

Lipid composition in response to temperature changes in blue mussels *Mytilus edulis* L. from the White Sea

N.N. FOKINA, T.R. RUOKOLAINEN, I.N. BAKHMET AND N.N. NEMOVA

Institute of Biology, Karelian Research Centre of RAS, 185910 Pushkinskaja st., 11. Petrozavodsk, Russia

Alterations of membrane lipid composition (cholesterol, phospholipids and their fatty acids) in response to various temperature changes were studied in blue mussels Mytilus edulis L. from the White Sea. Lipid composition changes after acute temperature stress, especially a temperature drop, included a significant reduction of the membrane phospholipid content directly (1 h) after return to the initial temperature, which was presumably a consequence of a non-specific stress reaction in the mussels. A longer recovery period (24 h) as well as long-term temperature acclimation (14 days) induced changes in gill fatty acid composition (for instance, a rise in phospholipid unsaturated fatty acids under low temperature impact), indicating 'homeoviscous adaptation' to maintain the membranes in response to temperature fluctuations. Moreover, the gill cholesterol level in mussels varied especially at long-term temperature exposure.

Keywords: lipids, fatty acids, mussels, acute, long-term, temperature, adaptation

Submitted 14 November 2014; accepted 3 March 2015; first published online 17 April 2015

INTRODUCTION

Temperature is the most important factor for marine poikilothermic organisms whose metabolic rate is determined by its environmental variables. Different physiological, biochemical and molecular regulation mechanisms provide poikilotherm adaptation/acclimation to various temperature fluctuations (Hochachka & Somero, 2002). It is known that membranes play diverse and essential roles as physical barriers, controlling the transport of molecules, establishing ion gradients, and acting in membrane-based cell signalling (Hazel & Williams, 1990). Various kinds of environmental stress, including temperature, cause alterations in physical properties of cell membrane lipids that are reflected in the membrane dynamics (mainly fluidity). The membrane fluidity influences the adaptive response of the organism to environmental stresses by regulating the activity of membrane-associated enzymes, ion channels and pumps, receptors and others (Hazel & Williams, 1990; Hazel, 1995). The most consistent biochemical mechanism of poikilotherm acclimation at temperature fluctuations includes 'homeoviscous adaptation' of membrane fluidity, which is accompanied by an enlarged unsaturated fatty acid content resulting in increased fluidity at low temperature (Sinensky, 1974; Hazel & Williams, 1990; Cossins, 1994). The timeframe of 'homeoviscous adaptation' during temperature acclimation of bivalves is still unclear (Williams & Somero, 1996; Pernet *et al.*, 2006). The poikilotherm lipid and fatty acid composition response to long-term temperature acclimation was mostly investigated. It was found that changes in membrane lipid fatty acid composition occurred

after a period of acclimation, varying in duration from 1 week in case of warm acclimation to several weeks during cold acclimation (Hazel & Williams, 1990; Williams & Somero, 1996; Pernet *et al.*, 2008). It has been shown however that intertidal mussel membranes can be restructured within hours in response to temperature fluctuations during the tidal cycle (Dey & Farkas, 1992; Williams & Somero, 1996; Pernet *et al.*, 2007). Gills in filter-feeding mussels are more sensitive to various natural environmental effects (Baršienė *et al.*, 2012). Since bivalve mollusc gills represent the site of initial contact with environmental factors, modification of the lipid composition (mainly phospholipids and their fatty acid composition, as well as cholesterol) in the organ reflects the primary compensatory response to stress. The aim of this study was to compare the response of gill cholesterol and phospholipid levels and phospholipid fatty acids composition to acute (24 h) and long-term (14 days) temperature effects in blue mussels *Mytilus edulis* L. living in a stable aquaculture environment with no tidal effects.

MATERIALS AND METHODS

Sampling and experiments were carried out at the 'Kartesh' Biological Research Station of the Zoological Institute RAS (Chupa Bay, Gulf of Kandalaksha, White Sea).

Acute temperature experiment

Mussels *Mytilus edulis* L. were collected from mariculture collectors in Kruglaya Bay (Kandalaksha Gulf, White Sea) at a depth of 1.5–2.0 m in April (0–3°C) and August (8°C) of 2012. The animals were acclimated to laboratory conditions for 72 h, where water temperature corresponded to the temperature of

Corresponding author:

N.N. Fokina

Email: fokinann@gmail.com

natural water at the time of experiments. The water temperature in the aquarium was raised from 0–3 to 8°C (in April) and reduced from 8 to 0–3°C (in August). Samples were collected after 1 and 24 h exposure. Thereafter, the mussels were returned to the initial temperature conditions: 0–3 and 8°C. Water in the aquarium was aerated and changed daily, no supplementary feeding was provided. Gill samples (N = 5) were taken for the lipid composition analyses 1, 24 and 72 h later.

Long-term temperature experiment

Mussels *Mytilus edulis* L. were collected from aquaculture site 'Sonoströv' (<http://www.mariprod.ru>), White Sea (66°09'00" N 34°10'00" W) in August 2011. Upon arrival, animals were sorted by size and age (shell length 58.5 ± 3.1 mm, 3+ years old) and acclimated to laboratory conditions for 72 h prior to starting the experiment. Water temperature and salinity in aquaria corresponded to the natural water conditions at the time of experiments and were 15°C and 25 psu, respectively. The long-term temperature experiment involved acclimation (14 days) of the blue mussels to different water temperatures (5, 15 (for control) and 20°C). Animals were fed with 'Coraliquid' (Sera, Germany, <http://www.sera.de>) twice a day. After the experimental exposure periods were over, mussel gills (N = 5) were fixed in 96% ethanol for further analysis.

Lipid composition analysis

The research was carried out using the facilities of the Equipment Sharing Centre of the Institute of Biology, Karelian Research Center RAS.

Lipids were extracted from blue mussel, *Mytilus edulis*, gills with chloroform/methanol (2:1, v/v) following the method of Folch *et al.* (1957). The extracted lipids were spotted onto silica gel thin-layer chromatography plates 'Sorbfil' and separated into different fractions of lipid classes: phospholipids, cholesterol, triacylglycerols and cholesterol ethers, using petroleum ether/diethyl ether/acetic acid (90:10:1, v/v) as the mobile phase. The quantitative composition of total lipid fractions was measured in SF-2000 UV/Vis spectrophotometer (Saint-Petersburg, Russia) at wavelength 540 nm for phospholipids, triacylglycerols and cholesterol ethers fractions, as well as at 550 nm for the cholesterol fraction (Sidorov *et al.*, 1972; Engelbrecht *et al.*, 1974). Individual phospholipid fractions (phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine, lysophosphatidylcholine and sphingomyelin) were analysed by high-performance liquid chromatography in isocratic liquid chromatograph with UV detector 'Stayer' (Moscow, Russia) using silica gel column Nucleosil 100-7, 250 mm ('Elsico', Moscow, Russia) and mobile phase: acetonitrile/hexane/methanol/phosphoric acid (918:30:30:17.5, v/v). The detection wavelength was 206 nm (Arduini *et al.*, 1996). Peaks were identified by reference to retention times of authentic standards ('Sigma Aldrich' and 'Supelco', USA). Fatty acid methyl esters (FAME) from the phospholipid fraction were prepared using methanol and acetyl chloride according to Tsyganov's method (1971). FAME were separated in a gas-liquid chromatograph 'Kristall.4000' (Yoshkar-Ola, Russia) with flame ionization detector using columns 'Zebron ZB-FFAP' (50–0.32 mm) ('Phenomenex', USA) and helium as the mobile phase.

Mussel phospholipid FAME were identified by comparison with standard mixes ('Supelco', USA).

Statistical analysis

The results of the mussel lipid composition changes are given as mean \pm SD of five gill samples. Statistical analysis was carried out using StatSoft Statistica v 7.0. Normality of distribution for all datasets was tested with the Kolmogorov–Smirnov test. As a majority of the data was found not to meet homogeneity standards, the data were analysed using the non-parametric Mann–Whitney U as well as a Kruskal–Wallis test with a Tukey *post hoc* test (Hill & Lewicki, 2007). The differences were considered significant at $P < 0.05$.

RESULTS

Acute temperature effect

Temperature rise from 0–3 to 8°C during 1 and 24 h did not affect the levels of membrane lipids such as cholesterol, major (phosphatidylcholine and phosphatidylethanolamine) and minor membrane phospholipids (phosphatidylinositol, phosphatidylserine and sphingomyelin) as well as the phospholipid fatty acid composition (Figure 1). Return to the initial temperature (0–3°C) for 1 h caused an acute decrease in the concentration of all phospholipid fractions, whereas longer exposure of the mussels to 0–3°C (for longer than 24 h) led again to a rise in these phospholipids, as well as in n-3 polyunsaturated fatty acids (PUFA) (mainly eicosapentaenoic 20:5n-3 and docosahexaenoic 22:6n-3 acids, EPA and DHA, respectively).

Temperature lowered from 8 to 0–3°C (1 and 24 h) and subsequent return to the initial temperature conditions (8°C) had no effect on the level of cholesterol, phospholipids and their fatty acid composition in blue mussel gills except for sphingomyelin content, which was increased at exposure to 8°C temperature for 1 h (Figure 2).

Long-term temperature effect

In the temperature acclimation experiment (14 days) the greatest changes in gill membrane fatty acid composition were revealed under low (5°C) temperature impact (Figure 3). Unlike low temperature impact, which produced an increased level of unsaturated fatty acids including non-methylene-interrupted ones (NMIFA) and decreased the saturated fatty acid concentration in mussel gills, high temperature impact (20°C) caused a reduction in n-6 PUFA content. A rise in cholesterol level was noted at both temperature impacts (5 and 20°C). A significant increase in phosphatidylserine (PS) and sphingomyelin (SPH) content was observed in the mussels under high temperature (20°C) impact.

DISCUSSION

Gill membranes of bivalve molluscs represent the site of initial contact with environmental factors (temperature, salinity, etc.). Various kinds of environmental effects induce alterations in membrane physical properties (mainly fluidity)

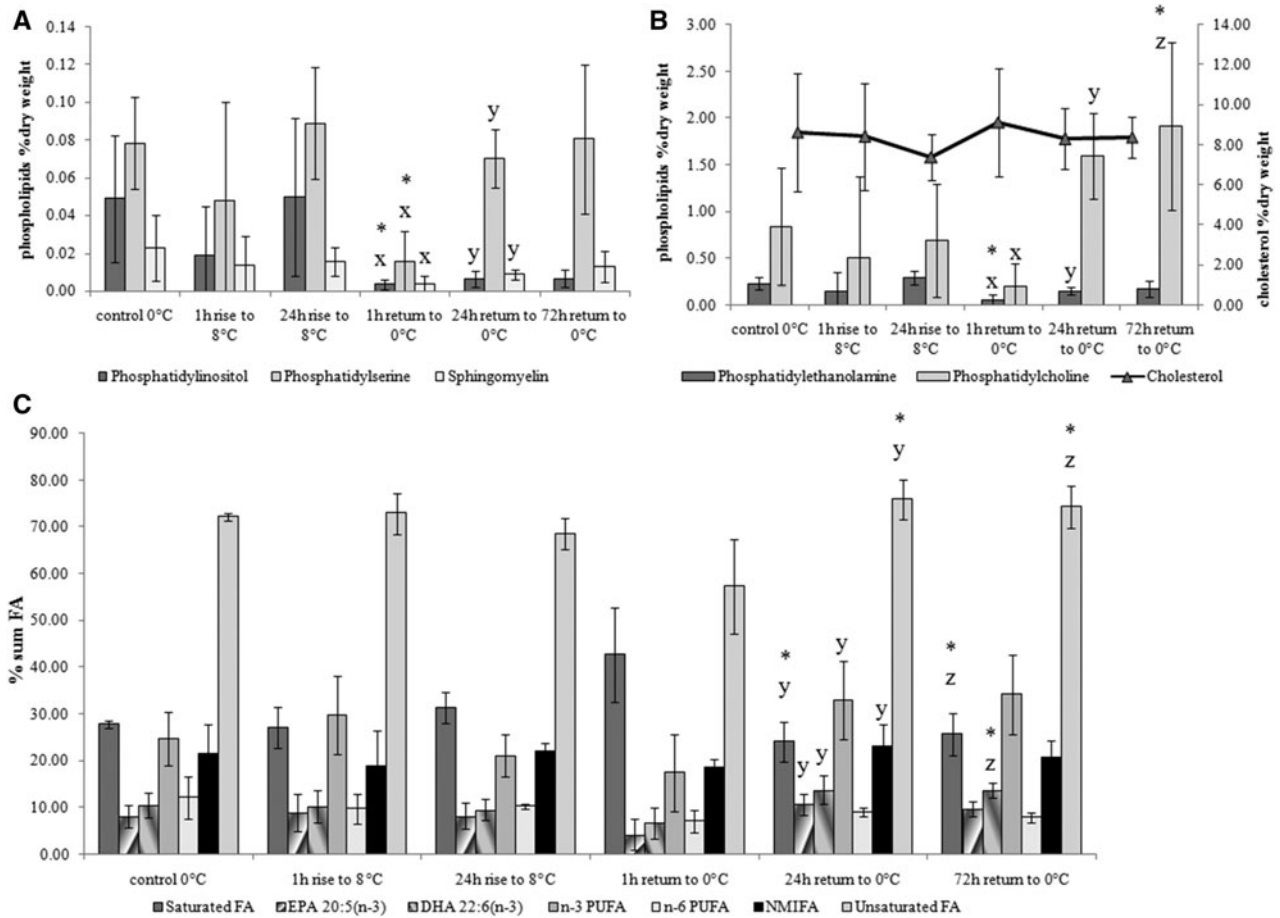


Fig. 1. Changes in minor (A: phosphatidylinositol, phosphatidylserine and sphingomyelin) and major (B: phosphatidylcholine, phosphatidylethanolamine, cholesterol) membrane lipids and phospholipid fatty acid composition (C) of blue mussels *Mytilus edulis* gills in response to acute temperature rise from 0–3 to 8°C and return to the initial 0–3°C temperature. Values are means \pm SD (N = 5). Significant results in comparison of datasets are indicated by alphabet letters and asterisks. Differences indicated with alphabetic letters were estimated by the non-parametric Mann–Whitney U test, $P < 0.05$: (x) – comparison of 24 h rise to 8°C with 1 h return to 0°C; (y) – comparison of 1 h return to 0°C with 24 h return to 0°C; (z) – comparison of 1 h return to 0°C with 72 h return to 0°C; other variants of pair-wise comparisons showed no significant differences. Differences indicated with asterisks – were estimated by Kruskal–Wallis test with a Tukey *post hoc* test, $P < 0.05$ (same pairs of comparison).

reflected in ion permeability and the activity of membrane-associated proteins (Hazel & Williams, 1990; Los & Murata, 2004). Cholesterol and phospholipids are the main lipid components of biological membranes. The ratio of phospholipids to cholesterol is considered as an indicator of membrane fluidity (Vance & Vance, 2002). Cholesterol is an essential component in animal membranes with multiple effects on their fluidity. Cholesterol is known to increase the order of the surrounding fatty acid chains in membranes (Crockett, 1998; Pernet *et al.*, 2006). It should be noted that in some bivalve molluscs an increased gill cholesterol level was described under short-term temperature changes, whereas in response to long-term temperature acclimation modifications of phospholipids containing PUFA were observed (Pernet *et al.*, 2006; Parent *et al.*, 2008). It was suggested that the use of cholesterol as a modulator may not require any expenditure of ATP or other reduced cofactors (Crockett, 1998) and hence the modulation of the phospholipid: cholesterol ratio probably provides a metabolically less expensive mechanism of membrane remodelling than *de novo* synthesis of PUFA-rich phospholipids (Pernet *et al.*, 2006; Parent *et al.*, 2008). In contrast to these previous reports, the increased cholesterol content in the gills of

White Sea blue mussels was probably due to its additional synthesis, required to stabilize gill membrane fluidity under long-term (14 days) exposure to both low (5°C) and high (20°C) temperature, whereas in response to short-term (1 day) temperature stress changes in phospholipid content and their fatty acid composition were noted. A decrease in major membrane phospholipids as well as their minor fractions under rapid temperature change (1 h) from 8 to 0–3°C presumably reflected a non-specific stress reaction of blue mussels to abrupt temperature drop. Prolonged exposure of the mussels to 0–3°C for 24 h caused an increase in the content of a majority of phospholipids and their n-3 PUFA. It is well known that *Bivalves* cannot *de novo* synthesize long-chain PUFA (such as EPA and DHA), which need to be obtained from the diet (Pernet *et al.*, 2006). It was suggested that the incorporation and elimination of essential fatty acids in membrane lipids in bivalve molluscs is strongly regulated (Delaunay *et al.*, 1993; Soudant *et al.*, 1998; Parent *et al.*, 2008; Pernet *et al.*, 2008). The adaptive mechanism of fatty acid membrane lipids regulation (mainly PUFA-rich phospholipids) to counteract the short-term low temperature effects in the gills of White Sea blue mussels is perhaps less energy expensive than additional synthesis of cholesterol,

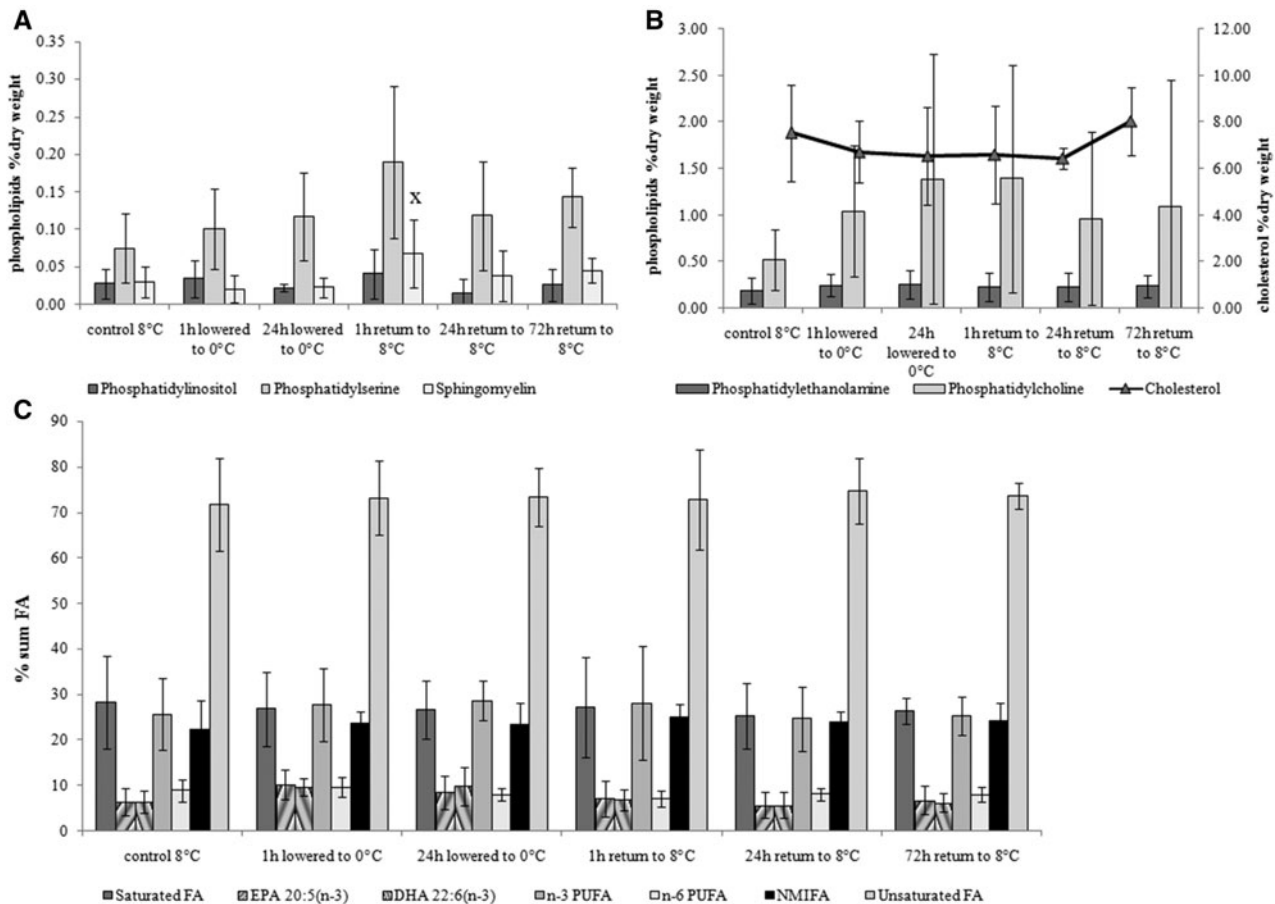


Fig. 2. Changes in minor (A: phosphatidylinositol, phosphatidylserine and sphingomyelin) and major (B: phosphatidylcholine, phosphatidylethanolamine, cholesterol) membrane lipids and phospholipids fatty acid composition (C) in blue mussel *Mytilus edulis* gills in response to acute temperature drop from 8 to 0–3°C and return to the initial temperature of 8°C. Values are means \pm SD (N = 5). Significant results in comparison of datasets are indicated by alphabet letters. Differences indicated with alphabetic letters were estimated by the non-parametric Mann–Whitney U test, $P < 0.05$; (x) – comparison of 24 h lowered to 0°C with 1 h return to 8°C; other variants of pair-wise comparisons showed no significant differences.

whereas to acclimate to a long-term low temperature exposure blue mussels use both these mechanisms. Moreover, blue mussel acclimation to 5°C temperature during 14 days promoted an increase in the level of gill phospholipid unsaturated fatty acids together with non-methylene-interrupted fatty acids (NMIFA), which are believed to be also involved in regulation of membrane fluidity in poikilothermic organisms (Rabinovich & Ripatti, 1991; Pernet *et al.*, 2006; Barnathan, 2009). Bivalve molluscs are known to be able to *de novo* synthesize NMIFA (Zhukova, 1991). Apparently, blue mussels use the ability for additional synthesis of these fatty acids, which allows them to regulate membrane fluidity under low temperature impact.

Increased PS and SPH content in the gills of mussels acclimated at 20°C and in response to a temperature rise from 0–3 to 8°C can be presumed to indicate changes in the membrane structure and properties (mainly fluidity). A similar response of these phospholipids was noted in our previous study on the experimental effect of salinity on lipid and fatty acid composition of blue mussels *Mytilus edulis* L. from the White Sea (Nemova *et al.*, 2013). It was assumed that in the situation of seawater desalination an increased content of SPH, which plays a multiple role in regulation of cell functions (Vance & Vance, 2002), contributes to the acclimation of blue mussels to low salinity (Nemova *et al.*, 2013). Moreover, the rise in PS content in

mussel gills exposed to both low and high seawater salinities was supposed to modify the activity of membrane-associated enzymes, ion channels and pumps (Nemova *et al.*, 2013). Since these phospholipids are involved in different aspects of cell metabolism, including cell volume regulation, we assume that PS and SPH in conjunction with cholesterol can contribute to adjusting membrane fluidity to prevent the adverse effect of high temperature. It is known that high temperature stress can cause damage to membrane lipids, which can increase the permeability of cell membranes (Kültz, 2005) and induce osmotic changes in gills (Nordlie, 2009).

Thus, the changes in lipid composition in blue mussel *Mytilus edulis* gills in response to a rapid temperature change (1 h) is assumed to be a consequence of non-specific physiological reaction of the bivalve to environmental stress (such as shell valves closing). On the other hand, a recovery period longer than 24 h after a temperature decrease, as well as long-term temperature acclimation (14 days), led to modifications in the gill fatty acid composition (for instance, increased unsaturated fatty acids content under low temperature influence) indicating ‘homeoviscous adaptation’ to maintain the membrane integrity and permeability in mussel gills under temperature fluctuations. Keeping in mind the cellular stress response described by Kültz (2005), one may assume changes in the mussel gill lipid composition in response to acute temperature

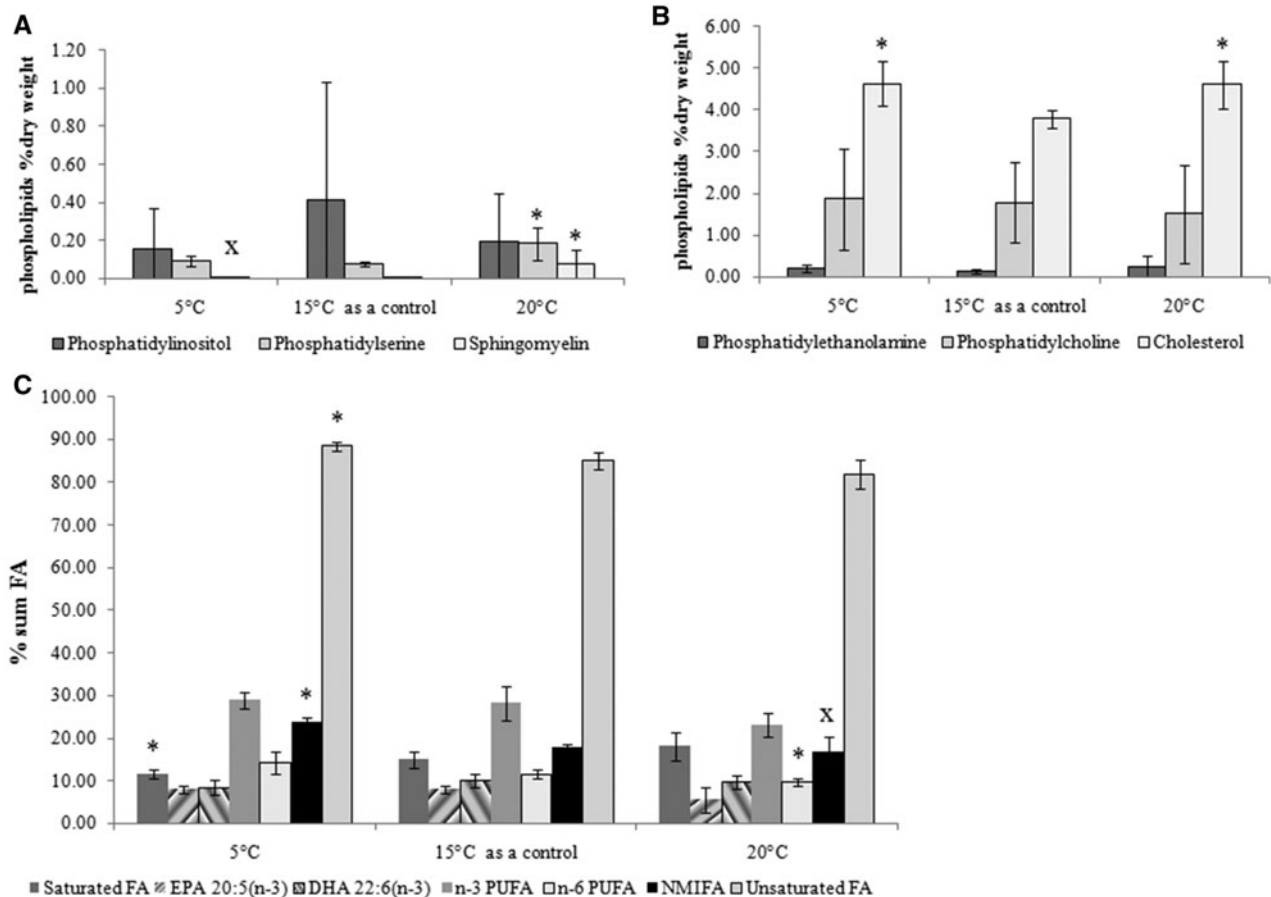


Fig. 3. Changes in minor (A: phosphatidylinositol, phosphatidylserine and sphingomyelin) and major (B: phosphatidylcholine, phosphatidylethanolamine, cholesterol) membrane lipids and phospholipid fatty acid composition (C) in blue mussel *Mytilus edulis* gills in response to long-term temperature impact. Values are means \pm SD (N = 5). Significant results in comparison with the control are indicated by asterisks and alphabet letters. Differences indicated with asterisks were estimated by the non-parametric Mann–Whitney U test, $P < 0.05$. Differences indicated with alphabetic letters – were estimated by the Kruskal–Wallis test with a Tukey *post hoc* test, $P < 0.05$: (x) – comparison of 5°C with 20°C; other variants of pair-wise comparisons showed no significant differences.

impact represent its physiological adaptive mechanism and serve the protection of cell membranes (termed ‘cellular stress response’), whereas the lipid composition reaction to long-term temperature influence is to maintain and restore the cell membrane homeostasis under new environmental conditions (termed ‘cellular homeostatic response’).

ACKNOWLEDGEMENTS

The authors are grateful to staff of the White Sea Biological Research Station ‘Kartesh’ of the Zoological Institute of RAS for the possibility to carry out research at the station. We are also deeply grateful to I.V. Sukhovskaya, PhD, researcher at the Laboratory of Ecological Biochemistry, Institute of Biology, Karelian Research Centre of RAS for assistance in conducting the experiments and sampling of mussels.

FINANCIAL SUPPORT

The study was supported by federal funding for governmental-order project theme No. 51.3, No. 01201358735 and grants from the Russian Presidential programme ‘Leading Scientific Schools’ SS 1642.2012.4 and SS-1410.2014.4, the

Russian Foundation for Basic Research No. 12-04-93081-Norv_a and No. 12-04-32205-mol_a, the RAS Presidium programme ‘Living Nature’ (Reg. no 01201262107).

REFERENCES

- Arduini A., Pescechera A., Dottori S., Sciarri A.F., Serafini F. and Calvani M. (1996) High-performance liquid chromatography of long-chain acylcarnitine and phospholipids in fatty acid turnover studies. *Journal of Lipid Research* 37, 684–689.
- Barnathan G. (2009) Non-methylene-interrupted fatty acids from marine invertebrates: occurrence, characterization, and biological properties. *Biochimie* 91, 671–678.
- Baršienė J., Rybakovas A., Garnaga G. and Andreikėnaitė L. (2012) Environmental genotoxicity and cytotoxicity studies in mussels before and after an oil spill at the marine oil terminal in the Baltic Sea. *Environmental Monitoring and Assessment* 184, 2067–2078.
- Cossins A.R. (1994) Homeoviscous adaptation of biological membranes and its functional significance. In Cossins A.R. (ed) *Temperature adaptation of biological membranes*. London: Portland Press, pp. 63–76.
- Crockett E.L. (1998) Cholesterol function in plasma membranes from ectotherms: membrane-specific roles in adaptation to temperature. *American Zoologist* 38, 291–304.

- Delaunay F., Marty Y., Moal J. and Samain J.F.** (1993) The effect of monospecific algal diets on growth and fatty acid composition of *Pecten maximus* (L) larvae. *Journal of Experimental Marine Biology and Ecology* 173, 163–179.
- Dey I. and Farkas T.** (1992) Temperature shifts induce adaptive changes in the physical state of carp (*Cyprinus carpio* L.) erythrocyte plasma membranes *in vitro*. *Fish Physiology and Biochemistry* 10, 347–355.
- Engelbrecht F.M., Mari F. and Anderson J.T.** (1974) Cholesterol. Determination in serum: a rapid direction method. *South African Medical Journal* 48, 250–256.
- Folch J., Lees M. and Sloan-Stanley G.H.** (1957) A simple method for the isolation and purification of total lipids animal tissue (for brain, liver, and muscle). *Journal of Biological Chemistry* 226, 497–509.
- Hazel J.R.** (1995) Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? *Annual Review of Physiology* 57, 19–42.
- Hazel J.R. and Williams E.E.** (1990) The role of alterations in membrane lipid-composition in enabling physiological adaptation of organisms to their physical environment. *Progress in Lipid Research* 29, 167–227.
- Hill T. and Lewicki P.** (2007) *Statistics: methods and applications*. Tulsa, OK: StatSoft, <http://www.statsoft.com/textbook/>
- Hochachka P.M. and Somero G.N.** (2002) *Biochemical adaptation*. Princeton, NJ: Princeton University Press.
- Kültz D.** (2005) Molecular and evolutionary basis of the cellular stress response. *Annual Review of Physiology* 67, 225–257.
- Los D.A. and Murata N.** (2004) Membrane fluidity and its role in the perception of environmental signals. *Biochimica Biophysica Acta* 1666, 142–157.
- Nemova N.N., Fokina N.N., Nefedova Z.A., Ruokolainen T.R. and Bakhmet I.N.** (2013) Modifications of gill lipid composition in littoral and cultured blue mussels *Mytilus edulis* L. under the influence of ambient salinity. *Polar Record* 49, 272–277.
- Nordlie F.G.** (2009) Environmental influences on regulation of blood plasma/serum components in teleost fishes: a review. *Reviews in Fish Biology and Fisheries* 19, 481–564.
- Parent G.J., Pernet F., Tremblay R., Sevigny J.M. and Ouellette M.** (2008) Remodeling of membrane lipids in gills of adult hard clam *Mercenaria mercenaria* during declining temperature. *Aquatic Biology* 3, 101–109.
- Pernet F., Tremblay R., Comeau L. and Guderley H.** (2007) Temperature adaptation in two bivalve species from different thermal habitats: energetics and remodelling of membrane lipids. *Journal of Experimental Biology* 210, 2999–3014.
- Pernet F., Tremblay R., Gionet C. and Landry T.** (2006) Lipid remodeling in wild and selectively bred hard clams at low temperatures in relation to genetic and physiological parameters. *Journal of Experimental Biology* 209, 4663–4675.
- Pernet F., Tremblay R., Redjah I., Sévigny J.M. and Gionet C.** (2008) Physiological and biochemical traits correlate with differences in growth rate and temperature adaptation among groups of the eastern oyster *Crassostrea virginica*. *Journal of Experimental Biology* 211, 969–977.
- Rabinovich A.L. and Ripatti P.O.** (1991) The flexibility of natural hydrocarbon chains with non-methylene-interrupted double bonds. *Chemistry and Physics of Lipids* 58, 185.
- Soudant P., Marty Y., Moal J., Masski H. and François Samain J.** (1998) Fatty acid composition of polar lipid classes during larval development of scallop *Pecten maximus* (L.). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 121, 279–288.
- Sidorov V.S., Lizenko E.I., Bolgova O.M. and Nefedova Z.A.** (1972) *Fish lipids. 1. Analysis technique*. Petrozavodsk: Karelian Branch of the USSR Academy of Science.
- Sinensky M.** (1974) Homeoviscous adaptation – a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. *Proceedings of the National Academy of Sciences USA* 71, 522–525.
- Tsyganov E.P.** (1971) A method of direct lipid methylation after TLC without elution with silica gel. *Lab Delo* 6, 490–493. [in Russian]
- Vance D.E. and Vance J.E.** (eds) (2002) *Biochemistry of lipids, lipoproteins and membranes*. 4th edition. Amsterdam: Elsevier.
- Williams E. and Somero G.** (1996) Seasonal-, tidal-cycle- and microhabitat-related variation in membrane order of phospholipid vesicles from gills of the intertidal mussel *Mytilus californianus*. *Journal of Experimental Biology* 199, 1587–1596.
- and
- Zhukova N.V.** (1991) The pathway of the biosynthesis of nonmethylene-interrupted dienoic fatty acids in mollusks. *Comparative Biochemistry and Physiology Part B Comparative Biochemistry* 100, 801–804.

Correspondence should be addressed to:

N.N. Fokina
 Institute of Biology, Karelian Research Centre of RAS, 185910
 Pushkinskaja st., 11. Petrozavodsk, Russia
 email: fokinann@gmail.com