Dependence of *Arctodiaptomus salinus* (Calanoida, Copepoda) halotolerance on exoosmolytes: new data and a hypothesis

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Abstract

Arctodiaptomus salinus is a widespread species in different regions including Mediterranean. Long-term study has shown the presence of the species in the Crimean lakes under the salinity from 5 to 210 ‰. The logarithm of the species density in a lake is inversely proportional to salinity. Earlier experimental studies have shown that the upper salinity limit of the species is 70-100 ‰. Authors explain this discrepancy of field and experimental data that at salinities above 70-100 ‰ copepods consume exoosmolytes, mainly with food. *A. salinus* is an osmokonformer; to survive under osmotic stress it utilizes mechanisms of the cellular level – accumulates the osmolytes. Every time when we found *A. salinus* under high salinities, we observed unicellular green algae *Dunaliella salina* bloom. *D. salina* intensifies the synthesis of osmolytes under high salinities, which can be up to 80 % of the dry weight of the cells. Probably *A. salinus* gets enough osmolytes by consuming *Dunaliella* to live under salinities above 70 ‰. Conclusions: The halotolerance limit of *A. salinus* is not determined purely by its physiology; the metabolism of community, as a whole, determines the upper limit of halotolerance of animals-conformers.

Introduction

A restricted number of Copepoda species have conquered hypersaline waters (Bayly & Boxshall 2009). One of the most widespread species among them is *Arctodiaptomus salinus* (Daday 1885) (Calanoida), Palaearctic species; its biogeographical area includes North Africa, Asia, and Europe (Alonso 1990, 2010; Rokneddine & Chentoufi 2004; Zhao et al. 2005; Marrone 2006; Demeter & Marrone 2009; Hamaidi et al. 2010; etc.). The species is common and abundant in the Crimea (Ukraine) – the biggest peninsula in the Black Sea (Balushkina et al. 2009; Belmonte et al. 2012; Anufriieva & Shadrin 2014). There are over 50 hyper-saline lakes, both marine and continental origin, in the Crimea (Kurnakov et al. 1936; Shadrin 2009). Lakes are shallow and polymixic, vary in size, in the range of fluctuations of abiotic factors and in biota inhabiting them. In lakes of marine origin there are the ratios of ions similar as in sea water, but the lakes of continental origin are sulfate lakes. Results of multiyear studies of lakes are given in earlier published papers (Shadrin 2009; Belmonte et al. 2012; Senicheva et al. 2008; etc.). The data on *A. salinus* halotolerance in these lakes are given at first time basing on our long-term study. Experimental studies of *A. salinus* give significantly lower limits of a salinity resistance of the species than values observed in nature. The purpose of the review

is to summarize a set of experimental and field data and offer plausible and verifiable hypothesis to explain the discrepancy between the results of experiments and observations in nature.

Material and methods

The results of our multiyear study of *A. salinus* in salt lakes of the Crimea (2004-2012) were used in this paper as well as published data of other researchers. In the lakes we collected the quantitative zooplankton samples by filtering 50-100 liters of water through Epstein net (pore size 110 µm), and then samples were fixed with 4 % formalin. Totally more than 200 samples were taken and analyzed. During sampling we measured the salinity, temperature, and pH. The number of animals was determined by direct counting under microscope with subsequent recalculation on the volume of filtered water. Standard statistical methods were used for the analysis of our dataset.

Results

More than 200 samples from 32 Crimean water bodies were collected and analyzed during 2004 to 2012. *A. salinus* was found in 54 samples of 17 reservoirs. The species was found in samples from March to December (in January and February we didn't collect samples). The species is common and abundant in the talassohaline as well as in atalassohaline-sulfate lakes. The maximum salinity at which the species was found was 210 ‰ (Lake Tobechik, 23.04.2009 and Lake Adzhigol, 08.08.2012), and minimum salinity - 5 ‰ (Freshwater Pond near v. Zavetnoye, 01.05.2009). *A. salinus* was encountered most frequently under salinities from 5 to 80 ‰ (Fig. 1), but at the salinities 100-210 ‰ it was also repeatedly noted. The maximum densities were observed at salinities 14-25 ‰ (to 340,000 ind./m^3), and under salinities more than 100 ‰ abundance of *A. salinus* not exceed 550 ind./m^3 (Fig. 1). Correlation analysis showed a reliable linear inverse relationship between logarithm of species density and salinity at a significance level of 0.0005. Dependence can be approximated by the equation:

$$y = - 0.0105x + 3.9242,$$

where y - lg of density, ind./m^3, x - salinity, ‰.

Our data lead us to conclude that pH (6.7 - 9.2) and temperature (4 - 32 °C) is not the important limiting factors for the species presence and abundance in the Crimean lakes.

Discussion

It is known that the minimal salinity at which the species can develop in nature is about 0.6 ‰ (Hamaidi et al. 2010), and it can become the dominant in plankton at 2 ‰ (Kipriyanova et al. 2007; Krupa et al. 2008). Experiments give the same value of minimal salinity (Svetlichny et al. 2012).

Presence and reproduction of the species at salinities up to 150-250 ‰ was observed in lakes of Siberia (Russia) (Vesnina 2003; Litvinenko et al. 2009) and Kazakhstan (Krupa 2010). In Lake Balkhash (Kazakhstan) there was found that *A. salinus* is a dominant species under salinities from 1 to 6 ‰, and there is a direct relationship between logarithm of species density and salinity (Krupa et al. 2008); value of the correlation coefficient was close to one found in our study. Taking into account that result and our data we can conclude this relationship is not linear in total salinity range. There is the positive relation between species density and salinity in range 1- 6 (10) ‰, and there is negative relationship under salinities higher then 15-20 ‰.

Halotolerance of *A. salinus* was evaluated in the experiments with *A. salinus* from long-term laboratory culture (Svetlichny et al. 2012). There was shown that adults survive under salinity of 1 ‰ to 70 ‰, but above 50 ‰ they don't reproduce. From other experiments it was concluded that the optimal salinity range for the species is 15-30 ‰ (Rokneddine & Chentoufi 2004). Our experiments with *A. salinus* from Lake Yanyshskoye under high microalgae concentration (blooming) showed that its upper limit of halotolerance was 100-110 ‰ (Anufriieva & Shadrin 2014).

Based on the experiments and observations on various water bodies the researchers often conclude that the species normally can exist and grow at salinities no higher 50-70 ‰. Does it mean that it is really upper limit of *A. salinus* halotolerance? What do we need to do with the many observations on lakes of the Crimea, Kazakhstan, Western Siberia, when there were the observations on mass development and even outbreaks of *A. salinus* at salinities well above 100 ‰, and at least up to 200 ‰?

It is possible to propose the various explanations, including one that we are dealing with the sibling species. However, a most plausible, in the opinion of the authors, an explanation lies elsewhere. It was shown that *A. salinus* is an osmoconformer (Svetlichny et

al. 2012), as most of Calanoida (Bayly & Boxshall 2009). The osmoconformers haven't the mechanisms of salt concentration regulation in the body fluids at the organismal level; an osmotic regulation occurs at the cellular level by increasing the intracellular concentration of organic compatible osmolytes - small organic molecules (Imhoff 1986; Detkova & Boltyanskaya 2007). Two defining characteristics of protecting osmolytes are that they stabilize proteins against denaturing stresses, and their presence in the cell does not largely alter protein functional activity. Osmolytes are either synthesized in the cell, or transported into it from the external environment. (Yancey 2001; Detkova & Boltyanskaya 2007). Such osmolyte use is widespread, from some Archaea to mammalian tissues such as kidney and brain (Yancey 2001; Nevoigt & Stahl 1997; Detkova & Boltyanskaya 2007). Animal - osmoconformers of different taxons solve the problem in the same way (Yancey 2001; Athamena et al. 2011). Increasing of a synthesis and a concentration of free amino acids (alanine, proline and glycine) during an acclimation to high salinity was shown for representatives of the two groups of copepods - Harpacticoida and Calanoida (Burton 1991; van der Meeren et al. 2008; Lindley et al. 2011). The free amino acids are not only osmolytes of copepods; they provide up to 42 % of the total cell osmolarity (Goolish & Burton 1989). It should be noted that at least some arthropods may use different types of osmolytes, such as proline and trehalose (disaccharide), and, if necessary, accumulate exogenous osmolyte (sorbitol) in their cells (Patrick & Bradley 2000). It is shown that coral polyps use the osmolytes synthesized by symbiotic algae (Yancey et al. 2010).

Life at high salt concentrations is energetically expensive. The upper salt concentration limit at which different bacteria, algae, animals can live in nature appears to be determined to a large extent by bioenergetic constraints (Oren 2011). The main factors that determine whether a certain type of organism can live at high salinity are the amount of energy generated during its dissimilation processes and the amount of energy needed for osmotic adaptation. For example, most halotolerant unicellular green alga *Dunaliella* in response to salinity stress synthesizes glycerol; its content in the cell is proportional to the medium salinity and can reach 80 % of the dry weight of cells (Ben-Amotz et al. 1982; Chen & Jiang 2009). The representative of Harpacticoida *Tigriopus californicus* (Baker, 1912), when moving out of the water with a salinity of 17 ‰ into sea water (35 ‰), increases the synthesis of free amino acids, spending on it to 23 % of total energy expenditure (Goolish & Burton 1989).

If we assume that *A. salinus* costs of a synthesis of osmolytes with increasing salinity grow in this way, it is clear that the species is unlikely to meet its energy needs under salinities higher 60-70 ‰. How then we to be with numerous presence of this species at much higher salinities? Yes, the species can not exist if it is forced to synthesize the osmolytes itself, but it can consume them from the environment. All our findings of the species at very high salinities occurred during intense outbreaks of *Dunaliella salina* (Teodoresco, 1905); the water in the reservoirs was of a red-orange (Senicheva et al., 2008; our unpublished data). We remember that osmolytes can be up to 80% of the dry weight of microalgae cells at very high salinities. Also in such situation there are high concentrations of various dissolved osmolytes in DOM (Zavarzin 2003). Therefore, it is logical to assume that in these conditions, *A. salinus* feeding on *Dunaliella*, and perhaps also absorbing the osmolytes from DOM, don't need to spend its energy resources to maintain the necessary level of osmolytes in their cells. Experiments, for instance (Svetlichny et al. 2012), were conducted with *A. salinus* feeding on microalgae, which did not contain high concentrations of osmolytes as algae were cultured at low salinities (Svetlichny, personal communication). This, probably, explains the results of many experiments, which showed that high level of halotolerance of *A. salinus* is to 50-70 ‰.

Figure 1 shows that all cases of *A. salinus* presence in plankton are grouped into two discrete clusters in salinity gradient. In the left cluster a halotolerance of the species is determined by maximum of its metabolic capabilities, and in the right cluster it is determined by a concentration of osmolytes produced by *Dunaliella*. The gap between the clusters can be explained by the fact that *A. salinus* at salinities higher 50-70 ‰ can not itself provide the necessary energy for the synthesis of osmolytes and *Dunaliella* has not yet given a bloom and the concentration of osmolytes in its cells is not
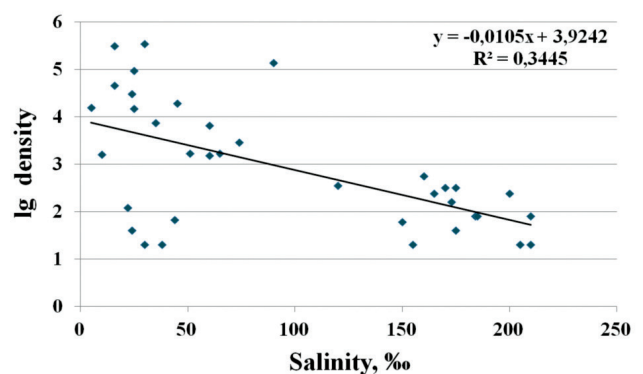

Fig. 1. Relationship between *A. salinus* density and salinity

high enough. As a result, *A. salinus* comes out of the plankton. High halotolerance of other Copepoda species are also likely to be explained by similar reasons. All our observations of mass development of Harpacticoida *Cletocamptus retrogressus* (Shmankevich, 1875) at 290 ‰ occurred during *Dunaliella* blooms when water was also orange-red (Anufriieva & Shadrin 2012).

These facts and arguments lead us to advance the hypothesis that the upper limit of salt tolerance of *A. salinus*, and probably, other osmoconformers, depend on a concentration of osmolytes in water and food objects. This hypothesis can be verified easily by experiments. Halotolerance of a particular species is not purely a physiological peculiarity of it; to some extent, its halotolerance is determined by a community functioning as a whole. From the ecosystem point of view, it is important to emphasize that osmolytes are some "socialized" osmoregulators of community.

Many organisms readily transport the osmolytes from the environment into the cells, getting in this way their resistance to osmotic stress, which is primarily typical for unicellular organisms (Zavarzin 2003; Khakhinov et al. 2007). Discussing the physiological capabilities of a species to exist in every environment, we must not overlook metabolism of community as a whole.

Acknowledgments

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