

Calanipeda aquaedulcis and *Arctodiaptomus salinus* are exceptionally euryhaline osmoconformers: evidence from mortality, oxygen consumption, and mass density patterns

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ABSTRACT: Mortality, respiration rate, and body mass density of the estuarine copepods *Calanipeda aquaedulcis* and *Arctodiaptomus salinus* and their eggs were studied over a wide range of salinities. Empirical hydrodynamic modeling was applied to calculate body mass density using the sinking speed of the copepods. According to the index of median lethal salinity (LS_{50}), the salinity tolerance ranges of *C. aquaedulcis* and *A. salinus* reared at 18 psu were 0.1 to 50 and 0.1 to 35 psu, respectively. No significant effect of salinity on the specific oxygen consumption rate was found in the range from 0.1 to 40 psu in both species, while mean body mass density increased iso-osmotically with water salinity from (mean \pm SD) 1.053 ± 0.007 to 1.077 ± 0.005 g cm⁻³ in *C. aquaedulcis* and from 1.039 ± 0.004 to 1.062 ± 0.007 g cm⁻³ in *A. salinus*. However, body density contrast and sinking speed were stable in both species, allowing them to avoid buoyancy problems with changing salinity. The mean mass density of resting eggs in *A. salinus* gradually increased iso-osmotically following gradual changes in salinity. In contrast to resting eggs, the mass density of subitaneous eggs was not altered by changes in the surrounding salinity, suggesting a hysteresis response in these eggs.

KEY WORDS: *Calanipeda aquaedulcis* · *Arctodiaptomus salinus* · Salinity tolerance · Respiration rate · Body mass density

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INTRODUCTION

Calanipeda aquaedulcis Kritschagin, 1873 and *Arctodiaptomus salinus* Daday, 1885 are typical representatives of holoplanktonic calanoid copepods abundant in estuaries and shallow coastal marine waters. Ephemeral and semi-permanent estuarine and coastal ecosystems are strongly affected by local environmental events (rain, evaporation, river flow, tides), resulting in frequent and unpredictable salinity changes (Ganning 1971, Ranta 1982, Devreker et al. 2008). To survive in fluctuating environments, copepods require behavioural and physiological adaptations.

Copepods in oceanic and coastal waters are known to be principally osmoconformers (Mauchline 1998), i.e. their body fluids are isotonic to the external medium. In osmoconformers, changes in environmental salinity lead to changes in internal osmolarity (Lance 1965), body density (McAllen et al. 1998, Knutsen et al. 2001), and behavior (Seuront 2006).

Some copepods may possess homeostatic mechanisms which permit physiological compensatory osmoregulation. Osmoregulatory responses to salinity alterations in organic osmolyte content are found in the marine copepod *Eurytemora affinis* (Roddie et al. 1984) and estuarine copepods *Tigriopus californicus* (Goolish & Burton 1989) and *Temora longicornis*

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(Tang et al. 2000). The ability to regulate the inorganic ion content of hemolymph is found in the benthic copepod *Tisbe reticulata* (Battaglia & Bryan 1964) and pelagic copepods *Calanoides acutus* and *Rhincalanus gigas* (Sartoris et al. 2010). However, the maintenance of ionic content and body density associated with a decrease in salinity and density of surrounding fluid would pose problems for females of species that carry eggs in an ovisac. The mass density may directly influence the ability of adults and their eggs to maintain a vertical position in favorable water layers.

Due to their ubiquitous distribution in brackish, marine, and hyper-saline environments, *Calanipeda aquaedulcis* and *Arctodiaptomus salinus* are appropriate candidate species to investigate the mechanisms allowing copepods to compensate for salinity stress. Little is known about the salinity tolerance and capacity for osmoregulation in these species. The objective of this study was to investigate the effects of gradual salinity changes on (1) mortality of *C. aquaedulcis* and *A. salinus*, in order to estimate the salinity tolerance range; and (2) respiration rate, body and egg mass density, and density contrast, in order to evaluate the ability of these copepods to osmoregulate across a wide range of salinities.

MATERIALS AND METHODS

Experimental set-up and effect of salinity variation on mortality

The experiments were conducted on adult females and males of *Calanipeda aquaedulcis* and *Arctodiaptomus salinus* from cultures. These copepod species have been in culture at the Institute of Biology of the Southern Seas (Sevastopol) since 2007. The cultures were originally generated from individuals collected from salt lakes (located near the Black Sea coast of the Kerch peninsula, Crimea), and are continuously cultivated in 0.45 μm filtered Black Sea water (FSW) of 18 psu at room temperature (20–22°C) and fed *ad libitum* a mixture of the microalgae *Isochrysis galbana*, *Prorocentrum minimum*, and *P. micans*.

Laboratory studies of the effects of salinity on mortality, copepod body and egg mass density and oxygen consumption of both species were conducted from spring 2010 to spring 2011. To estimate the salinity tolerance range (for survival), 20 to 30 actively moving individuals of *Calanipeda aquaedulcis* or *Arctodiaptomus salinus* were placed in 100 ml

beakers (1 gender and species per beaker in 3 to 5 replicates) containing FSW (18 psu) and then subjected to a gradual salinity decrease or increase over periods ranging from 6 to 8 h, which conformed to the duration of tidal cycles in estuaries of the Mediterranean region (Roddie et al. 1984, Devreker et al. 2008) and the time needed for the regulation of organic osmolyte concentration, body volume and oxygen consumption following osmotic stress in copepods (Goolish & Burton 1989).

To study the effect of salinity changes on copepod mortality, 3 treatments were used:

(1) Salinity was gradually increased at a rate of about 3 psu h^{-1} over 6 and 8 h, from 18 to 35 and 40 psu for *Arctodiaptomus salinus* and *Calanipeda aquaedulcis*, respectively. A further increase in salinity at the same rate led to a pronounced decrease in locomotor activity; therefore, the copepods were acclimated at these salinities for 5 d before further increasing the salinity above 35 and 40 psu. After that, only actively swimming and feeding individuals were exposed to further increases in salinity at a rate of 3 to 4 psu h^{-1} up to two different final salinities of 50 and 60 psu in *Calanipeda aquaedulcis*, and up to 40 and 70 psu in *Arctodiaptomus salinus*.

(2) Salinity was gradually decreased over 6 h from 18 to 1 psu at a rate of about 3 psu h^{-1} . Preliminary experiments revealed that a decrease in salinity from 18 to 0.2 psu in 1 d resulted in the loss of the majority of the *Calanipeda aquaedulcis* specimens due to body swelling; they were therefore kept for about 20 h at 1 psu. Salinity was then gradually decreased further to 0.1–0.2 psu during 5 h at a rate of about 0.2 psu h^{-1} .

(3) After long-term (up to 3 mo) maintenance in fresh water (0.2 psu), copepods were gradually acclimated to 6, 14, 18, 22, 30 and 40 psu over 6 to 8 h at a rate of 1 to 5 psu h^{-1} .

During all experiments, the copepods experienced the gradual salinity alteration by being transferred every hour to the next salinity increment. Three to 5 replicates were used for each salinity treatment in both species. Low- and high-salinity waters were prepared by stepwise addition (and steady inter-fusion) of distilled water or artificial sea salt to FSW of 18 psu. Water salinity (S , psu) was measured by a Hach conductivity meter (SensIon 5). Throughout the periods of acclimation to low or high salinity, the copepods were fed with the microalga *Haemotococcus pluvialis* or a mixture of the microalgae *Dunaliella tertiolecta*, *Monochrysis lutheri*, and *Prorocentrum micans*, respectively.

The number of live (actively swimming and containing food in the guts) and dead individuals was counted during the gradual salinity changes and after the salinity changes during long-term maintenance in water of increased or decreased salinity (5 to 10 d of maintenance at the final salinity). Copepod mortality (M , %) was calculated as: $M = 100 D / (D + s)$, where D is the number of dead individuals, and s is the number of surviving individuals. The salinity tolerance range in *Calanipeda aquaedulcis* and *Arctodiaptomus salinus* was estimated taking into account the lethal salinity values affecting 50% of the original number (LS_{50}) (Hopper 1960, Parra et al. 2005, Chen et al. 2006) in 5 to 10 d. We chose this period because the process of salinity acclimation in crustaceans usually takes at least 3 d (Lance 1963, Khlebovich 1981, Lee & Petersen 2003).

Oxygen consumption rate

Actively swimming adult females and males of each species ($n = 200\text{--}300$) were separated from the culture kept at 18 psu and divided into 2 groups, using a wide-mouth pipette. One group underwent a gradual salinity decrease from 18 to 0.1–0.2 psu (as in Treatment 2), while the other group experienced a gradual salinity increase from 18 to about 40 psu (as in Treatment 1). Thereafter, both groups were kept at the final salinities for 1 wk and fed ad libitum. Prior to the experiments, the copepods were placed into 100 ml beakers containing filtered (mesh size of 0.45 μm) FSW and deprived of food for approximately 2 h to allow emptying of the guts. Oxygen consumption rate was measured separately for each gender and species.

Respiration rate (R , $\mu\text{g O}_2 \text{ ind.}^{-1} \text{ h}^{-1}$) was determined using the closed sealed chamber method, with experimental and control syringes of 2.0 ml used as the respirometers. Three to 5 females or 6 to 8 males (to equalize copepod weight per volume of respirometers because females are heavier than males) of *Calanipeda aquaedulcis* and *Arctodiaptomus salinus* per 1 ml of FSW were gently transferred by a pipette into the experimental syringe, which was filled with FSW supplied through a protective sieve disc (mesh size 200 μm) at the confluent outlet.

To obtain identical oxygen, salinity, and seston content, we connected the control and experimental syringes with a plastic tube and pumped the water through it back and forth several times. The syringes were then separated, closed by stoppers, and placed into a dark chamber at $20 \pm 0.5^\circ\text{C}$. The

incubation period was ca. 2 h for *Arctodiaptomus salinus* and 3 h for *Calanipeda aquaedulcis*. At the end of the exposure, a water sample from the experimental or control syringes was transferred to a flow chamber with a variable volume (up to 0.3 ml) created from a truncated all-glass syringe and joined to a luminescent dissolved oxygen sensor (Hach LDO™). Details concerning the oxygen concentration and respiration rate measurements are described in Svetlichny & Hubareva (2005) and Svetlichny et al. (2010).

Body size, volume and wet weight

The total length (L_{tot} , mm) and length and width of the prosome (l_{pr} and d_{pr} , mm) of *Calanipeda aquaedulcis* and *Arctodiaptomus salinus* were measured under a light microscope fitted with an eyepiece micrometer. Body volume (V_b , mm^3) was calculated using the equation $V_b = 0.47 L_{\text{tot}}^{0.21} l_{\text{pr}}^{0.93} d_{\text{pr}}^{1.86}$. This equation was based on the empirical relationship between wet weight and body proportions determined in 80 Copepoda species (Svetlichny 1983a) and approximated to the cubic equation $V_b = kL_{\text{tot}}^3$, where k is the volume/size coefficient expressed in the present study by the function $k \sim f [l_{\text{pr}} / L_{\text{tot}} (d_{\text{pr}} / L_{\text{tot}})^2]$ as $k = 0.47 (l_{\text{pr}} d_{\text{pr}}^2 / L_{\text{tot}}^3)^{0.93}$ for both females and males of calanoid and cyclopoid copepods (see Fig. S1 in the supplement at www.int-res.com/articles/suppl/m470p015_supp.pdf) with body shapes varying from spherical (e.g. *Pachos punctatum*) to elongated ellipsoid (e.g. *Rhincalanus nasutus*). Wet weight (WW, mg) was calculated as $WW = V_b \rho_b$, where ρ_b (mg mm^{-3}) is the mean body mass density.

Copepod sinking speed and body mass density

Sinking speed and body mass density were measured at $22 \pm 0.5^\circ\text{C}$ in females of *Calanipeda aquaedulcis* and *Arctodiaptomus salinus* from the culture kept at 18 psu: (1) after gradual salinity decrease to 1–2 psu (Day 1) and 0.2–0.3 psu (Day 2) and (2) after gradual salinity increase to 30 psu (Day 1) and 40 psu (Day 2). Sinking speed and mass density of copepods were also measured at the final salinities at the end of 1 wk. At least 15 individuals of each species and gender were studied at each salinity. The effect of 8 d of starvation on mass density of *C. aquaedulcis* (the more susceptible species to food limitation in comparison with *A. salinus*) was studied at 18 psu.

To determine sinking speed, individuals were anesthetized with 1:5000 MS-222 Sandoz until complete immobilization was achieved (not more than 2 min) and were transferred into a 30 cm long, vertically oriented graduated tube (\varnothing : 2 cm) filled with seawater of the given density and temperature (described in the previous paragraph). When introduced into the tube, the animals always assumed one of their typical geometric positions: when the antennules were spread, the long axis of the body was horizontal or vertical (with the abdomen down), and when the antennules were folded along the body, the long axis was vertical (with the head down).

To minimize the effect of water thermal convection, the tube was completely submerged in a cylinder (\varnothing : 5 cm) with circulating water of a constant temperature (21.5 to 22.5°C). The sinking speed of each individual was calculated as the mean (of 10 replicates) time of sinking 20 cm down the tube, observed through a binocular microscope focused on the central axis of the tube. Previous studies (e.g. Stepanov & Svetlichny 1981) have shown that the wall of a vessel affects the sinking speed of copepods only if the animals are located near the wall (closer than 10 body lengths). Therefore, we did not take into consideration the result of the experiment when the distance between the copepod body (diameter of ca. 0.25 to 0.45 mm) and the wall of the vessel was <5 mm.

Body mass density was determined by $\rho_b = \rho_w(1 + C_D S U^2/2gV_b)$, where ρ_w is the water density (g cm^{-3}); S is the body cross-sectional area (cm^2); U is the sinking speed (cm s^{-1}); g is the acceleration due to the gravity (cm s^{-2}); and C_D is the hydrodynamic drag coefficient. S was expressed as $S = \pi d_{pr}^2/4$, and C_D was determined as $C_D = c/Re^n$, where Re is the Reynolds number, n is the regression slope, and c is the hydrodynamic shape factor. Taking into account that $Re = d_{pr}U/\nu$, where ν is the kinematic molecular viscosity ($\text{cm}^2 \text{s}^{-1}$), C_D can be calculated as $C_D = c \nu^n/d_{pr}^n U^n$. Finally, the equation for calculation of body mass density in copepods can be expressed as $\rho_b = \rho_w(1 + c \pi \nu^n d_{pr}^{2-n} U^{2-n}/8gV_b)$.

To determine the hydrodynamic coefficients c and n , previous data on the hydrodynamic characteristics of passive sinking for 16 copepod species (Svetlichny 1983b) were recalculated (see Table S1 in the supplement). Three types of copepod body orientation during sinking were studied: sinking with the head down and the antennules folded along the body (O_I), sinking with the abdomen down and antennules spread at an angle of 90° to the body axis (O_{II}), and sinking dorsally in a horizontal position with spread

antennules (O_{III}). When $Re < 1.0$, $n = 1.0$ in every type of body orientation, and when $1.0 < Re < 10.0$, $n = 0.87$ both for O_I and O_{II} and 0.82 for O_{III} .

The coefficient c was expressed as a function of the ratio $l_{an} l_{pr} : d_{pr}^2$, where l_{an} is the length of 1 antenna. We found that for orientation O_I , $c = 2.27 l_{an} l_{pr}/d_{pr}^2 + 52.8$; O_{II} , $c = 5.1 l_{an} l_{pr}/d_{pr}^2 + 70.9$; and O_{III} , $c = 6.17 l_{an} l_{pr}/d_{pr}^2 + 72.4$ (Fig. S2 in the supplement).

The density of seawater was calculated taking into account the temperature (measured with an accuracy of $\pm 0.1^\circ\text{C}$) and salinity (determined by a conductivity probe SensIon 5, accuracy of ± 0.1 psu) using Oceanological Calculator 2.1 (V. S. Arkhipkin).

Egg sinking speed and mass density

All measurements of egg sinking speed and mass density were performed at $22 \pm 0.5^\circ\text{C}$. Subitaneous eggs of *Calanipeda aquaedulcis* were collected from females cultivated at a constant salinity of 18 psu. In addition, subitaneous and resting eggs (colored grey just after laying and changing to pink color after several weeks of cold storage) were obtained from 2 groups of *Arctodiaptomus salinus* females kept in fresh (0.2 psu) and brackish (18.2 psu) water. Sinking speed and density were measured in the eggs of *A. salinus* after gradual salinity increase or decrease during 6 h in the range of 0.2 to 18.2 psu at a rate of 2 to 3 psu h^{-1} . The ovisacs were carefully separated from anesthetized females via dissecting needle, and the eggs were surgically removed from the ovisac and washed in filtered water of the experimental salinity. Eggs ($n = 1-3$) from each ovisac (not less than 10 ovisacs were used) were transported separately by a thin dropper to the measuring tube. The measurements of egg sinking speed were carried out using the same method as that for sinking adult females (see 'Materials and methods; Copepod sinking speed and body mass density') but with a measuring tube of a smaller diameter (0.8 cm).

The eggs of the studied species were spherical, and the Reynolds number during their sinking was low (maximum values of Re were 0.04 and 0.08 for *Calanipeda aquaedulcis* and *Arctodiaptomus salinus*, respectively). Consequently, the mass density of these copepod eggs (ρ_{egg} , g cm^{-3}) was determined from sinking speed following the Stokes equation (Happel & Brenner 1965): $\rho_{egg} = (3\nu\pi d_{egg} U + \rho_w g V_{egg})/g V_{egg}$, where d_{egg} is the egg diameter measured under a compound microscope at 400 \times . Egg volume was calculated as $V_{egg} = \pi d_{egg}^3/6$.

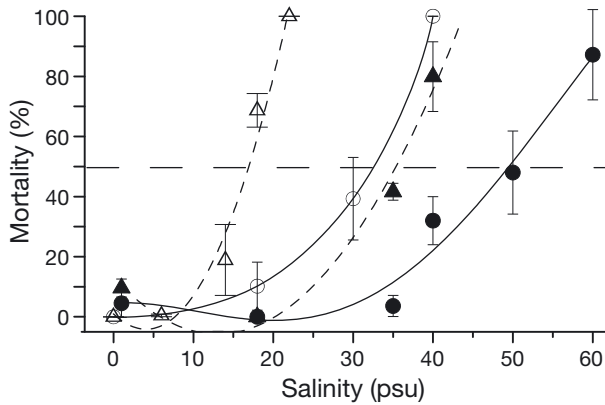


Fig. 1. *Arctodiaptomus salinus* and *Calanipeda aquaedulcis*. Salinity tolerance ranges of *A. salinus* (short-dashed lines) reared at 0.1 (Δ) and 18 (\blacktriangle) psu and *C. aquaedulcis* (solid lines) reared at 0.1 (\circ) and 18 (\bullet) psu. The salinity tolerance range in females and males of both species was between 0.1 psu and the points denoting 50% mortality (horizontal long-dashed line) after 5 d of exposure to a given salinity. Solid and short-dashed lines are the polynomial regression curves. Values are means \pm SD

Statistical analyses

Statistical evaluation of data was conducted by 1-way analysis of variance and Student's *t*-test. Values presented in the figures and tables are means \pm SD.

RESULTS

Long-term tolerance after gradual salinity changes

Mortality of males and females of *Calanipeda aquaedulcis* reared at 18 psu did not exceed 5% during 10 d after gradual salinity decrease or increase in the range of 0.2 to 35 psu, whereas after the gradual salinity increase from 18 to 40 psu and the subsequent increase to 50 psu (after 5 d exposure at 40 psu), mortality significantly ($p < 0.001$) increased to 32 ± 8 and $48 \pm 14\%$, respectively (Fig. 1). Of the *C. aquaedulcis* individuals preliminarily acclimated over 5 d to 35 psu, $87 \pm 15\%$ died during the 10 d (5 days of acclimation to 35 psu + 5 days of acclimation to final salinity) following the salinity increase to 60 psu.

After 2 months of acclimation to fresh water (0.2 psu), $10 \pm 8\%$ of *Calanipeda aquaedulcis* that had been subjected to a gradual salinity increase from 0.2 to 18 psu at a rate of 4 psu h^{-1} died during the 10 d exposure at final salinity. After a salinity increase from 0.2 to 30 psu at the same rate, $39 \pm 13\%$ of *C. aquaedulcis* died during the 8 d exposure, whilst an

increase in salinity from 0.2 to 40 psu at a rate of 5 psu h^{-1} resulted in 100% mortality on Day 1 of the exposure experiment.

In the experiment with the gradual salinity decrease from 18 to 0.2 psu, the mortality of *Arctodiaptomus salinus* was $10 \pm 3\%$ after 10 d of maintenance at the final salinity. We observed sharp increases in mortality, up to 42 ± 3 and $80 \pm 15\%$, after gradual (over 8 h) salinity increases from 18 to 35 psu and from 18 to 40 psu, respectively, during 5 to 10 d of maintenance at the final salinities. Few individuals of *A. salinus* acclimated to 40 psu during 10 d survived in the hyper-saline water of 70 psu (not shown in Fig. 1).

In *Arctodiaptomus salinus* cultivated in fresh water (0.2 psu) for 3 mo, mortality was $18.9 \pm 12\%$ after a gradual salinity increase from 0.2 to 14 psu at a rate of 2 psu h^{-1} , whereas after a gradual salinity increase from 0.2 to 18 psu at the same rate, the mortality of this group reached $69 \pm 6\%$. None of the copepods survived an abrupt salinity increase from 0.2 to 22 psu during 8 h. No egg production was observed in *A. salinus* and *Calanipeda aquaedulcis* kept at salinities higher than 40 and 50 psu, respectively. Moreover, nauplii did not hatch from the eggs of females transferred to hyper-saline water with the ovisacs.

Effect of salinity on adult respiration rate

While at 39.2 psu the respiration rate of female *Arctodiaptomus salinus* ($0.131 \pm 0.018 \mu\text{g O}_2 \text{ ind.}^{-1} \text{ h}^{-1}$) was 17% higher ($p < 0.001$) than that at 0.18 and 18.7 psu ($0.109 \pm 0.019 \mu\text{g O}_2 \text{ ind.}^{-1} \text{ h}^{-1}$), there were no statistical differences ($p > 0.05$) in the weight-specific respiration rates at all salinities studied (Table 1).

In females and males of *Calanipeda aquaedulcis*, respiration rates (0.079 ± 0.011 and $0.057 \pm 0.009 \mu\text{g O}_2 \text{ ind.}^{-1} \text{ h}^{-1}$, respectively) were significantly higher (30 and 40%; $p < 0.001$) at 0.1 psu than at 18 psu; however, there were no statistical differences ($p > 0.05$) between the weight-specific respiration rates of females and males of these species at 0.1, 18, and 40 psu.

Effect of salinity on copepod sinking speed and body mass density

At 18.3 psu, sinking speed of *Arctodiaptomus salinus* O_{II} females (abdomen down and antennules spread) was $0.23 \pm 0.04 \text{ cm s}^{-1}$ and did not differ sig-

Table 1. *Arctodiaptomus salinus* and *Calanipeda aquaedulcis*. Respiration rates at different salinities expressed as the rate per capita (R) and as the wet weight-specific rate (R/WW) at an experimental temperature of $20 \pm 0.5^\circ\text{C}$. n: number of experiments. Values are means \pm SD

Salinity (psu)	n	Wet weight, WW (mg)	Respiration rate, R ($\mu\text{g O}_2 \text{ ind.}^{-1} \text{ h}^{-1}$)	R/WW ($\mu\text{g O}_2 \text{ mg}^{-1} \text{ h}^{-1}$)
<i>A. salinus</i> , female				
0.18	10	0.096 ± 0.024	0.109 ± 0.016	1.11 ± 0.18
18.7	30	0.108 ± 0.011	0.109 ± 0.019	1.02 ± 0.17
39.2	6	0.115 ± 0.017	0.131 ± 0.018	1.15 ± 0.25
<i>C. aquaedulcis</i> , female				
0.1	11	0.049 ± 0.007	0.079 ± 0.011	1.59 ± 0.25
1.0	2	0.046 ± 0.004	0.064 ± 0.008	1.38 ± 0.17
18.0	31	0.042 ± 0.009	0.055 ± 0.011	1.32 ± 0.33
40.0	6	0.048 ± 0.005	0.069 ± 0.015	1.45 ± 0.45
<i>C. aquaedulcis</i> , male				
0.1	7	0.020 ± 0.003	0.057 ± 0.009	3.02 ± 0.52
1.0	2	0.019 ± 0.003	0.040 ± 0.004	2.08 ± 0.18
18.0	20	0.014 ± 0.001	0.034 ± 0.014	2.37 ± 0.96
40.0	4	0.015 ± 0.005	0.055 ± 0.023	3.76 ± 1.61

nificantly ($p > 0.05$) from the sinking speed of O_{II} females acclimated for 1 wk to 0.18 psu ($0.23 \pm 0.03 \text{ cm s}^{-1}$) and 39.2 psu ($0.20 \pm 0.04 \text{ cm s}^{-1}$) at the same temperature (22.5°C). However, mean body mass density, which was $1.052 \pm 0.006 \text{ g cm}^{-3}$ at 18.3 psu, significantly ($p < 0.001$) decreased to $1.039 \pm 0.004 \text{ g cm}^{-3}$ with a salinity decrease down to 0.18 psu and, in contrast, increased to $1.062 \pm 0.006 \text{ g cm}^{-3}$ with a

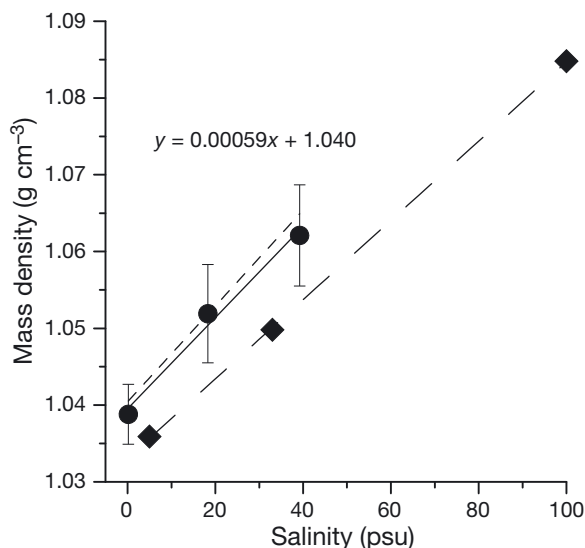


Fig. 2. *Arctodiaptomus salinus*. Effect of salinity on body mass density of females reared at 18 psu (●). Short-dashed line: theoretical change in body mass density in the case of ideal osmoconformity of *A. salinus* with a water content of 75.9% of the body volume. For comparison, the long-dashed line with diamonds shows the relationship between body mass density and salinity in *Tigriopus brevicornis* (McAllen et al. 1998). Values are means \pm SD

salinity increase up to 39.2 psu with a linear correlation described by the equation $\rho_b = 0.00059 S + 1.040$ (Fig. 2).

In *Calanipeda aquaedulcis*, orientation of the body and position of the antennules against the body axis was definitively correlated with water salinity. At low salinity, anesthetized females principally folded their antennules along the body and sank in the water head down due to gravity. At intermediate (18 psu) and high (30–40 psu) salinities, narcotized females sank mainly with the body orientated horizontally with the dorsal side down and the antennules spread. At that horizontal position, the sinking speed of *C. aquaedulcis* females varied insignificantly ($p > 0.05$) from 0.094 ± 0.026 to $0.118 \pm 0.011 \text{ cm s}^{-1}$ in the

salinity range of 0.25 to 42.5 psu. In contrast, mean body mass density decreased in 1 d after salinity decrease from 18.1 to 2.3 psu (Day 1), and then down further from 2.3 to 0.25 psu (Day 2) from 1.067 ± 0.006 to 1.055 ± 0.004 and $1.053 \pm 0.007 \text{ g cm}^{-3}$, respectively, and increased to 1.077 ± 0.005 and $1.079 \pm 0.007 \text{ g cm}^{-3}$ in 1 d after salinity increase from 18.1 to 39.2 psu (Day 1) and 42.5 psu (Day 2) (Fig. 3). The linear equation for this correlation was $\rho_b = 0.00060 S + 1.054$.

Mass density of *Calanipeda aquaedulcis* females was $1.042 \pm 0.009 \text{ g cm}^{-3}$ after 1 mo of acclimation at 0.2 psu, and $1.064 \pm 0.007 \text{ g cm}^{-3}$ after 1 wk of maintenance at 40 psu. In *C. aquaedulcis* females kept at 22°C and 18 psu, mass density decreased from 1.067 ± 0.006 to $1.055 \pm 0.002 \text{ g cm}^{-3}$ after 2 d of starvation and to $1.040 \pm 0.0055 \text{ g cm}^{-3}$ after 8 d of starvation, whereas sinking speed decreased from 0.145 ± 0.019 to 0.107 ± 0.01 and $0.074 \pm 0.0096 \text{ cm s}^{-1}$, respectively.

A gradual salinity increase over 8 h from 18.0 to 42.5 psu caused a linear increase in the mass density of females starved for 8 d to $1.053 \pm 0.002 \text{ g cm}^{-3}$ in accordance with the equation $\rho_b = 0.00053 S + 1.030$.

Effect of salinity on egg mass density

Our results summarized in Table 2 show that at 18.2 psu, the mass density of subitaneous eggs was significantly ($p < 0.001$) higher in *Calanipeda aquaedulcis* ($1.074 \pm 0.009 \text{ g cm}^{-3}$) than in *Arctodiaptomus salinus* ($1.061 \pm 0.006 \text{ g cm}^{-3}$). The mass density of

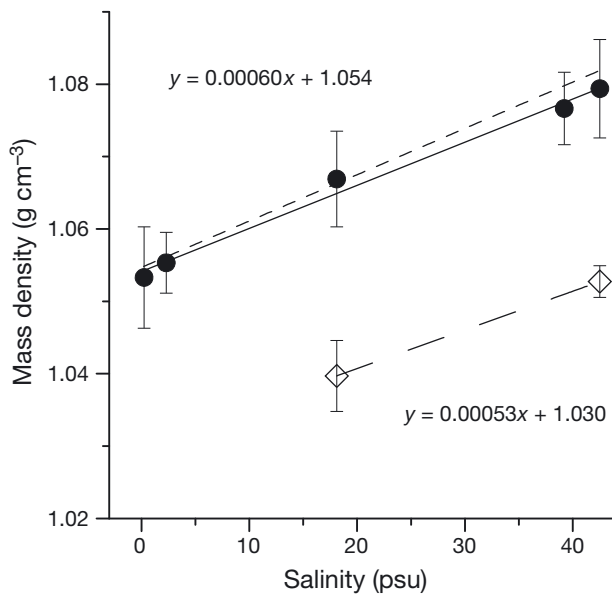


Fig. 3. *Calanipeda aquaedulcis*. Effect of salinity on body mass density of females reared at 18 psu and fed with algae ad libitum (●, solid line) and after 8 d of starvation (◇, long-dashed line). Short-dashed line: theoretical change in body mass density in the case of ideal osmoconformity of *C. aquaedulcis* with a water content of 80.7% of the body volume. Values are means \pm SD

resting grey ($1.077 \pm 0.007 \text{ g cm}^{-3}$) and resting pink ($1.087 \pm 0.005 \text{ g cm}^{-3}$) eggs of *A. salinus* was significantly ($p < 0.001$) higher than that of subitaneous eggs.

Table 2. *Arctodiaptomus salinus* and *Calanipeda aquaedulcis*. Mass density of subitaneous and resting eggs of *A. salinus* and subitaneous eggs of *C. aquaedulcis* at different salinities and $22 \pm 0.5^\circ\text{C}$. Values are means \pm SD

Species	Egg type	Salinity of culture (psu)	Experimental salinity (psu)	Egg diameter (μm)	Mass density (g cm^{-3})
<i>A. salinus</i>					
Subitaneous		0.2	0.2	144 ± 7	1.046 ± 0.003
		18.2	0.2 ^a	140 ± 8	1.058 ± 0.005
		18.2	2.3 ^b	143 ± 7	1.053 ± 0.003
		2.3	18.2 ^a	138 ± 4	$1.049 \pm .0002$
		2.3	18.2 ^b	134 ± 6	1.063 ± 0.007
		18.2	18.2	144 ± 7	1.061 ± 0.006
Resting grey		0.2	0.2	148 ± 11	1.067 ± 0.008
		0.2	18.0 ^a	143 ± 7	1.076 ± 0.004
		18.2	18.2	143 ± 6	1.077 ± 0.007
Resting pink		18.2	18.2	138 ± 6	1.087 ± 0.005
<i>C. aquaedulcis</i>					
Subitaneous		18.2	18.2	108 ± 7	1.074 ± 0.009

^aJust after gradual salinity change; ^b1 d after gradual salinity change

At 0.2 psu, mass densities of subitaneous and resting eggs (1.046 ± 0.003 and $1.067 \pm 0.008 \text{ g cm}^{-3}$, respectively) of *Arctodiaptomus salinus* were significantly ($p < 0.001$) lower than those at 18.2 psu.

A salinity increase from 0.2 to 18 psu at a rate of 2 to 3 psu h^{-1} resulted in a significant ($p < 0.001$) abrupt increase in the density of resting eggs laid in fresh water, up to $1.076 \pm 0.004 \text{ g cm}^{-3}$. In contrast, significant changes in the density of subitaneous eggs laid by *Arctodiaptomus salinus* at 18.2 psu were found only in the 24 h after their gradual transition to 0.2 psu (Table 2), and after the reverse gradual transition to 18.2 psu at the same rate (2 to 3 psu h^{-1}).

DISCUSSION

Salinity tolerance of *Calanipeda aquaedulcis* and *Arctodiaptomus salinus*

Calanipeda aquaedulcis and *Arctodiaptomus salinus* are representatives of the 2 'closest relatives': the families Pseudodiaptomidae and Diaptomidae (Grindley 1984). According to Grindley (1984), *C. aquaedulcis* originated from the sea and represents an intermediate stage in adaptation to freshwater environments (mainly coastal and estuarine areas). *A. salinus* is well adapted to highly mineralized inland water bodies. Despite these differences, the salinity ranges of their habitats are similar. In the brackish Black, Azov, and Caspian Seas (Garber 1951, Kovalev et al. 2006), *C. aquaedulcis* is found at salinities of 6 to 18 psu, whereas in the coastal waters of the Mediterranean Sea, it is distributed within a salinity range of 7 to 35 psu (Ramdani et al. 2001, Brucet et al. 2006, Alfonso & Belmonte 2011). This species occurred in the Aral Sea at 12 psu but disappeared when salinity increased to 57 psu (Aladin & Plotnikov 2008). *A. salinus* inhabits temporary salt lakes of Spain at salinities higher than 7 psu (Comin & Alonso 1988), and up to 25.5 psu (Jiménez-Melero et al. 2007). High salinity (up to 40 psu) is typical for the habitats of *A. salinus* in permanent inland waters of Sicily (Marrone et al. 2006) and temporary salt marshes of Morocco (Rokneddine & Chentoufi 2004), as well as in salt lakes of the Eastern Crimea (Shadrin et al. 2008) and Siberia (Ermolaeva 2010, Tolomeev et al. 2010).

Such osmotic relations of organisms with their environment characterize primary mar-

ine euryhaline osmoconformers that have a salinity tolerance range of 8 to 40 psu (Khlebovich & Aladin 2010). Those authors considered salinities of 5 to 8 psu as the critical values limiting the distribution of marine species. However, our experimental data on median lethal salinity (LS_{50}) of *Calanipeda aquaedulcis* and *Arctodiaptomus salinus* acclimated to 18 psu showed that their salinity ranges were within 0.2 to 50 and 0.2 to 35 psu, respectively (Fig. 1). It is important to emphasize that such a wide range of salinity tolerance in these species is apparently genetically determined, as they were maintained at a constant salinity of 18 psu for longer than 4 yr before the experiment.

In the freshwater treatments, copepods actively fed on the freshwater alga *Haemotococcus pluvialis* and laid eggs that hatched into viable nauplii. We did not find any significant differences in the swimming rate of copepods (L. Svetlichny et al. unpubl.) acclimated to fresh and brackish water (18 psu). In *Calanipeda aquaedulcis* and *Arctodiaptomus salinus* acclimated to fresh water, mortality did not exceed the 50% level (LS_{50}) during salinity changes from 0.2 to 30 psu and from 0.2 to ca. 18 psu, respectively (Fig. 1). Thus in our experiments, *C. aquaedulcis* reared both in fresh and brackish water could tolerate gradual salinity changes up to 30 psu, whereas *A. salinus* tolerated only salinity alterations limited to a range of about 18 psu. For comparison, *Pseudodiaptomus annandalei* from a lagoon near Wukan Bay tolerated gradual salinity increases from 22 to 35 psu and decreases from 22 to 5 psu (Chen et al. 2006). *Acartia tonsa* (Lance 1963, Cervetto et al. 1999, Hubareva et al. 2008) and *Eurytemora affinis* (Lee & Petersen 2003) successfully survived gradual salinity changes over 20 psu under experimental conditions.

The extremely wide range of salinity tolerance in *Calanipeda aquaedulcis* (0.2 to 50 psu) can be compared with the resistance to salinity alterations in intertidal harpacticoids from the genera *Tigriopus* and *Tisbe* living in the range of 0 to 60 psu (Finney 1979) and the calanoid *Eurytemora affinis* surviving in a salinity range of 0 to 40 psu (Roddie et al. 1984, Lee et al. 2007). However, a wide salinity tolerance range in harpacticoid Copepoda is due to inorganic (Battaglia & Bryan 1964) and biochemical osmoregulation, when intracellular organic osmolytes are used for the regulation of cell volume in response to changes in salinity (Goolish & Burton 1989).

Despite the fact that up to 15% of *Calanipeda aquaedulcis* specimens survived during their maintenance at 60 psu for 10 d, and some *Arctodiaptomus salinus* specimens survived for more than 14 d at

salinities up to 70 psu, we did not observe hatching of nauplii from the ovisacs of *A. salinus* and *C. aquaedulcis* females at salinities higher than 50 psu. This was likely due to osmotic effects during hatching. Copepod nauplii emerged from eggs after cracking of the outer membrane caused by active absorption of water through the inner membrane (Marshall & Orr 1972). Apparently, the hatching process is inhibited in eggs of *A. salinus* and *C. aquaedulcis* at salinities higher than 50 psu due to osmotic effects. According to Rokneddine & Chentoufi (2004), the reproductive potential of *A. salinus* from the Zima salt marsh in Morocco decreased 5-fold with a salinity increase to the upper boundary of its tolerance range (35 psu). In other estuarine holoplanktonic copepods, the critical salinity for survival of their juvenile stages was also found to be rather low: 20 psu (Ishikawa et al. 1999) and 25 psu (Lee et al. 2007) in *Eurytemora affinis*, and 35 psu in *Pseudodiaptomus annandalei* (Chen et al. 2006).

Effect of salinity on adult respiration rate

The results of numerous studies investigating the effect of salinity on respiration rates of copepods are contradictory (Anraku 1964, Gyllenberg & Lundqvist 1978, Newrkla 1978, Roddie et al. 1984, Goolish & Burton 1989, McAllen & Taylor 2001, Isla & Perissinotto 2004, Calliari et al. 2006) and cannot be explained without taking into account the energy expenditure for osmoregulation and locomotory activity of the animals. Theoretically, the minimum osmotic work for ion transport constitutes only 1% of the total metabolic energy requirements in brackish and freshwater animals (Potts 1954). However, Goolish & Burton (1989) showed that the daily energy required for adjusting metabolism to osmotic stress in *Tigriopus californicus* acclimated to constant salinity amounted to 11.6% of the total energy respired. Generally, in copepods, total metabolism of active individuals exceeds 3 to 6-fold the basal metabolism of immobilized individuals (Svetlichny & Hubareva 2005); therefore, adaptive changes in locomotory activity of copepods should affect respiration to a greater extent than the process of osmoregulation.

According to McAllen & Taylor (2001), both oxygen consumption rate and activity level decreased 2-fold in *Tigriopus brevicornis* exposed to a salinity increase from 33 to 90 psu, suggesting a decrease in energy requirements for sustained locomotion in more saline water. Earlier, McAllen et al. (1998) demonstrated that *T. brevicornis* was an exception-

ally euryhaline osmoconformer over the range of 10 to 100 psu. However, McAllen & Taylor (2001) showed that there were signs of osmoregulation in *T. brevicornis* at the lowest salinities tolerated by this species (5 psu).

Although in our experiments the respiration rate of *Arctodiaptomus salinus* females was 17% higher at 39.2 psu than at 0.18 or 18.7 psu, weight-specific respiration rates showed no significant differences at all salinities in the range 0.18 to 39.2 psu. Also, there was no evidence of salinity-associated differences in weight-specific respiration rates of *Calanipeda aquaedulcis* females and males at 0.1, 18.7, and 40 psu. The obtained accuracy of mean weight-specific oxygen consumption rates ranged from 16 to 22% coefficient of variation (CV) in *A. salinus* females, 12 to 31% CV in *C. aquaedulcis* females, and 8 to 41% CV in *C. aquaedulcis* males. The last-mentioned accuracy (8 to 41% CV) seems to be too low to claim an osmoregulatory capacity in the studied copepods, as it is comparable with the cost of this process (11.6% of the total energy respired) in *Tigriopus californicus* (Goolish & Burton 1989), but it is sufficient to conclude the absence of a salinity impact on their locomotory activities.

Effect of salinity on body mass density, sinking speed, and density contrast

Lowndes (1942) and Gross & Raymond (1942) were the first to measure mass density of copepods. For this purpose, Lowndes (1942) weighed a batch of adult copepods using 'density bottles' of 0.5 ml. The results obtained for 4 copepod species were converted to a 'sinking factor,' which is the same as the density contrast between the organisms and seawater multiplied by 1000 (Knutsen et al. 2001). Gross & Raymond (1942) determined the mass density of a single *Calanus finmarchicus* using the density bottle method, introducing the copepods to different mixtures of gum arabic and seawater of given density. Usually, with the density bottle method, the organisms are killed or anesthetized, and then a liquid of a certain density where the organisms are neutrally buoyant must be found (Aleyev & Khvorov 1985). For the measurement of copepod body mass density, McAllen et al. (1998) used a solution of sea salts and deionized water. The density bottle method introducing adult copepods to different mixtures of seawater and glycerol was also used by Greenlaw (1977) and Matsukura et al. (2009). Kögeler et al. (1987) and Visser & Jonasdottir (1999) used the density gradient

column method with solutions of diluted seawater and sugar. The density contrast of antarctic copepods was measured using a dual-density method (Chu & Wiebe 2005), where the densities of 2 fluids (natural seawater and distilled water) without copepods and mixtures of the fluids with copepods were determined. However, using hyper- or hypo-osmotic solutions might force the water in the body of a copepod to be transported across the cell membranes, causing changes in body weight and volume.

Lowndes (1942) raised the question of errors due to osmotic imbalance between the solution and the organism changing the relative water content of the body tissues. To determine mass density of zooplankton, Greenlaw & Johnson (1982) proposed to use a hypotonic solution of distilled water and colloidal silica. They observed no apparent changes in the buoyancy of live or preserved zooplankton organisms over time spans of seconds to minutes.

To exclude osmotic factors, Knutsen et al. (2001) used the Stokes hydrodynamic equation modified for the sinking of an elongated ellipsoid in water to calculate the mass density of *Calanus finmarchicus* and *C. glacialis*. Prior to the measurement of sinking speed, the antennae of each anesthetized animal were removed from the body to conform to the ellipsoidal shape. Previously, a similar method was used to determine mass density in 16 copepod species (Svetlichny 1981) based on an empirical study of species-specific hydrodynamic drag coefficients (C_D) determined during experiments with passive sinking of immobilized individuals that had been weighed in seawater using a special microbalance (Svetlichny 1983b).

In this study, we recalculated the hydrodynamic data of Svetlichny (1983b) and expressed C_D in the form of the power regression equation $C_D = c/Re^n$, where n is the regression slope and c is the hydrodynamic shape factor depending on the ratio of $l_{an} l_{pr} : d_{pr}^2$ and body position during sinking (see 'Materials and methods' and the supplement at www.int-res.com/articles/suppl/m470p015_supp.pdf). Using C_D and body volume calculated from the relation $V_b \sim L^{0.21} l_{pr}^{0.93} d_{pr}^{1.86}$ (see 'Materials and methods' and the supplement), we can determine mass density of the majority of calanoids or cyclopoids with body shapes varying from spherical with short antennules to elongated ellipsoids with very long antennules, taking into account only the values of sinking speed in the water and L , l_{an} , l_{pr} , and d_{pr} parameters. Despite the fact that the methods mentioned above (see 'Discussion' in Knutsen et al. 2001) should be treated as indicative rather than absolute, measure-

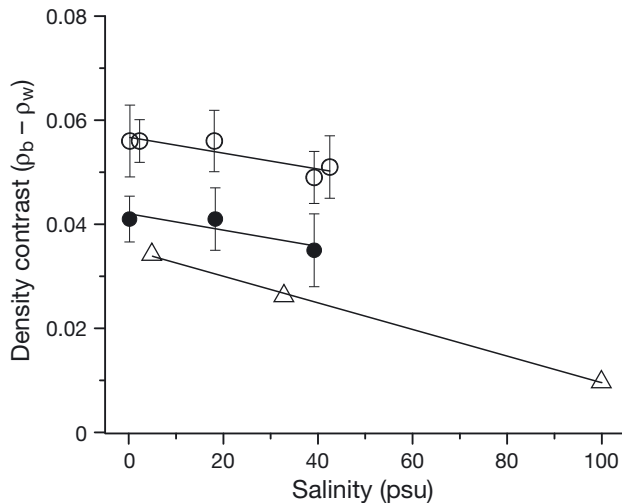


Fig. 4. *Arctodiaptomus salinus*, *Calanipeda aquaedulcis*, and *Tigriopus brevicornis*. Effect of salinity on density contrast of *A. salinus* (●) and *C. aquaedulcis* (○) reared at 18 psu, and for comparison, *T. brevicornis* (△, calculated from McAllen et al. 1998). Values are means \pm SD

ments on the basis of sinking speed allow the determination of copepod mass density with respect to their natural seawater habitat.

According to our results, the mass density in *Calanipeda aquaedulcis* acclimated to 18 psu increased from 1.053 ± 0.007 to 1.079 ± 0.007 g cm⁻³ with a gradual increase in salinity in the range of 0.2 to 42.5 psu whilst that in *Arctodiaptomus salinus* increased from 1.039 ± 0.004 to 1.062 ± 0.006 g cm⁻³ in the range of 0.2 to 39.2 psu. During no less than 1 wk at extreme salinities, the mass density in both species remained nearly the same. Unlike mass density, density contrast in the studied species did not differ significantly ($p > 0.05$) in the same salinity ranges and amounted to 0.056 ± 0.007 and 0.051 ± 0.006 at 0.2 and 42.5 psu, respectively, in *C. aquaedulcis*, and 0.041 ± 0.005 and 0.035 ± 0.007 at 18 and 39.2 psu, respectively, in *A. salinus* (Fig. 4), indicating osmoconformity of these species. To examine the possibility of iso-osmoticity in these species, we calculated theoretically expected changes in the mass density of the studied copepods when body fluid was iso-osmotic to the surrounding water. For *C. aquaedulcis* and *A. salinus* reared at 18.0 psu and 22°C, measured values of dry weight (0.0077 and 0.0254 mg) were equal to 19.3 and 24.1% of the values of wet weight (0.0400 and 0.1053 mg), and water content amounted to 80.7 and 75.9% of the mean wet weight and 85.1 and 79.1% of the body volume (0.0375 and 0.1001 mm³), respectively. This is in accordance with the values of water content in other copepods (Ikeda et al. 1990, 2004, 2006).

In euryhaline copepods, rapid salinity changes result in adaptive compensatory alterations in the body volume (Goolish & Burton 1989, McAllen et al. 1998). However, according to Goolish & Burton (1989), the body volume of *Tigriopus californicus* recovered in 1 h following hyperosmotic stress and 4 h after hypo-osmotic stress.

We did not find significant differences between the values of body size and volume in *Calanipeda aquaedulcis* and *Arctodiaptomus salinus* after a gradual salinity increase in the range of 0.2 to 40 psu and 0.18 to 39.2 psu, respectively. The mass densities of *C. aquaedulcis* and *A. salinus* calculated (within these salinity ranges) under the assumptions that body volume is constant and that water content is iso-osmotic to the surrounding water, are within the limits of 1.055 to 1.081 and 1.040 to 1.065 g cm⁻³, respectively, which are close to measured values (Figs. 2 & 3).

In *Tigriopus brevicornis*, mass density also linearly increased from 1.036 to 1.085 g cm⁻³ (McAllen et al. 1998) with a salinity increase from 5 to 100 psu (Fig. 2). Based on the pattern of changes in the osmolality of the body fluids, the authors considered this species to be a euryhaline osmoconformer. However, according to data presented by McAllen et al. (1998, their Table 2) on water and body density, the density contrast of *T. brevicornis* uniformly decreased 1.3-fold in the range of 5 to 33 psu and 3.5-fold in the range of 5 to 100 psu (Fig. 2) because body density increased more slowly than water density during the salinity increase. This result and the low slope of the regression line of the sodium ion in *T. brevicornis* (McAllen et al. 1998, their Fig. 2) indicate that this species is not an ideal conformer. In contrast, *Eurytemora affinis* (Seuront 2006) did not exhibit any significant differences between sinking speeds of males, non-ovigerous females, and ovigerous females in the salinity range of 0 to 35 psu, indicating that this species is as strong an osmoconformer as *Calanipeda aquaedulcis* and *Arctodiaptomus salinus*.

Mass density and dry weight changes during starvation in *Calanipeda aquaedulcis*

Mean mass density of copepods of all species depends on such things as their body composition, characteristics of their life cycle, feeding, starvation, and stresses. In deep-water copepods of the genus *Calanus* that accumulate wax esters in the oil sac before diapause, the mass density decreased from

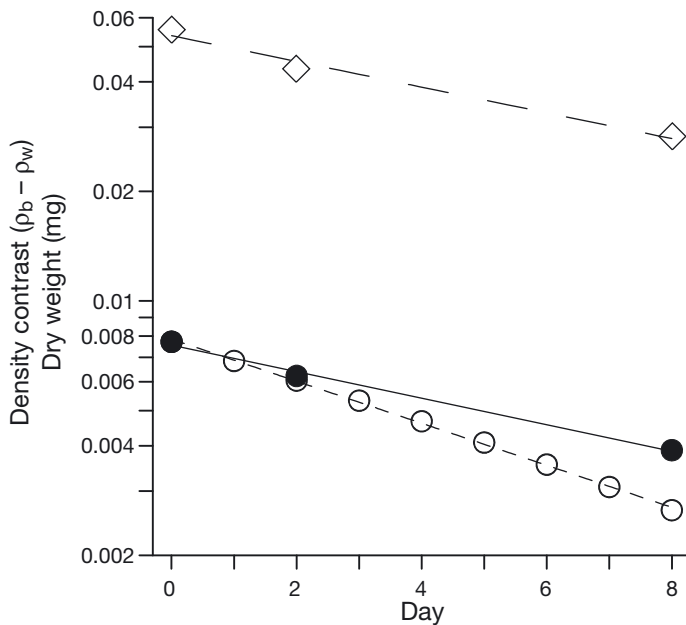


Fig. 5. *Calanipeda aquaedulcis*. Changes in density contrast (◇, long-dashed line) and body dry weight calculated on the basis of body mass density (●, solid line) and respired energy losses converted to dry weight (○, dashed line) during starvation

1.06 to 0.99 g cm⁻³ with an increase in lipid content at seawater density of 1.016 g cm⁻³ (Svetlichny et al. 1998) and from 1.052 to 1.025 g cm⁻³ at seawater density of about 1.022 g cm⁻³ (Visser & Jonasdottir 1999). Hence, individuals with a lipid content of 60% of dry weight in the Black Sea or 30% of dry weight in the Norwegian Sea may have neutral buoyancy. Wax esters are accumulated slowly and could be involved in metabolic processes as a last energy resource, later than other lipids, probably allowing calanoid copepods to keep positive buoyancy during long-term diapause. Estuarine and freshwater copepods (including *Arctodiaptomus salinus* and *Calanipeda aquaedulcis*) accumulate mainly triacylglycerols in the form of droplets distributed in the body (Ventura & Catalan 2005, Guerrero et al. 2007, Tolomeev et al. 2010). However, in contrast to wax esters, triacylglycerols can be rapidly synthesized during feeding and utilized at starvation, thus influencing the body mass density. The pattern of changes in the mass density due to losses of low-density and high-density body components may indicate a metabolic path used during starvation.

According to our results, the mass density of *Calanipeda aquaedulcis* at 22°C decreased from 1.067 ± 0.006 to 1.055 ± 0.002 g cm⁻³ after 2 d of starvation and to 1.040 ± 0.005 g cm⁻³ after 8 d of starvation. During the entire period of starvation, sinking speed

and density contrast decreased about 2-fold from 0.145 to 0.074 cm s⁻¹ and from 0.0555 to 0.0283 g cm⁻³, respectively (Fig. 5), while body volume did not change significantly. Consequently, if during starvation only low-density lipids were utilized, the mass density would increase due to uptake of water. Thus, the decrease in mass density could occur as a result of utilization of high-density body components such as proteins with densities > 1.35 g cm⁻³.

We estimated the dry weight (DW) losses in *Calanipeda aquaedulcis* (corresponding to changes in the mass density during starvation) by accepting their initial DW as 0.0077 mg (19.3% of WW, see above, this section) and body volume of 0.038 mm³. DW was equal to 0.0062 mg after 2 d of starvation and 0.0039 mg after 8 d of starvation. To examine estimated DW losses, we calculated the expected losses in DW during starvation of *C. aquaedulcis* in accordance with their respired energy. The mean respiration rate in *C. aquaedulcis* (our results) at 20°C was 0.055 μg O₂ ind.⁻¹ h⁻¹ (or 0.041 μmol O₂ ind.⁻¹ d⁻¹). So, according to the temperature coefficient for copepods ($Q_{10} = 2.0$) formulated by Ikeda et al. (2001), mean respiration rate in *C. aquaedulcis* at 22°C would be 0.048 μmol O₂ ind.⁻¹ h⁻¹.

To convert the daily consumed oxygen into energy units, we can use the oxyenthalpic equivalent of 480 kJ mol⁻¹ O₂ (Gnaiger & Forstner 1983), assuming that the combusted substrate was a mixture of lipids, proteins, and carbohydrates. To convert energy dissipation rate to DW losses, we used the copepod mean DW energy content of 26 J mg⁻¹ DW (Ikeda et al. 2006). Changes in respiration rate due to a decrease in DW were estimated according to the equation: $R_{t-1}/R_t = (DW_{t-1}/DW_t)^{0.8}$, where t is time (d). Calculated this way, DW of starved *Calanipeda aquaedulcis* decreased during 8 d of starvation from 0.0077 to 0.0027 mg with a higher regression slope than that calculated in accordance with changes in the body mass density (Fig. 5). This may be due to the fact that in starving animals, the respiration rate decreases more quickly than body weight.

Consequently, the indirect method of determining mass density based on sinking speed gives the opportunity to conduct long-term observations of live copepods and may be improved using video recordings of the behavior of intact individuals. For example, studies on changes in the mass density of dead copepods based on the sinking speed of carcasses allowed us to determine the decomposition rate in intact individuals inside conical tubes with constantly ascending seawater flows (Stepanov & Svetlichny 1981).

Effects of salinity on egg mass density

At 18 psu, the mass density of subitaneous eggs in *Calanipeda aquaedulcis* ($1.074 \pm 0.009 \text{ g cm}^{-3}$) was significantly ($p < 0.001$) higher than in *Arctodiaptomus salinus* subitaneous eggs ($1.061 \pm 0.006 \text{ g cm}^{-3}$), and the density contrast in *C. aquaedulcis* eggs exceeded by 27% that in *A. salinus* subitaneous eggs. The same tendency was observed in females of these species (1.067 ± 0.006 and $1.0529 \pm 0.006 \text{ g cm}^{-3}$, respectively), probably due to higher lipid content in eggs and females of *A. salinus*. In comparison with the mass density of subitaneous eggs, mass density of resting eggs of *A. salinus* was significantly ($p < 0.001$) higher: 1.077 ± 0.007 and $1.087 \pm 0.005 \text{ g cm}^{-3}$ in grey and pink eggs, respectively.

According to our data, the mass density of eggs was higher than the mass density of females and seawater. It is important to estimate the energy cost of bearing eggs in sac-spawning copepods. At 22.5°C and 18.3 psu, the sinking speed of non-ovigerous *Arctodiaptomus salinus* females was $0.23 \pm 0.04 \text{ cm s}^{-1}$, whereas that of females with 12–14 and 20 eggs in the ovisac was 0.286 ± 0.021 and $0.335 \pm 0.011 \text{ cm s}^{-1}$, respectively, i.e. 1.46-fold higher. At a low Reynolds number, the hydrodynamic power F is proportional to U^2 , hence the cost of sustained locomotion in the heaviest *A. salinus* females due to a large egg sac will be approximately 2-fold higher than that in non-egg-carrying females.

According to Seuront (2006), the sinking speed of ovigerous females of *Eurytemora affinis* was approximately 1.7-fold higher than that of non-ovigerous females at the same water salinity. The clutch size in this species reaches 75 eggs clutch⁻¹ (Beyrend-Dur et al. 2009), which explains the stronger (compared to our data) difference between sinking speeds of non-ovigerous and brooding females. Hence, the cost of the egg-brooding strategy in comparison with the broadcast strategy is likely to be increased due to energy demands for sustained locomotion and increased predation on adult females, since the large egg masses can cause a decrease in swimming speed (Seuront 2006).

To study the osmoregulatory ability in eggs of *Arctodiaptomus salinus*, we estimated changes in the mass density of resting and subitaneous eggs in the range of 0.2 to 18.2 psu. The mean mass density in resting eggs of *A. salinus* reared at 0.2 psu increased from 1.067 to 1.076 g cm^{-3} just after 6 h of a gradual salinity increase to 18.0 psu, while the density contrast (0.069 and 0.065 g cm^{-3} at 0.2 and 18.2 psu, respectively) was nearly stable and similar to the

density contrast in resting eggs of females reared at 18.2 psu (Table 2). To examine whether the changes in egg mass density conformed to the changes in the inner water mass density, we calculated the water content and density of dry mass of *A. salinus* resting eggs and compared the obtained values to the literature data. The water content (C_w) as a share of the egg volume can be determined by solving the following linear equations:

$$\begin{cases} \rho_{b1} = [\rho_{DM} V_{egg}(1 - C_w) + \rho_{w1} V_{egg} C_w] / V_{egg} \\ \rho_{b2} = [\rho_{DM} V_{egg}(1 - C_w) + \rho_{w2} V_{egg} C_w] / V_{egg} \end{cases}$$

where ρ_{DM} is the density of dry mass, ρ_w is the water density, and V_{egg} is the egg volume, which is constant before and after salinity changes (see Table 2). The solution for C_w can be obtained as:

$$C_w = (\rho_{b1} - \rho_{b2}) / (\rho_{w1} - \rho_{w2})$$

The estimated value of C_w for resting eggs of *Arctodiaptomus salinus* was 0.67, and therefore ρ_{DM} was calculated as 1.207 g cm^{-3} . In accordance with the obtained constant values of C_w and ρ_{DM} , egg mass density at 0.2 and 18 psu should be 1.067 and 1.076 g cm^{-3} , respectively, as it had been calculated from measured sinking speeds. For comparison, the mass density of resting eggs in the closely related *Centropages tenuiremis* at a salinity of 28 psu was equal to 1.1486 g cm^{-3} (Wang et al. 2005), while C_w and ρ_{DM} calculated from the values of DW (165 ng egg⁻¹) and egg diameter (79.69 μm) were 0.52 and 1.285 g cm^{-3} , respectively.

The mean mass densities in subitaneous eggs of *Arctodiaptomus salinus* obtained from females reared at 0.2 and 18.2 psu were also different (1.046 and 1.061 g cm^{-3} , respectively, Table 2), whereas the values of density contrast showed no essential difference (0.048 and 0.049 g cm^{-3} , respectively), as in resting eggs. However, in contrast to resting eggs, the changes in mass density of subitaneous eggs did not occur immediately after the changes in salinity of the surrounding water, suggesting a hysteresis response to salinity in this type of eggs.

CONCLUSIONS

According to our experimental results, the range of salinity tolerance (conditions under which the specimens survive and reproduce over a long period of time) is within 0.2 to 50 psu for *Calanipeda aquaedulcis* and within 0.2 to 35 psu for *Arctodiaptomus salinus*. Over a time period of about 8 h, corresponding to the duration of tidal cycles, *C. aquaedulcis* and *A.*

salinus can withstand gradual salinity alterations of about 30 and 18 psu, respectively. Adapted to survive at extreme low and high salinities, these species are potentially able to colonize both fresh and marine environments. The absence of changes in respiration rate and iso-osmotic changes in copepod body density revealed in our study are evidence of the capability of the studied species to acclimate to both fresh water and hypersaline conditions without special mechanisms of osmotic regulation. These findings are in disagreement with the existing opinion that the salinity tolerance range within 0 to 50 psu characterizes euryhaline amphiosmotic osmoregulators that originated from freshwater environments (Khlebovich & Aladin 2010). Copepods from the Families Pseudodiaptomidae (*C. aquaedulcis*) and Diaptomidae (*A. salinus*) should be considered to be of marine origin and represent different stages of adaptation to freshwater environments (Grindley 1984).

Confusingly, *Calanipeda aquaedulcis* (the specific epithet means 'fresh water') withstood higher increases in salinity and acclimated more easily to higher salinities than *Arctodiaptomus salinus* (the specific epithet means 'salty'). Again, despite the fact that the majority of researchers rank *C. aquaedulcis* among marine species and *A. salinus* among freshwater species, we could not locate any literature record on findings of the former species in the open sea (only in estuarine areas), or on findings of the latter species in freshwater habitats, to support the above rankings. Moreover, both species can be found in nature within similar ranges of salinity, albeit in different habitats. Thus, the specific epithets that were derived from past descriptions of the copepod species without knowing the peculiarities of their ecological patterns can be rather confusing and may require adjustment (Boero 2011).

In this paper we have also demonstrated that the direct observations on changes in copepod body density following changes in water salinity allow us to resolve the question of whether a species is an osmoconformer or an osmoregulator. We have also demonstrated that it is possible to determine copepod body density by measuring the velocity of passive sinking of anesthetized animals. Such a methodological approach circumvents the effects derived from using a method that involves copepod volume-weight characteristics. In the supplement we present the equations and recalculate our previous data for the determination of body volumes and hydrodynamic characteristics of copepods required for the calculation of their body mass density. In order to apply these formulae to numerous copepod

species without taking into consideration their species specifics, the formulae were unified to the basic copepod body proportions and space orientation during passive sinking.

Acknowledgements. This work was partially supported by the project PERSEUS (FP7-287600). We thank G. S. Minyuk and T. V. Rauen for help in the maintenance of the phytoplankton cultures used for feeding the copepods, and L. O. Aganesova for supplying the initial copepod cultures. We are grateful to the anonymous reviewers for their numerous helpful comments and suggestions.

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Editorial responsibility: Anna Pasternak,
Moscow, Russian Federation

Submitted: December 14, 2011; Accepted: June 26, 2012
Proofs received from author(s): November 28, 2012