Nonspecific stress response to temperature increase in *Gammarus lacustris* Sars with respect to oxygen-limited thermal tolerance concept

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This study is the first time when dynamics of HSP70 and lipid peroxidation products was assessed, along with activity of lactate dehydrogenase in gradual temperature increase impacting *Gammarus lacustris* Sars amphipods from the saltwater lake Shira (Khakassia Republic). The animals were exposed to gradual temperature increase (rate of increasing 1°C/h, duration of exposure 33 hours) starting from mean annual temperature of the lake (7°C). It was shown that gradual increase of ambient temperature causes a complex of biochemical reactions in *G.lacustris*, which are expressed in lower lactate dehydrogenase activity and launching of lipid peroxidation. The HSP70 level did not change significantly during the entire exposure to gradual temperature increase. Within the concept of oxygen-limited thermal tolerance, accumulation of the most toxic lipid peroxides (triene conjugates and Schiff bases) in phospholipids occurs at the same time and temperature of exposure as accumulation of lactate. It shows that the main criterion of overriding the temperature threshold is transition to anaerobiosis, confirmed by elevated lactate level, and development of cellular stress, which is expressed as accumulation of lipid peroxidation products. The obtained data confirm the earlier hypothesis that compared to freshwater lake population, the increased thermotolerance of *G. lacustris* from the saltwater lake is caused by differences in energy metabolism and energy supply of nonspecific cellular stress-response mechanisms.

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Abstract

This study is the first time when dynamics of HSP70 and lipid peroxidation products was assessed, along with activity of lactate dehydrogenase in gradual temperature increase impacting *Gammarus lacustris* Sars amphipods from the saltwater lake Shira (Khakassia Republic). The animals were exposed to gradual temperature increase (rate of increasing 1°C/h, duration of exposure 33 hours) starting from mean annual temperature of the lake (7°C). It was shown that gradual increase of ambient temperature causes a complex of biochemical reactions in *G. lacustris*, which are expressed in lower lactate dehydrogenase activity and launching of lipid peroxidation. The HSP70 level did not change significantly during the entire exposure to gradual temperature increase. Within the concept of oxygen-limited thermal tolerance, accumulation of the most toxic lipid peroxides (triene conjugates and Schiff bases) in phospholipids occurs at the same time and temperature of exposure as accumulation of lactate. It shows that the main criterion of overriding the temperature threshold is transition to anaerobiosis, confirmed by elevated lactate level, and development of cellular stress, which is expressed as accumulation of lipid peroxidation products. The obtained data confirm the earlier hypothesis that compared to freshwater lake population, the increased thermotolerance of *G. lacustris* from the saltwater lake is caused by differences in energy metabolism and energy supply of nonspecific cellular stress-response mechanisms.

Introduction

Studying the interaction of organisms with the environment and identification of their mechanisms of adaptation to changing living conditions is one of the most important ecological research trends, which is particularly topical in terms of investigating the problem of global climate change (Pörtner, 2008, 2009; IPCC, 2014; Deutsch, 2015). Temperature is one of the factors that determine functioning and stability of ecosystems. In hydrobionts, metabolic rate and condition of their cellular protection system are closely related to the ambient temperature. Hence, temperature changes can impact metabolism of thermosensitive organisms and, hence, their survival (Timofeyev, 2010). In stress, consumption of energy and constructive metabolism products grows, and the organisms launch their protection systems. Among protection systems in hydrobionts, a great role belongs to nonspecific mechanisms of stress-adaptation, which include antioxidative system, heat shock proteins (HSP), and other (Pörtner, Knust, 2007).

In recent years, surface temperature of lakes throughout the world has grown significantly (about 0.34 °C within 10 years) (O'Reilly et al., 2015). Such rapid warming is a drastic signal for the need to study comprehensively the impact of climate change on the status of water ecosystems, to assess the fauna vulnerability and adaptive capacity. In addition, this induces the need to develop new methods and tools for environmental protection. Studying thermal tolerance mechanisms and energy metabolism components in aquatic organisms in changing ambient temperature is of essential interest and relevance.

The research work was carried out in order to study the effect of ambient temperature gradual increase on nonspecific mechanisms of stress response in *Gammarus lacustris* Sars amphipods from the saltwater lake Shira (Khakassia Republic).

Materials and methods

Animals

Holarctic species of the amphipod *G. lacustris* Sars, 1863 (Sars, 1863) is a broadly spread opportunistic species a wide ecological valence that lives in lentic and stream ecosystems (Väinölä, 2007; Takhteev, 2015). This species reproduces basically during summertime. Though, it can also have several reproductive periods depending on the water body environmental conditions and ecological characteristics (Timoshkin, 2001). From the previous research works, preferable temperature for this species is 15–16°C (Timofeyev, 2010). It is an euryhaline species, at the same time it is highly tolerant to a wide range of environment pH variations (6.2–9.2) (Timofeyev, 2010). Also, *G. lacustris* is highly tolerant to hypoxia, especially in low water temperature. Thereby, this species is a regular inhabitant of eutrophic water bodies. Food spectrum of *G. lacustris* is broad. Being an opportunistic species, in standard conditions it prefers detritus and plant food (Gladyshev et al., 2000).

Amphipod sampling

The amphipods were caught in July 2013 in saltwater lake Shira in the western part of Siberia (Khakassia Republic, Russia) (54°29'7.25" N, 90°12'1.49" E). This lake is one of the most saline water bodies (15–17 ‰), where *G. lacustris* inhabits. Chemical composition is presented as the following anion-cation ratio (mg/L): Cl^- - 2100, Na^+ - 2880, K^+ - 37, Mg^{2+} - 1080, CO_3^{2-} - 174, Ca^{2+} - 51, SO_4^{2-} - 8010, HCO_3^- - 998, pH of the environment is close to 8.7 (Kalacheva et al., 2002). This lake is meromictic, and *G. lacustris* takes the top of trophic pyramid occupying the bentoplanktonic niche, it is found both in littoral and in pelagic zone (Zadereev et al., 2010). The amphipods were sampled using standard hydrobiological method: with a plankton net, bottom to surface, from a boat – above about 7 meters depth. In summer, water temperature in the lake littoral zone can reach 28°C, mean annual temperature of water is 6–7°C (Rogozin et al., 2017).

Experimental design

In this study, we carried out the experiments exposing *G. lacustris* to gradual temperature increase. Prior to starting the experiment, amphipods were acclimated for 7 days at 7 ºC, in 2 L constantly aerated aquariums. The experimental animals were fed daily with potatoes *ad libitum*. Excess food was removed. During acclimation, the amphipods showed high motor activity and

no deaths, which can point at the fact that the acclimation conditions were not stressing for this species. In all experiments, we used active animals of the same size and age group free from mechanical damages and parasites (Atrashkevich, 2009). The temperature was increased at the rate of 1°C/h. Every two hours, fixation was conducted upon reaching a definite temperature – 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31 and 33 ºC. Samples (2–3 specimens per sample) were fixed in liquid nitrogen for further biochemical treatment (Sokolova, Pörtner, 2003).

Biochemical methods

Assessment of heat shock proteins 70 content

Total protein was isolated in 0.1 M Tris HCl (pH 7.6). The amount of protein in samples was determined using the M. Bradford method (Bradford, 1976) at 595 nm wavelength. HSP70 dynamics was determined using standard sodium dodecyl sulfate denaturating electrophoresis (SDS-PAGE) in 12.5% polyacrylamide gel, succeeded by the Western blotting technique (Laemmli, 1970). For HSP70 visualization, at first, the obtained membranes were incubated with antibodies to HSP70 (mouse antibodies to HSP70; Sigma-Aldrich, # H5147, 1:1000 dilution). Then, after washing off the unreacted antibodies, the membranes were incubated in solution of secondary antibodies conjugated with alkaline phosphatase (antibodies to mouse immunoglobulin IgG:AP Conj., Sigma-Aldrich # A3562, 1:1000 dilution). We used actin as reference protein. For actin visualization, the following antibodies were used: polyclonal antibodies to β-actin (rabbit antibodies Sigma-Aldrich #A266, 1:1000 dilution) and antibodies to rabbit immunoglobulin (Sigma-Aldrich #A9919, 1:1000 dilution). Semiquantitative analysis of the studied protein content on membranes was carried out using the ImageJ Package (v.1.41., Wayne Rasband, NIH, USA). Protein relative count is given in arbitrary units (arb. un.) calculated from the optic density of HSP70 binding sites divided by the optic density of actin binding sites.

Measurement of lactate dehydrogenase activity

Activity of lactate dehydrogenase (LDH) was measured using the enzymatic spectrophotometric method. The method is based on reaction of pyruvate converting into lactate. NADH in NAD^+ oxidation rate is proportional to LDH activity. Measurements were taken in buffered sodium phosphate solution (0.1 M, pH=7.5) using the LDH-Vital express kit (Vital–

Diagnostics Spb), as described in instruction, at 340 nm wavelength. Optic density was measured using the Carry 50 Conc UV/VIS analytical station (Varian, USA).

Measurement of lipid peroxidation product level

The levels of lipid peroxidation products were estimated from monochromatic light flux absorbed by lipid extract in UV spectrum according to the Deryugina et al. modified technique (Deryugina et al., 2010). Frozen specimens were ground in 1:1 heptane–isopropanol extraction mixture. Using the extraction mixture, homogenate volume was brought up to 4.5 ml. In order to separate fractions, 1 ml of distilled water was added, and afterwards, provided intensive stirring and incubation at 25°C for 30 min. After the phase separation, the isopropanol (lower) and heptane (upper) fractions were centrifuged for 2 min at 14 krpm. We added 97% ethanol (ratio 1:3) to the received supernatant, then, the solution optic density was measured using the Carry 50 Conc UV/VIS spectrophotometer (USA). Diene and triene conjugates, and Schiff bases were measured at the wave lengths of 232, 278 and 400 nm, respectively. Content of lipid peroxidation products was estimated in arbitrary units in terms of isolated double bonds as measured at 220 nm wavelength.

Statistical analysis

All the experiments were carried out with 3–8 biological replicates, and biochemical measurements for each sample were performed in triplicate (technical replicates) for each sample. Immunoblots were analysed using the ImageJ package (v.1.41., Wayne Rasband, NIH, USA). Normality was checked with the Kolmogorov-Simonov test. Data analysis was performed using the one-way ANOVA test, the Student-Newman-Keuls test was used as a post hoc-test. When the data distribution deviated from the normal, Kruskal-Wallis with Dunn test as a post hoc-test was used. With p-value <0.05, the differences were considered to be significant (to check statistical hypotheses with multiple testing, we also used the Bonferroni correction). Statistical data processing was performed with SigmaPlot package (version 12, Systat Software Inc., USA/Canada).

150 **Results**

151 In the performed study, it is shown that the HSP70 level did not change significantly
152 during the entire exposure to ambient temperature gradual increase from 7°C to 33°C (Fig. 1).

153 It is shown that the temperature gradual increase imposes effect on lactate
154 dehydrogenase, an important component of anaerobic metabolism (Fig. 2). During the exposure,
155 *G. lacustris* demonstrated a reliable 16-fold decrease of the enzyme activity from 151.57±3.80
156 nKat/mg of protein to 9.19±3.83 nKat/mg of protein on reaching 11°C. Within the range from
157 11°C to 21°C, the enzyme activity remained low. After that, we observed a short-term
158 reactivation up to 56.37±21.68 nKat/mg of protein and 75.97±27.69 nKat/mg protein in exposure
159 temperature of 25°C and 27°C respectively. However, at the exposure temperature over 27°C,
160 the enzyme activity was low again as related to control levels.

161 To assess the dynamic of oxidation processes under the temperature increase, we
162 measured the content of lipid peroxidation products such as diene conjugates, triene conjugates
163 and Schiff bases. These metabolites reflect various oxidation stages in an organism. From the
164 data obtained, in *G. lacustris* decreased the level of diene conjugates in neutral lipid fraction
165 (Fig. 3) when temperature reached 21°C (0.24±0.05) and 27°C (0.24±0.04). Changes of diene
166 conjugates content in phospholipid fraction were not observed till the end of the experiment.

167 It is shown that the ambient temperature gradual increase led to elevated triene conjugates
168 in *G. lacustris*, as related to control level, in composition of both phospholipids and neutral
169 lipids. Thus, the elevated levels of triene conjugates were observed in neutral lipids composition,
170 on reaching 31°C (two-fold as compared to the basal level, 0.25±0.03). With phospholipids, the
171 elevation occurred on reaching 31 and 33°C (0.24±0.03 and 0.24±0.02, respectively).

172 In analysis of the ambient temperature gradual increase effect on lipid peroxidation
173 endproducts (Fig. 5) in saltwater *G. lacustris*, both fractions showed changes in levels of Schiff
174 bases at 31°C. It should be noted that in case of neutral lipids, the reaction was short-term, while
175 in the phospholipid fraction, content of Schiff bases deviated from the basal level till the end of
176 exposure.

177

178 **Discussion**

179 To discover molecular underpinnings of the *G. lacustris* adaptive potential in dispersion
180 to new areas, and to assess the risks for the species populations habitating in contrast
181 environmental conditions in climate change, during this study we were revealing features of
182 some components of nonspecific cellular stress-response (NCSR) in saltwater population of the
183 species. According to early experimental data, the saltwater G. *lacustris* is more thermoresistant
184 than representatives of the same species freshwater population. Thus, LT50 (time of 50%
185 specimen's death under exposure at 30°C) in *G. lacustris* saltwater population amounted 22.8
186 hours, while LT50 in this species from a freshwater waterbody was noted after 7.7 hours of the
187 exposure. Estimation of death rate under exposure to ambient temperature gradual increase
188 revealed that deaths of 100% specimens in saltwater population of *G. lacustris* occurred two
189 hours later, compared to freshwater population from a water body in Irkutsk (Vereshchagina et
190 al., 2016).

191 From the data we obtained earlier, it was assumed that thermal tolerance of saltwater
192 population is associated with less metabolic cost of sustaining the osmotic pressure in a salt lake,
193 whose water is isoosmotic to hemolymph of freshwater amphipods. At the same time, the
194 freshwater population representatives live in hypoosmotic environment, and have to spend their
195 metabolic energy, which affects their ability to provide energy for NCSR (Vereshchagina et al.,
196 2016).

197 The components of NCSR are highly conservative; they are highly researched in a
198 number of model and nonmodel organisms (Lushchak, 2011; Elder, Seibel, 2015). Nevertheless,
199 molecular underpinnings of adaptation, which are caused by micro-evolution of NCSR
200 regulatory pathways, practically, are still not studied. Some of the well-studied and significant
201 NCSR components are heat shock proteins HSP70. They are essential in cell protection, when
202 not only temperature but also other environmental factors change. In terms of evolution, HSPs
203 are highly conservative proteins found in all organisms from bacteria to human. This evidences
204 that these proteins fulfil fundamental cellular functions. As molecular chaperones, HSPs
205 participate in multiple cellular processes including folding of protein molecules, and transport of
206 proteins through membranes; they also take part in renaturation of cellular proteins that were
207 partially denaturated by proteotoxic stressors (Tomanek, 2010; Shatilina et al., 2011). HSP70
208 participation in response to the changes of ambient temperature was shown with the example of

many organisms, where these proteins function as protectors preventing degradation of cellular proteins (Sørensen et al., 2003; Timofeyev, Steinberg, 2006). Stress-induced HSP70 synthesis is highly researched in model organisms. Therewith, partially denaturated proteins occurring in cells lead to activation of signal cascade and amplified gene expression of stress-induced *hsp70*. However, elevation of the stress-induced HSP70 is energy-consumptive; molecular and biochemical adaptation of the organisms to their temperature niches is often implemented through sustaining the pool of these proteins in the cells, in amount sufficient to protect cellular proteins from damage in significant variations of the environmental abiotic parameters (Bedulina et al., 2013). It is expressed in originally high constitutively-synthesized levels of HSP70 in cells, and there is no vibrant response to the stress. HSP70 constitutive levels, including both constitutive and stress-inducible HSP70 forms, can vary significantly both in different species and in separate populations of the same species adapted to different environmental conditions. Thus, our previous study showed that in *G. lacustris* from freshwater population (Irkutsk region), a significant 9-fold HSP70 elevation was observed in gradual temperature change, on reaching 31°C (Axenov-Gribanov et al., 2016). It is notable that multiple HSP70 accumulation in freshwater population was observed immediately prior to critical thermal death point of 100% specimens (Axenov-Gribanov et al., 2016). The results of current study demonstrate that such multiple elevation of HSP70 level does not occur in saltwater lake population. This shows that there are differences in the mechanisms for cellular regulation of stress-induced HSP70 synthesis, implemented in different populations of the same species. To reveal the nature of such differences is essential to understand the molecular underpinnings of the species phenotypic flexibility in adaptation to various environmental conditions.

Constitutive high HSP70 level, or other molecular chaperones, or other NCSR components in the cells of saltwater animals can be one of possible explanations of the demonstrated differences. Our previous study (Vereshchagina et al., 2016) showed the elevated levels of antioxidant enzymes (catalase and glutathione S-transferase) activity in saltwater *G. lacustris* compared to freshwater population. These high levels denote high constitutive level of NCSR in cells. Though, additional research is required to reveal the cause of the shown differences.

It is known that in ambient temperature variation, energy deficiency in cells grows due to malfunction of the electron transport chain; this forces the organisms to activate less efficient

energy recovery pathways. In particular, the anaerobic glycolysis pathway and changes in activity of lactate dehydrogenase (Axenov-Gribanov et al., 2016). During glycolysis, lactate dehydrogenase participates in reversible reaction of pyruvate–lactate conversion. In the presence of oxygen, pyruvate converts into acetyl coenzyme A and enters the Krebs cycle; however, in anaerobic conditions, or if the mitochondrial electron transport chain is damaged, pyruvate is reversibly converted into lactate (Devlin, 2011). During this study, is was shown that in Shira *G. lacustris*, activity of lactate dehydrogenase decreased 16-fold at the first stages of the experiment, when the temperature increased from 7 °C to 11 °C (Fig. 2). The decrease of lactate dehydrogenase activity can be induced by pyruvate levels increasing during glucose oxygen catabolism. In concentration over 4mM (Fregoso-Peñuñuri et al., 2017), pyruvate is capable of inhibiting the enzyme activity in crustaceans. Earlier, we showed elevation of adenosine triphosphate and depletion of glucose content in the first phases of exposure to gradual temperature increase (starting from 13 °C) (Vereshchagina et al., 2016). This also denotes activation of glucose oxygen catabolism. At 23 °C, lactate dehydrogenase activity increases up to control levels again, which can evidence the launched anaerobic glycolysis processes at these temperatures; though, when 27 °C is reached, the enzyme activity drops down again remaining low till the end of the experiment. It is notable that the multiple accumulation of lactate, as the main marker for anaerobiosis in crustaceans, was observed in this population only on reaching 31 °C. At the same time, the adenosine triphosphate level not showing significant decrease (Vereshchagina et al., 2016).

In our early studies, the decreased lactate dehydrogenase activity in gradual temperature increase was also observed in *G. lacustris* freshwater population on reaching 17 °C. Nevertheless, we observed direct correlation between elevation of lactate and decrease of ATP levels. This indirectly proves that anaerobic processes prevail over aerobiosis in freshwater population, with incomplete deactivation of aerobic process. Considerable and multiple lactate accumulation in freshwater population was noted on reaching 29 °C, which also came amid some decrease of lactate dehydrogenase activity (Axenov-Gribanov et al., 2016; Vereshchagina et al., 2016). Thus, the data received in this study support our early results concerning significant differences in energy metabolism regulation between *G. lacustris* salt and freshwater population.

One of possible causes of energy equilibrium displacement toward anaerobiosis can be the increasing oxidation processes in cells, and development of oxidative stress (Krone, 1994).

The latter often occurs with accumulation of oxygen active forms. In our study, this is indicated by the change in the levels of lipid peroxidation products – diene (primary products) and triene (secondary products) conjugates, and Schiff bases (endproducts) in *G. lacustris* (Fig. 3, 4, 5). It is notable that accumulation of the most toxic lipid peroxides (triene conjugates and Schiff bases) in phospholipids occurs at the same time and temperature of exposure as accumulation of lactate (Vereshchagina et al., 2016). This supports the concept of oxygen-limited thermal tolerance (Pörtner et al., 2017). The concept deals with molecular mechanisms of oxygen metabolism sustaining, which determine the limits of each species thermal tolerance. According to this concept, when environmental parameters deviate from optimal values, organisms switch their metabolism and increase the number of anaerobic energetic pathways. This leads to accumulation of lactate and other products of anaerobic metabolism (acetate, succinate and etc.) in tissues of animals. Development of cellular stress, changes in the structure and functions of cell membranes, and activation of lipid peroxidation processes occur at the same time.

In our study, in *G. lacustris* in neutral lipids (heptane fraction), decreased level of diene conjugates was observed when temperature reached 21°C (Fig. 3). Since no relevant growth was observed in triene conjugate and Schiff base levels, this can evidence that low-molecular antioxidants are included in antioxidant protection (Kenya et al., 1993; Mittler, 2002).

Conclusion

Thus, it was shown that the ambient temperature gradual increase causes a complex of biochemical reactions in the studied *G. lacustris*, which are expressed in lower lactate dehydrogenase activity and launching of lipid peroxidation. There is no multiple HSP70 elevation occurring, probably because of initially high pool of these proteins in cells, which is energy-efficient for organisms. The obtained data support the earlier hypothesis that the increased thermotolerance of *G. lacustris* from the saltwater lake Shira, as compared to freshwater lake population, is caused by the differences in energy metabolic processes and energy supply of NCSR mechanisms (Axenov-Gribanov et al., 2016; Vereshchagina et al., 2016). In developing global climate warming, this fact can be advantageous for saltwater *G. lacustris*. Besides, the studied biochemical reactions can be used as biomarkers for the stress status of aquatic organisms when their habitat temperature changes.

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Figure 1

HSP70 levels in Lake Shira *G. lacustris* amphipods in ambient temperature gradual increase.

HSP70 levels presented in arbitrary units, with respect to control level.

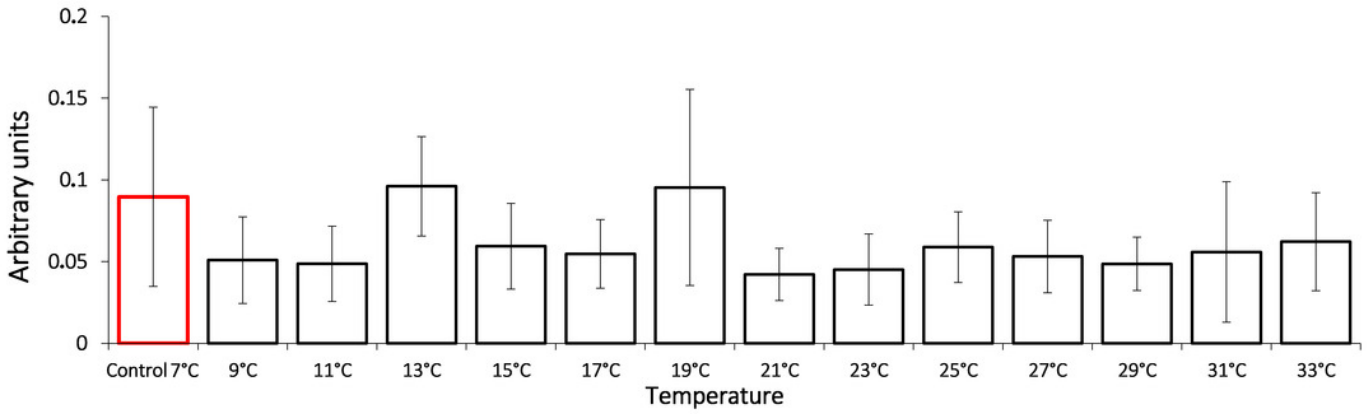

Figure 2

Lactate dehydrogenase activity (in nKat/mg of protein) in Lake Shira *G. lacustris* amphipods in ambient temperature gradual increase.

Asterisks (*) denote a significant change during the thermal exposure compared to the control animals $p<0.05$.

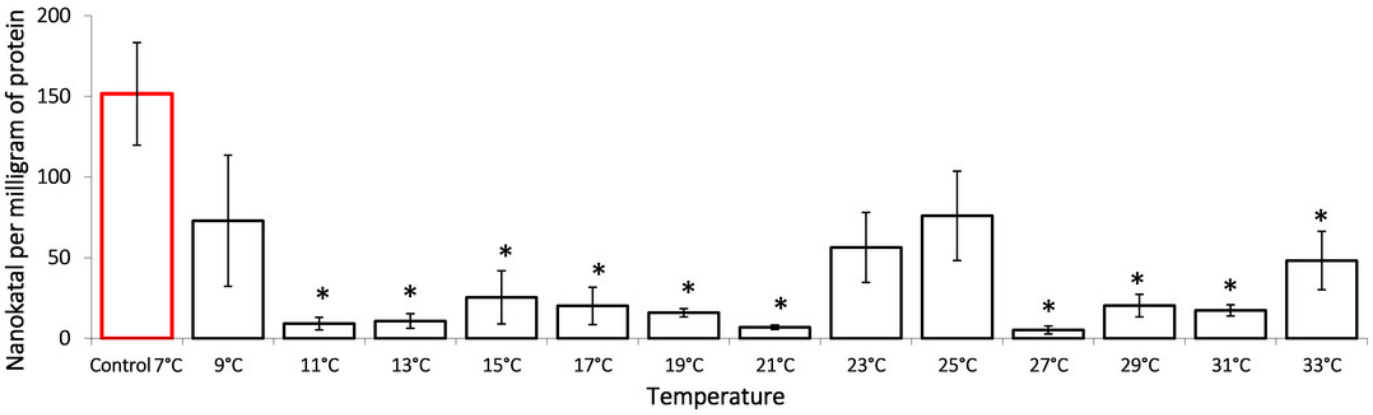

Figure 3

Levels of diene conjugate in neutral lipids (heptane fraction) and phospholipids (isopropanol fraction) in Lake Shira *G. lacustris* amphipods in ambient temperature gradual increase.

Asterisks (*) denote a significant change during the thermal exposure compared to the control animals $p<0.05$.

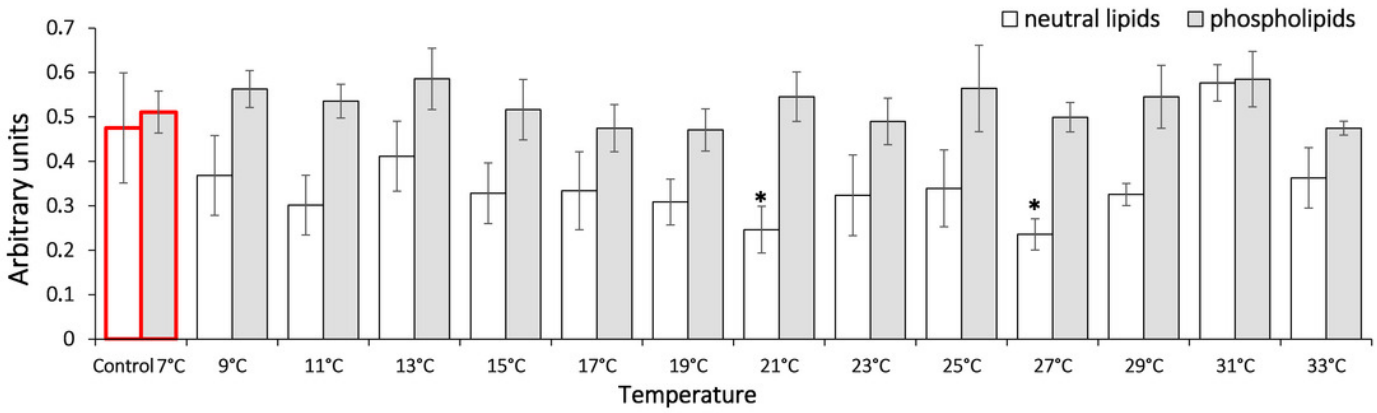

Figure 4

Levels of triene conjugate in neutral lipids (heptane fraction) and phospholipids (isopropanol fraction) in Lake Shira *G. lacustris* amphipods in ambient temperature gradual increase.

Asterisks (*) denote a significant change during the thermal exposure compared to the control animals $p<0.05$.

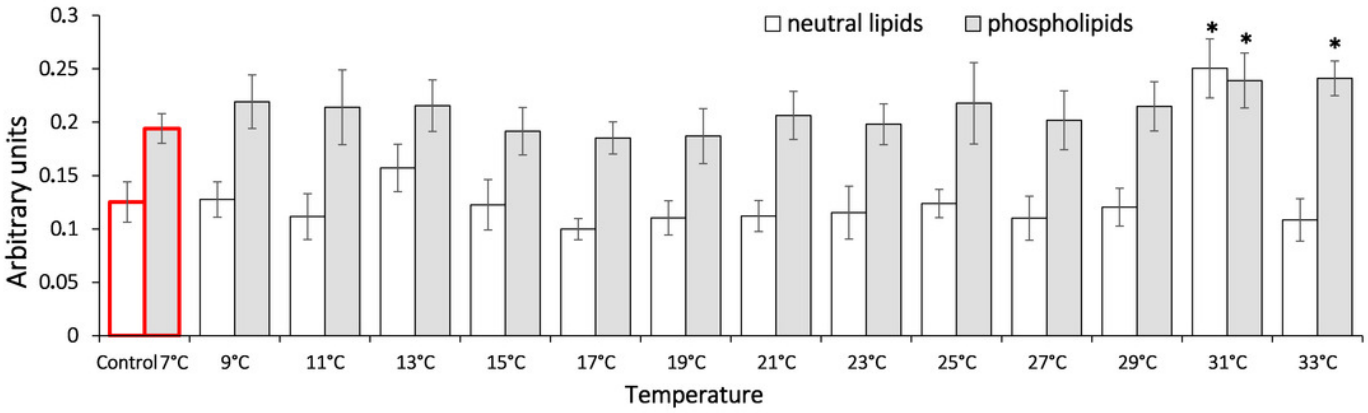

Figure 5

Levels of Schiff base in neutral lipids (heptane fraction) and phospholipids (isopropanol fraction) in Lake Shira *G. lacustris* amphipods in ambient temperature gradual increase.

Asterisks (*) denote a significant change during the thermal exposure compared to the control animals $p<0.05$.