter-Elvehjem homogenizer. Homogenates were centrifuged at 12 000 g for 30 min (at 4 °C). The resulting supernatant was further centrifuged at 100 000 g for 60 min (at 4 °C) to obtain the microsomal pellet. The enzymatic reaction was started by adding a reaction mixture (600 µl of *tris*-HCl buffer (pH 7.4), 1 µl of 1 mM 7-ethoxyresorufin and 20 µl of 60 mM NADPH (β-nicotinamide adenine dinucleotide phosphate) to 250 µl of supernatant. A baseline of fluorescence was recorded at Ex 510 nm and Em 586 nm. The fluorimeter was calibrated with 1 µl resorufine (50 µM in DMSO). Specific enzymatic activity was defined as the amount of substrate metabolized by the enzyme per minute per milligram of soluble protein (pg min^{-1} mg protein^{-1}).

For GST activity determination about 0.1–0.2 mg of the fish livers and muscles were individually homogenized in 1 ml of 50 mM *tris*-HCl buffer (pH 7.5) containing 5 mM EDTA by Potter-Elvehjem homogenizer. Homogenates were centrifuged at 100 000 g for 60 min at 4 °C. GST activity was evaluated with 1-chloro-2.4-dinitrobenzene as a substrate. Final reaction mixture comprised 0.125 M phosphate buffer (pH 6.5), 1 mM CDNB and 1 mM GSH. The reaction was started by adding 0.1 ml of the homogenate to 0.9 ml of the reaction mixture. The activity rate was measured as change in optical density at 340 nm ($\varepsilon = 9.6\ \text{mM}^{-1} \cdot \text{cm}^{-1}$). Specific enzymatic activity was defined as the amount of substrate metabolized by the enzyme per minute per milligram of soluble protein ($\text{nM} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$).

The supernatant remaining after GST assay was used for determination of reduced glutathione level. The concentration of GSH was determined using a modified procedure of V. H. Cohn, J. Lyle [1966] and P. J. Hissin, R. Hilf [1976]. Soluble proteins were precipitated from the supernatant using 5 % trichloroacetic acid. The resulting precipitate was separated by centrifugation at 2500 g for 15 min. The supernatant was led up by alkali to pH 8.5 and then 5 mM EDTA in 0.4 M *tris*-HCl buffer (pH 8.5) was added. The reaction was started by adding 0.01 % *ortho*-phthalaldehyde in methanol. After 15-min incubation at room temperature with mixing, the fluorescence of the reaction product was measured (Em at 420 nm and Ex at 350 nm). The final concentration of GSH was determined using different concentrations of reduced glutathione with 0.01 % *ortho*-phthalaldehyde as a standard. The relative concentration of glutathione GSH was expressed in micrograms GSH per mg of soluble protein ($\mu\text{g} \cdot \text{mg protein}^{-1}$).

The protein content of the samples was measured spectrophotometrically in the supernatant by recording the absorption at Ex 205 nm, using bovine serum albumin as a standard [Noble, Bailey, 2009; Sukhovskaya et al., 2010].

For lipid extraction and phospholipids analysis samples (0.3–0.5 g) were homogenized in 5 volumes of 96 % ethyl alcohol and fixed in the chloroform: methanol solvent system (2 : 1 v/v). Total lipids of muscles and liver samples were extracted following the method of J. Folch et al. [1957]. Total lipids were fractioned by ascending thin-layer chromatography in the petroleum ether: diethyl ether: acetic

acid solvent system (90 : 10 : 1 v/v) at room temperature [Stahl, 1965]. The position of phospholipids (PhL) was determined by standard phospholipid mixture (P3817 "Supelco", USA). The concentration of PhL in question was determined by spectrophotometry and expressed in % of dry tissue [Sidorov et al., 1972].

Data analysis. The statistical analyses were performed with R 3.1.1. Statistical Software. The non-parametric Mann-Whitney U-test was used to seek significant differences between the samplings. Relations between parameters were analyzed using Spearman's correlation coefficients. Data were presented as the median ± deviation of median.

RESULTS AND DISCUSSION

The water temperature and feeding regime are the key factors influencing fish growth performance and maturation processes and therefore their physiological status. During the cold season the overall level of the fish metabolism is low and growth processes are suppressed. When temperature increases, fish consume more feed and grow faster (Table 2). In this work, juvenile whitefish from three sampling periods were studied: before the ice break-up in March, in late spring in May and in the end of summer in August. In the sampling zone the water temperature during that time increased from 0.2 °C in March to 15.8 °C in August (see Table 1). Dissolved oxygen levels were relatively stable and favorable for the whitefish species.

C. muksun showed the considerable increase of growth rate in parallel with temperature and feed consuming rate (see Table 2). *C. lavaretus* demonstrated a different pattern: the rapid growth in spring (the growth rate being four times higher than that of *C. muksun*) followed by a marked decrease in August. These results probably reflect the differences in the biology of the two species. *C. muksun* is characterized by a longer life span and relatively late puberty that begins at the age of 6–7 years. In turn, *C. lavaretus* has greater growth rate during the first years of life and earlier puberty at the age of 3–5 years [Fish..., 2010]. It probably indicates that the sharp increase in *C. lavaretus* growth rate at spring is largely due to genetics than feeding.

Hepatosomatic index alters differently in the studied species. In *C. muksun* the liver portion in general was bigger than in *C. lavaretus,* with maximum share obtained in the beginning of the active feeding and growth period followed by a decrease in August. In *C. lavaretus* I_H was relatively constant with a slight decrease in the end of the study. The high values of hepatosomatic index (hepatomegaly) often indicate morphological disturbances in liver caused by different toxic effects, including imbalanced diet [Grigorjev, Sedova, 2008]. In this study there was no elevation of this parameter, which acknowledged a low negative impact of commercial feed on the farmed fish during the studied period.

Numerous studies have revealed that differences in sex and body size can interfere with enzymatic activities and complicate the interpretation of biomarker results [Van der Oost et al., 2003]. In this study the whitefish were immature, hence, the influence of sex and reproductive stage were left aside. Interconnections between growth processes and biomarkers response were investigated by correlation analysis and are presented in the Table 3. A moderate positive correlation (r = 0.5–0.7) was observed in *C. lavaretus* tissues between body size and both GST and GSH. In *C. muksun* these parameters also tended to rise with the fish

Table 2

Seasonal alterations of growth-related parameters in *C. muksun* and *C. lavaretus*

Growth-related parameters	Fish	March	May	August
SGR, %/day	*C. muksun*	–	0.3	0.8
	C. lavaretus	–	1.3	0.9
I_H, %	*C. muksun*	1.50 ± 0.10	1.86 ± 0.09	1.17 ± 0.10
	C. lavaretus	1.18 ± 0.12	1.10 ± 0.15	1.00 ± 0.17

Table 3

Correlations (Spearman's correlation coefficients) between the studied biochemical parameters and fish weight during the study

	EROD	GST	GSH	PhL
		Liver		
C. muksun	−0.07	0.37	0.70	−0.38
C. lavaretus	0.02	0.64*	0.66*	−0.23
		Muscles		
C. muksun	N.d.	0.44	0.49	−0.39
C. lavaretus	N.d.	0.58*	0.54*	−0.52

* Correlations are significant ($p < 0.05$, $n = 27$); N.d. – not determined.

weight, although the correlation coefficients were not significant.

The correlation analysis also indicated some coordinated actions of the studied biochemical parameters. A significant correlation was observed between GSH content and GST activity in the fish livers, which was moderate in *C. muksun* ($r = 0.42$) and strong in *C. lavaretus* ($r = 0.77$). GSTs are part of the phase II of the xenobiotic biotransformation pathway, which catalyses the conjugation of the toxic compounds and organic hydroperoxides with reduced glutathione. Moreover, glutathione also can non-enzymatically bind with variety of oxyradicals, including the main non-protein antioxidant which is important in defense against oxidative injuries [Presnell et al., 2013; Aquilano et al., 2014]. Thus, the obtained results can reflect the cooperative regulation of enzymatic and non-enzymatic components of antioxidant defense in the whitefish, a trend that has also been reported in other studies [Haluzová et al., 2011; Ibrahim, Harabawy, 2014].

At the same time, there were no significant correlations between EROD and GST activities in the whitefish livers. The EROD is the phase I xenobiotic biotransformation enzyme, which is responsible for the biotransformation (mainly oxidation) of numerous compounds, thus providing substrates for phase II enzymes. It was reported about possible simultaneous upregulation phase I and phase II battery of enzymes through the AhR signaling pathway [Nebert et al., 2000]. Such relationship between EROD and GST activity was confirmed during several aquatic field studies, mainly in the areas under anthropogenic pollution [Jiménez-Tenorio et al., 2008; Fonseca et al., 2011; Güngördü, Ozmen, 2011]. To our mind, poor coordination of biotransformation enzymes confirms the low contamination of water by hydrocarbon compounds.

Moderate increase of GST activity together with a decrease of PhL ($r = -0.62$) was detected in *C. muksun* liver. In accordance with previous studies [Hazel, Williams, 1990; Tocher et al., 2008] we expected that, as a potential source of lipid peroxides, phospholipids would positively correlate with antioxidants such as GST and GSH. At this point the obtained results indirectly indicated the low level of phospholipids peroxidation which could activate the antioxidant defense system in the whitefish tissues. The negative correlation in *C. muksun* was probably the result of a simultaneous increase of GST activity and decrease of PhL during the fish growth (see Table 3). Other biochemical parameters analyzed in the liver, as well as biomarkers in the muscles, exhibited no significant relationships.

Many studies have suggested that antioxidant defense enzymes and other biomarkers fluctuate significantly throughout the year with regard to environmental temperature and season-related growth rate [Pavlović et al., 2004, 2010]. In order to assess the natural variability of biotransformation phase I and II enzymes and PhL and GSH in juvenile whitefish, we examined their activity in different seasons. The fluctuations of the studied biomarkers in the whitefish liver and muscles are presented on the Fig. 1, 2, respectively.

There was no significant effect of season on the EROD activity in the liver of *C. muk-*

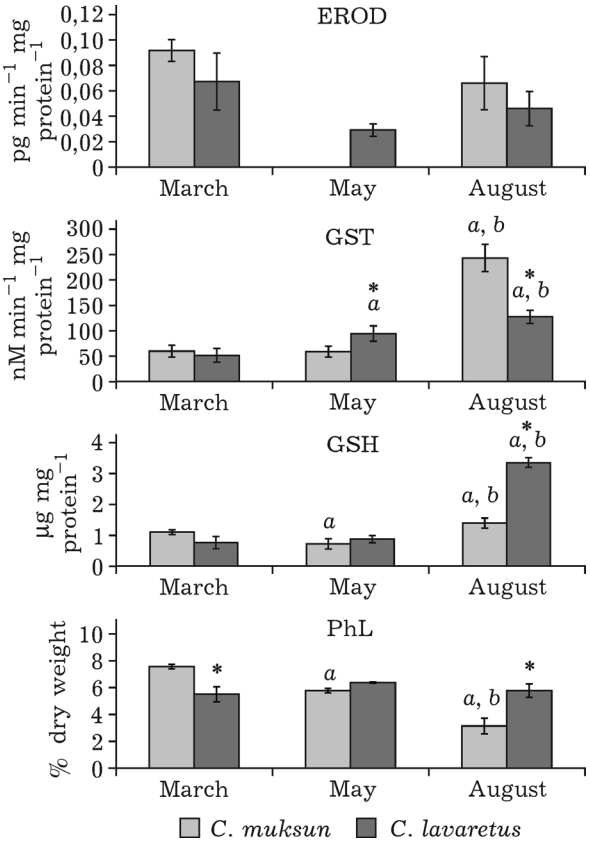

Fig. 1. Seasonal changes in biomarkers of liver of *C. muksun and C. lavaretus:* ethoxyresorufin-O-deethylase activity (EROD), glutathione *S*-transferase (GST) activity, concentration of reduced glutathione (GSH) and concentration of phospholipids (PhL).

a – differences are significant compared to March; *b* – differences are significant compared to May; * – differences are significant between *C. muksun* and *C. lavaretus* ($p < 0.05$)

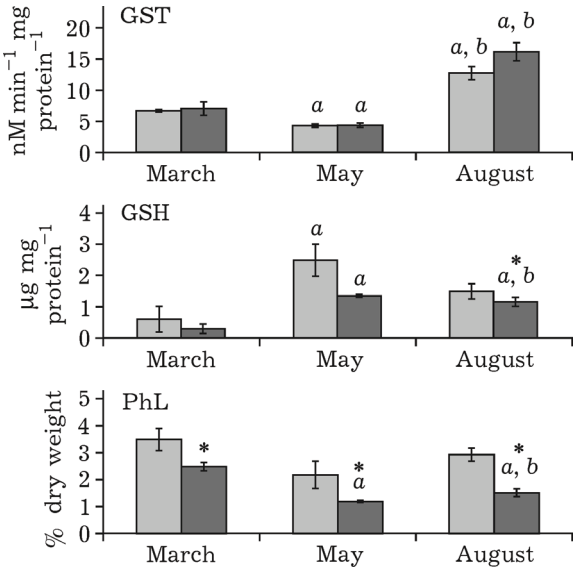

Fig. 2. Seasonal changes in biomarkers of muscles of *C. muksun* and *C. lavaretus:* glutathione *S*-transferase (GST) activity, concentration of reduced glutathione (GSH) and concentration of phospholipids (PhL).

a – differences are significant compared to March; *b* – differences are significant compared to May; * – differences are significant between *C. muksun* and *C. lavaretus* ($p < 0.05$)

sun and *C. lavaretus*. In both species hepatic EROD tended to decrease in August, with the minimum in May (data available for *C. lavaretus*).

Seasonal fluctuations of this enzyme have been investigated multiple times [Mathieu et al., 1991; Chiang et al., 2012; Koenig, Solé et al., 2012; Dévier et al., 2013]. It was shown that a causal relationship between EROD activity and annual environmental changes is somewhat unstable and highly dependent on fish species. For instance, for dab (*Limanda limanda*) from Seine Estuary (France) it was found that over the years the EROD activity differed in the same season [Dévier et al., 2013]. In the case of marked seasonality, EROD activity is considered to be related to sex steroid production [Chiang et al., 2012]. In order to eliminate the possible effect of reproductive status on EROD seasonal fluctuations the juvenile whitefish was taken for our study.

GST activities and reduced glutathione contents showed important seasonal variations in the whitefish liver and muscles. Hepatic GST activity in *C. lavaretus* and *C. muksun* was higher in the end of summer than during the cold season. In *C. muksun* liver this increase was detected later (in August), but it was twice more pronounced than in *C. lavaretus*. In the fish muscles significant differences in GST activity were obtained during the studied seasons. For both species it showed similar behavior, i. e. the decrease of the activity was recorded in May followed by significant induction in August. The hepatic glutathione level fluctuations were more pronounced in *C. muksun* livers with significant decrease in May and increase in August. In *C. lavaretus* liver the GSH content significantly rose in August (two times higher than that of *C. muksun*). Similarly, seasonal glutathione variations were de-

tected in the fish muscles. The minimum GSH content was detected in March and the maximum – at the beginning of the warm period.

Thus, the minimum GST activity and the concentration of glutathione in the tissues of *Coregonus* spp. occured in the spring months, while the maximum values were obtained in late summer, except for the GSH level in the muscles – there the maximum GSH concentration was recorded in late spring. Previously it was shown, that elevation of GST activity and GSH content during the study may partially be related to fish growth (see Table 3). Temperature is another essential factor that can affect these parameters. The results obtained by N. M. Aras et al. [2009] in the study of biomarkers of *Salmo trutta caspius*, *S. t. labrax* and *S. t. macrostigma* showed that the highest liver GST level was in autumn (in October) for all sub-species. During the warm period from April to July the GST activity in *S. t. labrax* liver increased similar to that of *Coregonus* sp. However in *S. t. caspius* and *S. t. macrostigma* the GST activity was minimal in summer. It is noteworthy, that in this study the level of lipid peroxidation showed similar changes during the study, with significant increases in autumn and decreases in summer [Aras et al., 2009]. S. Koenig and M. Solé [2012] showed that hepatic GST of a deep-sea fish *Alepocephalus rostratus* showed highest activity during autumn. The reduction of the activity at lower environmental temperature was detected in eelpout (*Zoarces viviparous*) in the Baltic Sea [Ronisz et al., 1999] and in mussel (*Mytilus galloprovincialis*) [Viarengo et al., 1999]. V. Machado and collaborators [2014] determined that the level of reduced glutathione is directly dependent on water temperature: in the liver of the Antarctic fish *Notothenia rossii* the GSH content increased while rising the temperature to 8 °C, although the GST activity and lipid peroxides level showed a down-regulation. It could be concluded that the regulation of the studied biochemical markers is a very complicated process, because together with the level of oxidation, others factors (temperature, reproductive status, development stage, etc.) can change these parameters, very often in different directions. Change of season means change of the whole complex of these factors, so a unique response from each of the fish species should be expected.

Different seasonal dynamics of PhL were noted for the liver of the studied fish. In *C. muksun* the concentration of PhLs tended to decrease from March to August, whereas for *C. lavaretus* no significant alterations were found. PhL values of the muscles of the two species showed similar changes during the study, with higher levels during the cold months and decreases in summer. In *C. lavaretus* the overall level of these components was lower during the observation. The different pattern of phospholipids behavior for these two species can be due to a varying efficiency of PhL assimilation from the feed. It is known that in case of insufficient dietary intake of PhLs, their synthesis in fish liver increases [Tocher et al., 2003]. Therefore, a higher level of hepatic PhL in *C. lavaretus* in comparison to *C. muksun* can indicate active synthesis of these molecules in the liver due to insufficient supply of dietary PhL. It was established that excessive alteration of membrane's fluidity caused by temperature changes is regulated by adjusting their lipid composition [Hazel, 1979; Hazel et al., 1991]. Hence, seasonal decline of phospholipids probably reflects nonspecific modifications of the biomembranes.

The obtained results describe seasonal variability of the studied parameters in the whitefish species. The phase II enzyme glutathione *S*-transferase and reduced glutathione varied more strongly during the study period. These data suggest that detoxification and antioxidant reactions can be caused by a factor not related with contamination, and interpretation of the responses of these biomarkers in biomonitoring studies requires accurate characterization of environment conditions.

CONCLUSIONS

The obtained data on the activity of biotransformation phase I and II enzymes and contents of low molecular weight antioxidant GSH and structural membrane component PhL showed a seasonal pattern of these biomarkers in the liver and white muscles of the whitefish species. Antioxidant biomarker's response (GST and GSH) was in general higher

in warmer season compared to cold periods and these changes were partially related to growth processes, which in turn also depended on temperature and annual cycle. Effects of other seasonal exogenous and endogenous factors on the studied parameters are also possible.

All in all, the observed fluctuations in the studied parameters, except for the PhL level, were very similar for *C. muksun* and *C. lavaretus*. This confirms the considerable similarity of metabolic processes in these closely related species. In turn, the content of phospholipids, as well as growth rate characteristics, indicate some interspecific differences. In the present work farmed fish was used as an experimental model, however further experiments will be useful for the accurate characterization of the natural variability of biomarkers in *Coregonus* spp. from wild populations.

The work was supported by State project N 51.3 (N 01201358735) and The Russian President's Grant "Scientific Schools" HSh-1410. 2014.4. The authors warmly thank the fish farm administration for the kindly provided materials for the experiments.

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