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Ecophysiological and behavioural responses to salinity and temperature stress in cyclopoid copepod *Oithona davisae* with comments on gender differences

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Abstract

The gender differences in reaction to salinity (3–50) and temperature (6°C–26°C) stress were studied in the thermophilic cyclopoid copepod *Oithona davisae*, introduced in the brackish temperate Black Sea since 2001. Both females and males possessed similar salinity tolerance ranges (6–40) irrespective of the salinity change rate, and females displayed a striking osmotic control upon sharp (18–40 and 40–18) salinity shocks. By contrast, the temperature response of males and females were different. Torpidity was recorded at a temperature below 10°C in males, collected both in the warm and cold seasons, as well as in summer-autumn females, whilst in females grown at the beginning of winter the locomotor parameters were high even at 6°C. The total metabolic rate of summer-autumn and winter females was determined by the level of basal metabolic rate and energy expenditures due to motor activity. In the winter females that maintained high activity at low temperature, the total and basal metabolic rates, differing by 2.3 times at all temperatures within the range of 8°C–28°C, varied in accordance with the temperature coefficient Q_{10} of about 2, whereas in summer-autumn females at low temperatures the total metabolic rate decreased to the basal level. The plasticity of both males and females to wide ranges in abiotic conditions provide an adaptive strategy to sustain the spreading of *O. davisae* in diverse environments.

Keywords: *Oithona davisae*; salinity; temperature; metabolic rate; behaviour; Black Sea.

Introduction

Oithona davisae Ferrari and Orsi, 1984 is a perennial, warm-water Indo-Pacific cyclopoid copepod (Uye & Sano, 1995) that has become globally distributed, mostly through the ship ballast waters, in coastal areas displaying a wide range of salinity and temperature (Zagami *et al.,* 2018). In its native habitat (Fukuyama Harbor, Inland Sea of Japan), *O. davisae* occurs at salinities varying in the range of 28.6–32.3, over a temperature range between 8.9°C and 28.2°C (Uye & Sano, 1995). However, after its introduction in new areas, this species was found at a salinity of 12 in the Sacramento-San Joaquin estuary (California) (Ferrari & Orsi, 1984), in the range 17.3–18.9 in the San Francisco Bay estuary (Lougee *et al.,* 2002), and in the range 21–37 in the lakes Faro and Ganzirri (Central area of the Mediterranean Sea) (Zagami *et al.,* 2018). Similarly, the temperature range over the introduced areas was wide, ranging from -1.8°C to 29°C (Zagami *et al.*, 2018 and references therein).

In the brackish Black Sea, *O. davisae* was first found in 2001, initially being misidentified as *Oithona brevicornis* (Zagorodnyaya, 2002), but later being re-examined and identified as *O. davisae* (Temnykh & Nishida, 2012). This species was not found between 2001 and 2004, but in 2005 the first *O. davisae* population outbreak was observed in the Sevastopol Bay (Gubanova *et al.,* 2014). Since then, this species has expanded in the basin and gradually occupied all coastal areas of the Black Sea (Yıldız *et al.*, 2017).

The remarkable haline and thermal windows occupied by *O. davisae* might sustain its ability to adapt to diverse environments, and consequently to spread worldwide. Laboratory studies (Svetlichny & Hubareva, 2014) showed that, despite the long-term (about 10 years) inhabitation of the Black Sea at a typical salinity of about 18 (Ivanov & Belokopytov, 2013; Leonov, 2005; Özsoy & Ünlüata, 1997), *O. davisae* females retained their ability to withstand gradual salinity changes over 24 hours in the range 3–40, such as those associated with tidal events. *O. davisae* females were also capable to osmoregulate after 24 h salinity changes in the range 12–40 (Svetlichny & Hubareva, 2014). However, during the transportation within ships ballast water, considered the most likely principal vector of introduction for this species (Zagami *et al*., 2018), *O. davisae* may experience more rapid salinity variations after an exchange of the ballast waters. Given its high spreading activity, it is consequently crucial to investigate whether and how (from physiological and behavioural standpoints) can this species withstand sharp salinity shocks.

The difference in temperatures between native and introduced areas is considered to be an important factor as well. In Fukuyama Harbor, the native habitat of *O. davisae*, a one-month decrease in temperature (down to 8.9°C) led to the pronounced reduction in population density, productivity of females, share of males and copepodites (Uye & Sano, 1995), indicating a thermophilic character of this species. Among the regions colonised by *O. davisae* over the last few decades (Zagami *et al.,* 2018), the Black Sea is the coldest one with long-term, low surface-water winter temperatures (6°C–8°C) (Oguz *et al.,* 2005). Although this species was additionally recorded in the cold waters of the northern Wadden Sea (North Sea) (Cornils & Wend-Heckmann, 2015) and in South Pacific (Chile) (Hirakawa, 1988), no evidence of its successful establishment in these cold environments is available to date.

Females constitute the main part of the adult *O. davisae* population in the Sevastopol Bay all year round, particularly during wintertime when males almost disappear (Svetlichny *et al*., 2016 and 2018). *O. davisae* is incapable of any type of diapause (Alekseev & Starobogatov, 1996; Marcus, 1996). This notwithstanding, the perennial population of this species survives the four-month-long low temperatures of the Sevastopol Bay thanks to the ability of the females, fertilised before the winter decrease in temperature, to postpone the production of eggs until the spring temperature rise (Svetlichny *et al.,* 2016). Such a delay may also be beneficial to naupliar development, which is inhibited at 12°C (Almeda *et al*., 2010).

The overarching goal of the present contribution is to advance the current understanding of the biological mechanisms allowing *O. davisae* to physiologically and behaviourally cope with different environmental conditions. To this aim, the salinity tolerance and the effect of temperature on *O. davisae* males and females were explored by investigating: a) the mortality rate due to gradual or sharp salinity changes; b) the effect of drastic osmotic shocks on body mass density and sinking speed; c) the changes in swimming behaviour upon variations in temperature. These experiments complement previously published evidence of the osmoregulatory response in *O. davisae* females to slow salinity changes (Svetlichny & Hubareva, 2014), shedding light into the females' ability to osmotically control their body density even under acute salinity stress. With reference to males, the data presented in this contribution represent the first investigation on their salinity tolerance. Based on earlier reports regarding life cycle parameters in the Black Sea (Svetlichny *et al.*, 2016; 2018), the working hypothesis is that *O. davisae* females and males display similar salinity tolerance, but differ in the acclimation to seasonal temperature changes.

An earlier assessment of the metabolic requests indicated that overwintering females abated oxygen demand and locomotory activity (Svetlichny *et al.,* 2016). The present investigation further delves into this aspect by measuring the basal metabolism in overwintering females, and the variations in the total and basal metabolism in summer-autumn *O. davisae* females. This approach allows an evaluation of the temperature-dependent metabolic demands over the temperature windows experienced by *O. davisae* in the Black Sea.

Overall, the results presented in this study reveal very complex physiological and behavioural mechanisms, allowing *O. davisae* males and females to face a wide gamut of abiotic conditions. The ability to withstand both sharp and gradual salinity changes, the osmotic plasticity, the flexible swimming repertoire and the season-associated metabolic costs all represent specific individual traits that, alone or in combination, may explain the successful worldwide spreading of this species.

Materials and Methods

Males and females of *O. davisae* were collected in the Sevastopol Bay (Black Sea) by horizontal hauls using a 100 µm plankton net. Upon collection, the copepods were transferred into a 1 L aquarium filled with room temperature seawater at a salinity of 18. Actively swimming adult males and females were captured with a pipette near the illuminated border of the aquarium and transferred into beakers with filtered aerated seawater (FSW) containing the cryptophyte algae (IBSS-CrPr54) *ad libitum* for preliminary acclimation to laboratory experiments for 24 h.

Effect of salinity changes on mortality, body density and sinking speed

The experiments were carried out in August–September 2015 on adult males and females that were freshly collected from the Sevastopol Bay at the typical salinity of 17–18 and at a temperature of 23°C–25°C. To study the effects of gradual and sharp salinity changes on the mortality of *O. davisae* males, two treatments were used (Fig. 1):

- Salinity Treatment 1 (S_Treat1): Adult males acclimated to a salinity of 18 were subject to a gradual decrease/increase in salinity at a rate of 2–3 h^{-1} up to the final endpoints at lower (3, 5, 6, 8, 10, 12) and higher salinities (30, 33, 36, 40, 43, 45 and 47).
- Salinity Treatment 2 (S_Treat2): Adult males were directly transferred from a salinity of 18 to the endpoint salinities (30, 37 and 45).

The mortality induced by sharp salinity changes were also tested in *O. davisae* females (Fig. 1) using the fol-

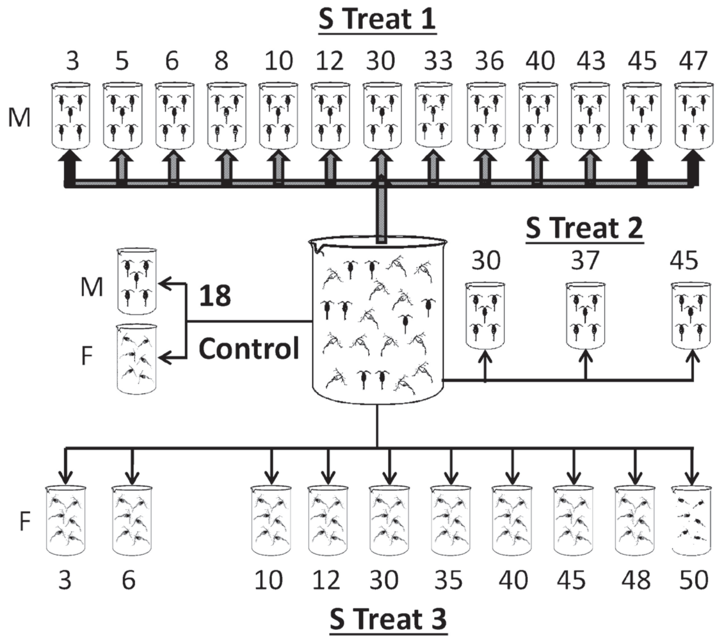

Fig. 1: Schematic of the experimental design adopted in the present study for salinity tolerance experiments in *Oithona davisae*. Solid thick lines and arrows indicate a gradual change in salinity (S_Treat1); thin lines show direct transfer to new salinities (S_Treat2 and S_Treat3). M and F indicate males and females, respectively. The salinity values in the figure are given as cumulative three-day mean ± 0.5.

lowing protocol:

- Salinity Treatment 3 (S_Treat3): Adult females acclimated to a salinity of 18 were directly transferred to the endpoint salinities (3, 6, 10, 12, 30, 35, 40, 45, 48 and 50). Specifically, S_Treat3 was implemented to complement a previous study (Svetlichny & Hubareva, 2014) on the effect of the gradual decrease/increase of salinity on females.

S_Treat experiments aimed at measuring the mortality rates of males and females upon changes in salinity resembling natural processes determining either a smooth variation in salinity (e.g., rain events, floods, tides) (S_Treat1) or a drastic transition (e.g., discharge of ballast waters) (S_Treat2 and S_Treat3). In the control salinity levels, the copepods were monitored at a salinity of 18. 20–30 individuals with 3–5 replicates were used in every experiment for each treatment. During all tests, *O. davisae* males and females were fed *ad libitum* with cryptophyte culture IBSS-CrPr54 at a mean concentration of 0.7 mg C mL^{-1}. Low- and high-salinity waters were prepared by stepwise addition of distilled water or artificial sea salt to filtered seawater (salinity: 18). The water salinity was measured by a Hach SensIon 5 (USA) conductivity meter.

The number of live and dead individuals was counted daily over three days upon completion and maintenance at the final salinities. Copepod mortality (m, %) was calculated as $m = 100\ d/(d + s)$, where d is the total cumulative number of dead individuals and s is the number of survived individuals. Salinity tolerance ranges were estimated taking into account the lethal salinity values affecting more than 50% of the initial number of individuals (LS$_{50}$).

To study the effect of sharp salinity changes (as for S_Treat2 and S_Treat_3) on the sinking behaviour and the mean body density of *O. davisae* females, two treatments were used:

- Salinity Treatment 4 (S_Treat4): Females were transferred from the salinity of 18 to 40;
- Salinity Treatment 5 (S_Treat5): Females acclimated for one week to the salinity of 40 were transferred to a salinity of 18.

The two salinity endpoints were selected considering the typical value for the Black Sea region (18) and the higher-end of the salinity tolerance window emerging from S_Treat3 experiments (see Results section for more details). Trials were conducted on 12 individuals that were placed in vertically oriented rectangular elongated chambers (20 ml) filled with seawater at the ambient temperature to minimise convection. Video observations were performed using a Nikon 1V1 (Japan) digital camera mounting a long-focus objective. Recordings were started immediately after the transfer of *O. davisae* into seawater with final salinity. Behavioural observations were performed periodically over 21.5 h and 16.7 h hours after being transferred to the water with higher (S_Treat4) and lower (S_Treat5) salinity, respectively.

The mean body mass density of *O. davisae* females was determined through the sinking speed of active specimens measured between the periods of active motion or during the sinking phases of immobile (salinity shocked) individuals. The sinking speed was recorded by periodically flipping the chambers and filming with a frequency of 59 fps (see section 2.2). The body mass density (ρ_b, g cm^{-3}) was determined through sinking speed (U, cm s^{-1}) in accordance with the empirical equation (Svetlichny *et* *al.,* 2012): $\rho_b = \rho_w(1 + c\pi v d_{pr} U/8gV_b)$, where ρ_w is water density (g cm^{-3}); c is the hydrodynamic shape factor; v is the kinematic molecular viscosity (cm^2 s^{-1}); d_{pr} is the prosome width (cm); g is the acceleration due to the gravity (cm s^{-2}) and V_b is the body volume (cm^3). The c of *O. davisae* was calculated using the empirical dependence on antennae length and body proportions of the copepods at different positions during passive sinking (Svetlichny & Hubareva, 2014), while V_b was calculated as $V_b = 0.47\ L_{tot}^{0.21}\ l_{pr}^{0.93}\ d_{pr}^{1.86}$, where L_{tot} is the total length (cm) and l_{pr} is the prosome length (cm) (Svetlichny & Hubareva, 2014). At least five repeated measurements were carried out on the same individual to obtain the average sinking speed measurements. Morphometric parameters, i.e. the total length, prosome width and length, were determined under a LOMO MBI 3 (USSR) light microscope.

Effect of temperature on moving activity of males and females

The effect of temperature on *O. davisae* motion parameters was studied in the males and females collected in September 2015 (22°C; summer-autumn population) and in February 2016 (8°C; winter population). Four treatments were used (Fig. 2):

- Temperature Treatment 1 (T_Treat1): The behaviour of summer-autumn females and males acclimated within 24 hours to 8ºC was studied after a direct transfer (i.e., without any gradual acclimation) to water at temperatures ranging from 7ºC to 13ºC (Fig. 2).
- Temperature Treatment 2 (T_Treat2): The behaviour of summer-autumn females and males was observed after a direct transfer from 22ºC to 16ºC, 20ºC, 26ºC, respectively (Fig. 2).
- Temperature Treatment 3 (T_Treat3): Winter males and females were observed after direct transfer from 8ºC to 6ºC–10ºC (Fig. 2).
- Temperature Treatment 4 (T_Treat4): The behaviour of winter males acclimated to 8ºC was investigated at 14ºC and 22ºC after 24 hours of acclimation to 14ºC, while the behaviour of females was studied after 24 hours of acclimation to 22ºC (Fig. 2).

To record the parameters of swimming behaviour (jumping frequency, potential distance covered in 1 min, mean speed of reposition during jumping), ca.10 individuals were placed into 20 mL rectangular aquaria and their spontaneous activity was recorded using a Nikon 1V1 (Japan) digital camera several times within half an hour. To maintain a constant temperature during video recordings, the aquaria were placed in a small transparent box (20×20×20 cm) in which a preset temperature was created with an accuracy of ± 0.5°C using cold holdover or heat elements. A frequency of 59 fps was set for the registration of the number of jumps during 1 min (in ten replicates) with a 4x magnification lens (Industar I-100U 110 mm, f/4.0; USSR) at low illumination. The duration, distance and mean speed of jump kicks during 5 s (in ten replicates) was observed using a frequency of 1,200 fps with a back collimated beam of light produced by a 5W LED. Video sequences showing the movements of specimens in the focal plane were only selected for frame-by-frame analysis.

Effect of temperature on respiration rate of O. davisae females

The effect of temperature on the seasonal-dependent total (R_t) and basal (R_b) metabolic rates of *O. davisae* adult females was studied. The experiments were carried out: in September 2015, testing R_t and R_b on freshly collected females (22°C) from the summer-autumn generation (Fig. 3, R_Treat1), acclimated over 24 h to 10ºC,

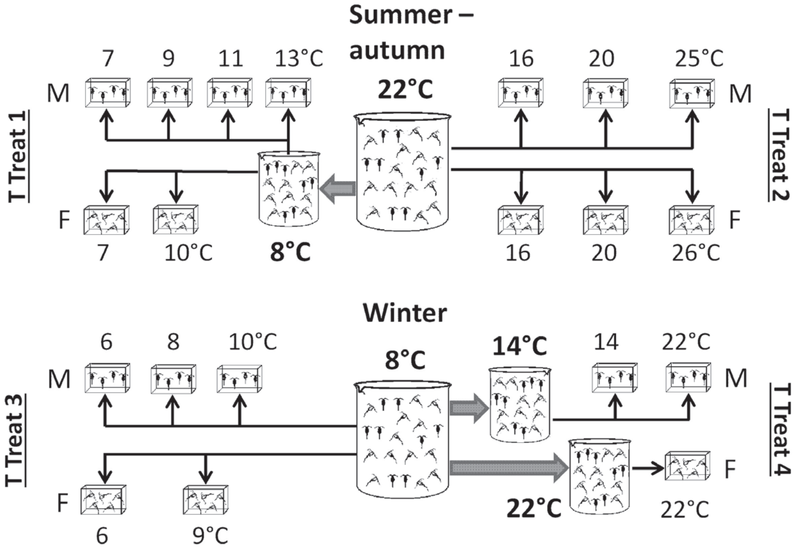

Fig. 2: Schematic of the experimental design adopted in the present study for experiments on temperature effect on the swimming behaviour of *Oithona davisae* males and females. Solid thick arrows indicate a 24 h acclimation of animals; thin lines show direct transfer to a new temperature. M and F indicate males and females, respectively. The temperatures in the figure are averages, rounded to the nearest °C.

15ºC, 20ºC, 28ºC and 32ºC; and in March 2016, measuring R_b of females collected at 8°C (winter population) and exposed to 18ºC, 20ºC and 28°C after 24 h acclimation. In our comparative analysis, we used data on the respiration rate of active winter *O. davisae* females measured at the same temperatures in March 2012 (Svetlichny *et al.,* 2016).

Prior to the experiments, actively swimming females without ovisacs were separated from the samples and placed into 500 mL beakers containing aerated filtered seawater at an ambient temperature (~ 22ºC and ~ 8ºC for summer-autumn and winter experiments, respectively) and a salinity of 18.

The respiration rate of copepods was determined in ten replicates using the closed sealed chamber method, with all-glass experimental and control syringes used as 1.0 mL respirometers. Approximately 60–80 active or approximately 100 magnesium chloride solution (final concentration of 9 g L^{-1}) anaesthetized females were gently transferred by a pipette into an experimental syringe (filled with FSW) supplied by protective sieve disc (mesh size: 100 µm) at the confluent outlet. While MS-222 was used for *O. davisae* as anaesthetic in previous works (Svetlichny *et al.,* 2016; Hubareva & Svetlichny, 2016), in this study a magnesium chloride solution was used as it is associated with milder and more stable anaesthesia (Svetlichny, personal observations). Almost all completely immobilised anaesthetized individuals restored their normal activity even after one-day maintenance in such a solution.

In order to obtain identical oxygen, salinity and random seston content, the control and experimental syringes were connected with a plastic tube through which water was gently pumped back and forth several times. Then, the syringes were separated, closed by the stoppers and placed into temperature-controlled chambers. The incubation periods were about 2–3 h and 3–4 h for active and anaesthetized females, respectively, which ensured a decrease in the oxygen content in the syringes, not exceeding 30% of its initial concentration. At the end of the exposure, the water sample from experimental or control syringes was transferred to a small measuring flow chamber (0.3 mL) joined to a luminescent dissolved oxygen sensor Hach LDO^{TM} (USA) (Svetlichny *et al.,* 2012). The oxygen consumption rate in copepods was calculated by the difference between the final oxygen content in the experimental and control syringes and expressed in units of weight-specific respiration rates (R_t and R_b, μgO_2 mg WW^{-1} h^{-1}), i.e. in the amount of oxygen per unit wet weight of copepods (WW, mg), which was calculated using the equation $WW=\rho_b V_b$. The temperature dependence of the respiration rate of *O. davisae* females was estimated using the temperature coefficient $Q_{10} = (R_1/R_2)^{10/t1-t2}$, where R_1 and R_2 are respiration rates corresponding to the temperatures t_1 and t_2, respectively (Prosser, 1961). For each temperature, the measurements were then converted to their equivalent value at the reference temperature of 20°C.

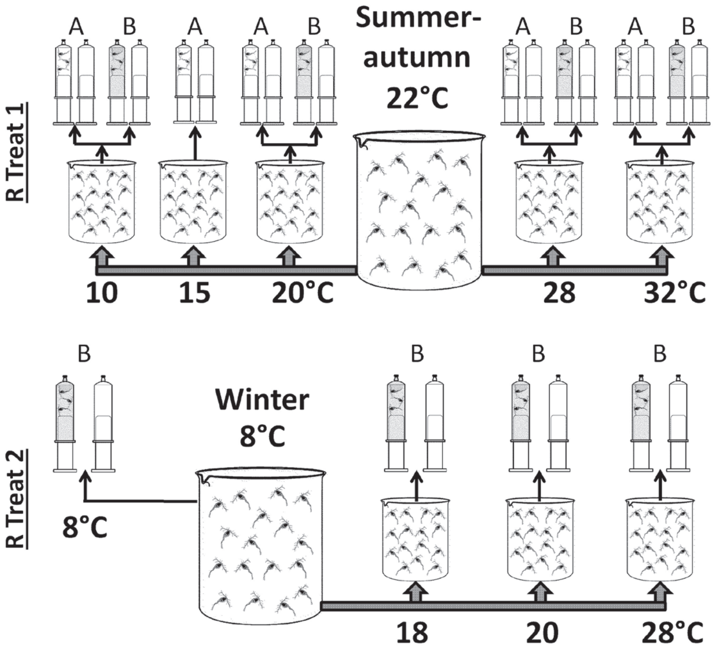

Fig. 3: Schematic of the experimental design adopted in the present study to test the effect of temperature on the respiration rate of *Oithona davisae* adult females from summer-autumn and winter generations. Solid thick arrows indicate a 24 h acclimation of animals; thin lines show direct transfer to a new temperature. A and B indicate the pairs of experimental (with animals) and control (without animals) syringes when determining the respiration rate of active (A; total energy metabolism) and anaesthetized (B; basal energy metabolism) females, respectively. For active females from the winter generation, data from Svetlichny *et al.* (2016) were used.

Statistical analyses of measured parameters

For all treatments, data were preliminarily checked for normality using the Shapiro-Wilk test (α=0.01) (Shapiro & Wilk, 1965; Yap & Sim, 2011) corroborated by normal and detrended QQ plots (Loy *et al*., 2016). Since all samples rejected the null hypothesis of normal distribution (data not shown), all comparative analyses were performed using non-parametric tests for independent samples. In particular, when comparing two-element populations, a Mann-Whitney *U* test (MW; Mann & Whitney 1947) was employed, whereas in the presence of three or more samples, a Kruskal-Wallis *H* test (KW; Kruskal & Wallis, 1952) was used in combination with a Dunn's *post hoc* multiple pairwise comparison (D*ph*; Dunn, 1964). For all tests, a confidence level α=0.01 was adopted for more robustness.

Results

Effect of salinity on mortality of males and females

In S_Treat1 and S_Treat2 control salinity levels (S=18), the mortality of *O. davisae* adult males scored 13.6 ± 2 %. After stepwise decrease/increase in salinity (S_Treat1) to 3 and 47, the mean mortality rates of males, over three days after final salinity had been reached, showed overall statistically different values (KW: $p=3.0\times10^{-4}$). The D*ph* pairwise comparison, in particular, highlighted that the control differed from S=5 and S=3 in the stepwise decrease trials, and from S=43, 45 and 47 in the stepwise increase trials (Fig. 4A). The salinity tolerance range based on a 50% mortality level thus amounted to 6–40. In the case of direct transfer from 18 to hyper-saline waters (S_Treat2), the critical salinity value varied between 40 and 45. Although KW returned $p=1.5\times10^{-2}$, a closer analysis of this specific dataset indicated the occurrence of a type II error based on which mortality at S=45 (scoring 100% in all replicates) could be considered statistically different from other tested cases (Fig. 4A).

To further investigate the dependence of salinity tolerance on the acclimation process, the mortalities at 30, 36–37 and 45 were compared between S_Treat1 and S_Treat2. The MW test indicated that, for the same S (both within and outside the salinity window of *O. davisae* males depicted in S_Treat1), the mortality was statistically similar independent of the acclimation history (gradual *vs.* sharp) of the individuals ($p=1.0\times10^{-1}$, 1.0×10^{0} and 6.0×10^{-1} for S=30, 36–37 and 45, respectively).

In *O. davisae* females exposed to a direct transfer from 18 to fresher/saltier waters (S_Treat3), the LS$_{50}$ was recorded at salinities of 3 and 45 (Fig. 4B) (KW reported $p=1.5\times10^{-5}$, supported by D*ph*). The associated salinity tolerance window was comparable to that resulting from a gradual exposure to different salinities as depicted in Hubareva & Svetlichny (2016) (Fig. 4B).

Effect of abrupt salinity changes on body density and sinking behaviour

The sharp salinity increase from 18 to 40 (S_Ttreat4) had a significant impact on the *O. davisae* females body density (KW: $p=1.0\times10^{-3}$). In particular, the D*ph* test pointed out that ρ_b increased sharply over the first few minutes (5–7) compared to control, from 1.040 ± 0.005 to 1.059 ± 0.005 g cm^{-3}. Females became torpid and their body shrank due to osmosis. However, in slightly more than 2 h (137 min), the body density re-attained the baseline values (Fig. 5): the bodies reshaped, specimens began to show moving activity whilst the ρ_b was restored to the initial values and remained constant up to the end of the experiment.

The transfer of *O. davisae* females from S_Treat4 (ac-

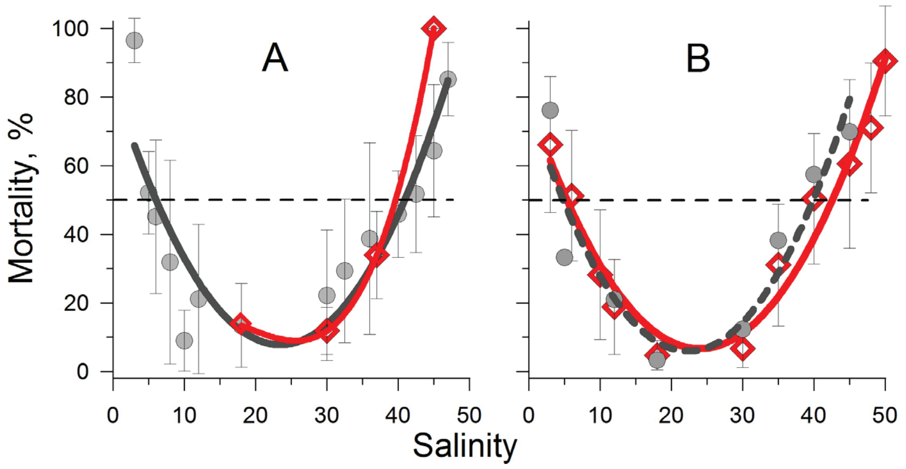

Fig. 4: Effect of salinity on the mortality of *Oithona davisae* males and females collected in the Black Sea at S=18. A: male mortality during 3 days after the gradual salinity decrease from 18 to 3 and increase from 18 to 47 at a rate of 2–3 h^{-1} (●, black solid line) (S_Treat1) and direct transfer (♦, red line) from 18 to 30, 37 and 45 (S_Treat2). B: female mortality during 3 days after the direct transfer from 18 to fresher/saltier water within the range of 3–50 (♦, red line) (S_Treat3) and over 3 days after the gradual salinity change (●, black dashed line, data from Hubareva & Svetlichny, 2016). All lines in A and B are polynomial regression curves. Values are reported as mean ± SD. LS$_{50}$ reference (horizontal black dashed line) is plotted as eye guidance.

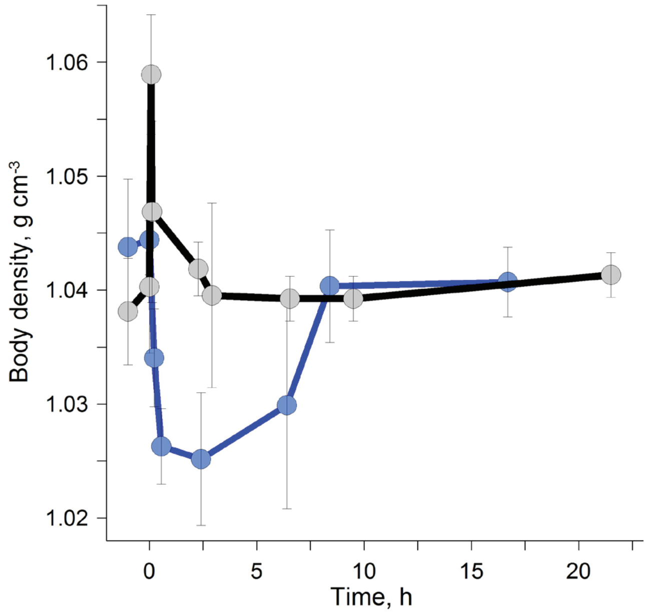

Fig. 5: Time scale dynamics of body mass density ρ_b in *Oithona davisae* females acclimated to S=18 psu and directly transferred to S=40 (●, black solid line; S_Treat4), and in females acclimated to S=40 and directly transferred to S=18 (●, blue solid line; S_Treat5). Values are reported as mean ± SD.

climated one week at the salinity of 40) to 18 (S_Treat5) showed an opposite pattern. The body mass density at first decreased rapidly from 1.044 ± 0.003 to 1.025 ± 0.006 g cm^{-3}, and then, in several hours (> 8 h), increased back up to the initial values (KW: p=3.4×10^{-7}; confirmed by the D*ph* test). The sinking speeds were different as well, following the same pattern of body density (KW: p=7.7×10^{-4}; confirmed by D*ph* test). It is worth underlining that, in both S_Treat4 and S_Treat5, a positive correlation between sinking speed and body density was scored in *O. davisae* females (data not shown).

Effect of temperature on the swimming behaviour of O. davisae males and females

The *O. davisae* males collected in September at 22ºC (T_Treat1 and T_Treat2) were not capable of swimming at 7ºC. At 9ºC copepods, they performed short sporadic reposition jumps at a mean speed of 0.12 ± 0.01 cm s^{-1}. After further rising of temperature, the mean speed increased (Fig. 6A) up to 1.45 ± 0.39 cm s^{-1} at 20ºC and to 0.98±0.17 cm s^{-1} at 25ºC, in accordance with the equation: Y = exp (0.14 X) 0.049 (r^2 = 0.84). Each parameter displayed statistical independence of temperature (KW: p<1.0×10^{-3} in all cases; confirmed by D*ph* test), with two only exceptions for swimming speed at 11°C and 13°C in T_Treat1, and at 6–8°C and 8–10°C in T_Treat2.

In individuals collected in February at 8ºC (T_Treat3 and T_Treat4), the mean swimming speed also increased from 0.08 ± 0.04 to 0.66 ± 0.32 cm s^{-1} in the temperature range of 6–22ºC according to the equation: Y = exp (0.13 X) 0.048 (r^2 = 0.94). Similarly, a strong temperature dependence in the frequency of jumps was recorded, from 4–9 jumps min^{-1} to 80–90 jumps min^{-1} (Fig. 6B). At a high temperature, the kick parameters in summer-autumn and winter males did not differ significantly. The potential distance covered in 1 min increased from 0.27 cm to 3.220 cm in the temperature range 6–22ºC in individuals from the winter population, and from 0.14 cm to 2.63 cm in the temperature range 9–25ºC in specimens from the summer-autumn population (Fig. 6C), with a shift in the values towards higher temperatures. In all cases, values were statistically different (KW: p<2.6×10^{-5} in all cases; confirmed by D*ph* test); only the swimming speed at 16°C and 25°C were similar.

In summer-autumn females grown at high temperature, the mean swimming speed, number of jumps per minute and the potential distance significantly (KW: p<2.3×10^{-7} in all cases; confirmed by D*ph* test) increased from 0.0014 ± 0.0007 cm s^{-1} to 0.07 ± 0.01 cm s^{-1}, from 2.2 ± 1.4 jumps min^{-1} to 60 ± 12 jumps min^{-1} and from 0.09 ± 0.05 cm min^{-1} to 3.60 ± 0.70 cm min^{-1}, respectively, when the temperature rose from 7ºC to 26ºC (T_Treat1

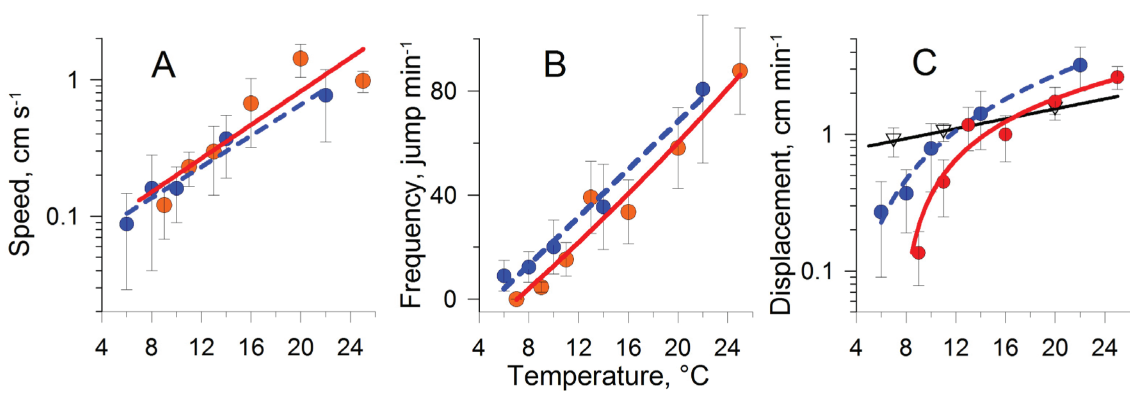

Fig. 6: Effect of temperature on the parameters of swimming behaviour in males from the winter (●, blue dashed line) and summer-autumn (●, red solid line) generations of *Oithona davisae* (values reported as mean ± SD). A: mean swimming speed, cm s^{-1}; B: mean frequency of jumps per minute, jumps min^{-1}; C: potential distance covered by individuals from winter (●) and autumn (●) generations. For mere comparative purposes, the linear trend in temperature-dependent passive sinking distance (cm min^{-1}) is shown (∇, black solid line). All lines in A, B and C are exponential, linear and polynomial regression curves, respectively.

and T_Treat2) (Fig. 7 A, B, C). All parameters were statistically similar only at 16ºC and 25°C. However, in winter females (T_Treat3 and T_Treat4), no significant changes were scored in these parameters (KW: $p>1.0\times10^{-1}$ in all cases), with a variation from 0.04 ± 0.02 cm s^{-1} to 0.06 ± 0.03 cm s^{-1}, from 32.8 ± 15.6 jumps min^{-1} to 46.4 ± 21.6 jumps min^{-1} and from 2.7 ± 1.2 cm min^{-1} to 3.7 ± 1.7 cm min^{-1}, respectively. There were no significant differences in the parameters of reposition jumps determined in winter females at 6ºC and 22ºC (Fig.7).

Effect of temperature on respiration rate of adult females

In active females from the summer-autumn generations (R_Treat1), the total weight-specific respiration rates (R_t) at the lowest temperature of 10ºC (0.38 ± 0.03 µg O_2 mg^{-1}h^{-1}) were close to that of the anaesthetized (basal metabolic rate, R_b) individuals (0.37 ± 0.06 µg O_2 mg^{-1}h^{-1}) (Fig. 8A). The increase in temperature (t, °C) from 10ºC to 15ºC resulted in a dramatic 2.3- fold increase in the R_t of active females to 0.91± 0.08 µg O_2 mg^{-1}h^{-1} and up to 2.71 ± 0.63 µg O_2 mg^{-1}h^{-1} at 32ºC, while in the anaesthetized specimens the R_b increased to 1.52 ± 0.12 µg O_2 mg^{-1}h^{-1}, strictly in accordance with the Arrhenius exponential law ($R_b = 0.19\ e^{0.067\,t}$) with the mean temperature coefficient Q_{10} = 1.92 ± 0.19. The maximum value of the $R_t : R_b$ ratio (2.3) was found at 20ºC.

In R_Treat2, when the experimental temperature rose from 8ºC to 28ºC, weight-specific respiration rates of both active and anaesthetized females from the winter generations increased from 0.54 ± 0.11 µg O_2 mg^{-1}h^{-1} to 2.27 ± 0.49 µg O_2 mg^{-1}h^{-1} and from 0.26 ± 0.04 µg O_2 mg^{-1}h^{-1} to 1.10 ± 0.08 µg O_2 mg^{-1}h^{-1}, respectively, according to the Arrhenius law, with the mean Q_{10} values of 2.06 ± 0.18 and 2.13 ± 0.24, respectively (Fig. 8A). The average $R_t : R_b$ ratio was 2.3 ± 0.3.

The difference between R_t and R_b, called as the metabolic scope of activity (R_s), increased about 30-fold from 0.04 µg O_2 mg^{-1}h^{-1} to 1.16 µg O_2 mg^{-1}h^{-1} in summer-autumn females and 4-fold from 0.28 µg O_2 mg^{-1}h^{-1} to 1.18 µg O_2 mg^{-1}h^{-1} in females from the winter generations (Fig. 8B).

For a proper statistical comparison, all data were

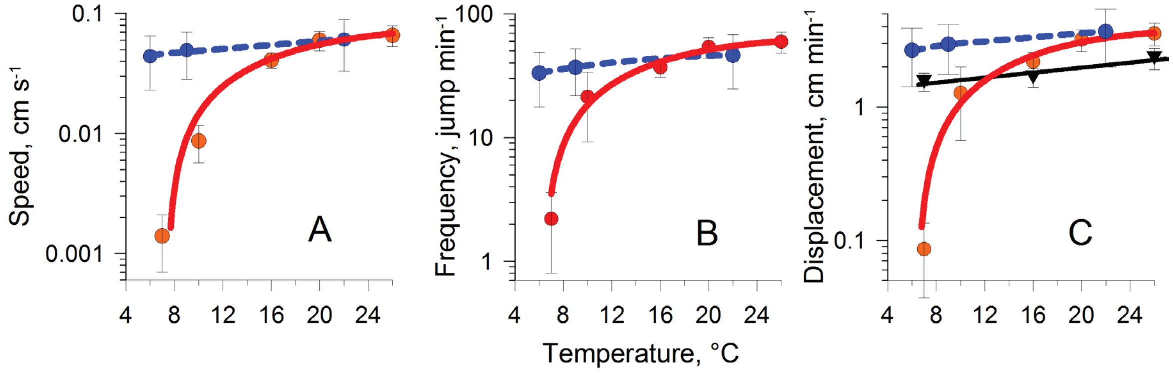

Fig. 7: Mean swimming speed, cm s^{-1} (A), frequency of jumps, jumps min^{-1} (B) and potential distance covered in 1 min, cm min^{-1} (C) in *Oithona davisae* females from the winter (●, blue dashed line) and summer-autumn (●, red solid line) generations. For mere comparative purposes, the linear trend in temperature-dependent passive sinking distance (cm min^{-1}) is shown (▼, black solid line). Solid red lines are polynomial regression curves, dashed blue and black lines are exponential regression curves. Values are reported as means ± SD

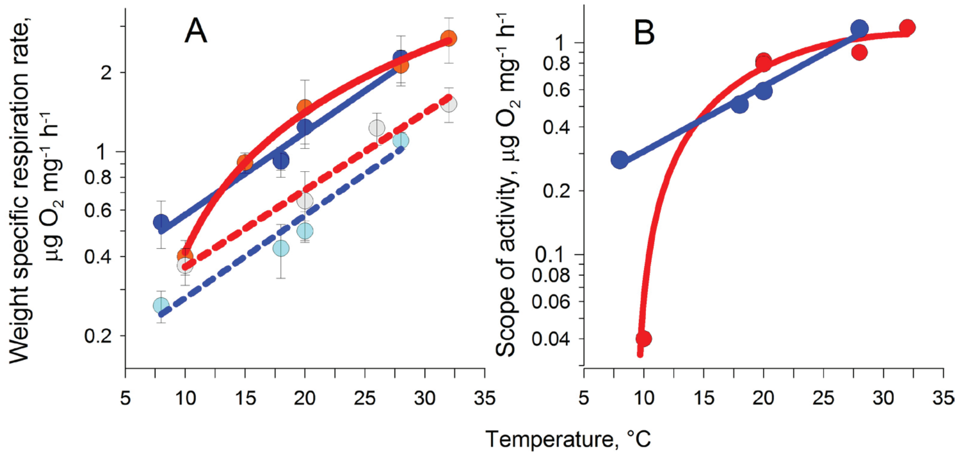

Fig. 8: A: effect of temperature on the weight-specific respiration rate of active (●, red solid line) and anaesthetized (○, red dashed line) *Oithona davisae* females collected during summer-autumn period, and in anaesthetized (○, blue dashed line) and active overwintering females (●, blue solid line) according to Svetlichny *et al.* (2016). B: effect of temperature on the metabolic scope of activity of summer-autumn (●, red solid line) and overwintering (●, blue solid line) females. All lines in panels A and B, except for the red solid lines, are exponential regression curves. Values are mean ± SD.

converted to the equivalent temperature of 20°C, with the only exception of the values at 10°C in R_Treat1, when active females turned out to be inactive as indicated above. For both treatments, active and anaesthetized females reported statistically different values (MW: $p=1.0\times10^{-8}$ and $p=3.3\times10^{-11}$ for R_Treat1 and R_Treat2, respectively), highlighting the increased metabolic demand of active metabolism.

Discussion

The present experimental study provides insight into the adaptive plasticity of the small cyclopoid copepod *O. davisae.* A broad salinity tolerance range (Svetlichny *et al.,* 2014; present study) and homeostatic response to sharp salinity stress (present study) has allowed this Indo-Pacific species to rapidly widen its biogeographical distribution in the last decades due to synanthropic introduction in many regions of the world, from brackish to hyper-saline waters (Zagami *et al.,* 2018). On the other hand, temperature turns out to be the leading factor limiting the distribution of *O. davisae* in cold seas. In particular, males inhabiting the Black Sea are unable to adapt to temperatures below 10/12°C.

The diet of *O. davisae* consists of small autotrophic and heterotrophic flagellates (Uchima & Hirano, 1988), which enables to avoid the trophic competition with the neritic native copepods that mostly consume the photosynthetic algae (Amelina *et al.,* 2017). Higher locomotor activity, metabolic and production rates facilitate the elimination of the congeneric cyclopoid species from the habitat (Isinibilir *et al.,* 2017).

The response of both sexes is temperature-dependent, males becoming unable to swim at very low temperatures while females keeping their ability to swim. The positive correlation with temperature is manifested for all the parameters tested in the study, i.e. swimming speed, jumping frequency and distance covered during swimming.

Salinity tolerance of males and females

Our experimental study on the effect of salinity on males of *O. davisae* shows that, after the gradual salinity changes (S_Treat1), the salinity tolerance range based on LS_{50} amounts to 6–40 (Fig. 4). A similar salinity tolerance range is reported for females experiencing similar stress (Svetlichny & Hubareva, 2014; Hubareva & Svetlichny, 2016). Moreover, our experiments show that both *O. davisae* males and females successfully survive under sharp stress changes in salinity (S_Treat2 and S_Treat3). The salinity tolerance range of this species in the case of the direct transfer to water of different salinity is similar to that scored under gradual changes, revealing that the salinity tolerance range is independent of the acclimation process. A similar salinity window has also been observed recently for *Pseudodiaptomus marinus* (Svetlichny *et al*., 2019), a calanoid species which, just like *O. davisae*, is renowned for its great ability to spread in environments covering a wide haline spectrum (Sabia *et al*., 2015; Uttieri *et al*., 2020). This shared trait suggests that haline flexibility and adaptability might represent a key to success in the introduction and establishment of non-indigenous species, allowing both species to adapt to environments characterised by different salinities.

The survival of *O. davisae* females at hypersaline levels (S=40, S_Treat3) provides a robust benchmark to test their osmoregulatory ability. Females rapidly numb and contract dorsoventrally after direct transfer from 18 to 40 (S_Treat4), then restore their shape and swimming ability in 2–3 h (Fig. 5); meanwhile, their ρ_b first increases to 1.059 ± 0.006 g cm^{-3}, before retaining the initial value of 1.040 ± 0.005 g cm^{-3}. A homeostatic response is registered also after the transfer of females from 40 to 18 (S_Treat5) (Fig. 5), although in this case the sign of body density variation is expectedly reversed, with an initial decrease from 1.044 ± 0.003 g cm^{-3} to 1.025 ± 0.006 g cm^{-3}, followed by the restoration of the initial value in approximately 8 h, a timespan almost three times that recorded in S_Treat4. This evidences the capability of *O. davisae* to control efficiently its water balance not only after slow gradual salinity variations (Svetlichny *et al.,* 2014) but even after abrupt changes in salinity. Similar rapid (over 1-3 h) homeostatic regulations upon hypo- and hyper-osmotic stress are observed in *Tigriopus californicus* (Goolish & Burton, 1989), but also in another species with great spreading ability, *Acartia tonsa* (Svetlichny & Hubareva, 2014). This suggests that the ability to withstand sharp haline shocks over few hours may provide *O. davisae* with a competitive advantage over other species, letting it rapidly adapt to environments with different abiotic conditions and sustaining its colonising capacity. The effects of sharp salinity changes have been tested here on females only; however, as males display numbness and recovery patterns similar to females in S_Treat2, it can be hypothesised that males would display responses analogous to those of females. Specifically targeted experiments will test this thesis.

Gender differences in the temperature acclimation of the parameters of moving activity

The mechanism of locomotion of adult females and males of *O. davisae* was previously described in detail in numerous studies (Uchima & Murano, 1988; Uchima & Hirano, 1988; Kiørboe, 2007a, b; Kiørboe, 2008; Kiørboe *et al.,* 2009; Kiørboe *et al.,* 2010; Jiang & Kiørboe, 2011; Heuschele & Kiørboe, 2012; Cheng *et al.,* 2014). Here we make some refinements for the Black Sea population, focusing mainly on the differences in the temperature dependence of the locomotion parameters of females and males. Both genders in *O. davisae* move in a jumping mode that is realised by a sequence of locomotor cycles or "kicks" consisting of power and return strokes performed by the swimming appendages (Burdick *et al.,* 2007). Relocation in females is due to a single or few kicks, while "Adult males advance by paddling, which is an erratic, curved movement consisting of continuous small hops" (Uchima & Hirano, 1988; Jiang & Kiørboe 2011).

In many copepod species, males showed significantly higher moving activity (Van Duren & Videler, 1995; Kiørboe & Bagøien, 2005; Uttieri *et al.,* 2007; Dur *et al.,* 2010; Michalec *et al.,* 2010) and respiration rate (Isla & Perissinotto, 2004; Svetlichny *et al.,* 2012) than females due to their substantial contribution to the mating process (high cost of investment in active mate-seeking behaviour). In the experiments of Kiørboe (2007, 2008) and Jiang & Kiørboe (2011) at 20ºC–22ºC, the mean swimming speed in *O. davisae* males (0.8–1.6 cm s^{-1}) isolated from females was 15-fold higher than that in females. However, in the proximity of virgin females, males' swimming speed reached up to 4 cm s^{-1} (Heuschele & Kiørboe, 2012) when following the female pheromone tracks. In our experiments with males isolated from females at 20ºC, the mean swimming speed (1.45 cm s^{-1}) from the summer-autumn population individuals is comparable to literature records but is 2-fold lower (0.66 cm s^{-1}) in individuals from the winter population. A decrease in temperature results in the reduction of the swimming speed and frequency of locomotor acts in both summer-autumn and winter males (Fig. 6A, B). At 6ºC–8ºC, the males' mean swimming speed decreases 10-fold to about 0.1 cm s^{-1}, which is only about twice the swimming speed of winter females at these temperatures. This can further reduce the mating efficiency of males, if at all possible at this temperature, considering that even at the optimum temperature the success of mating is dependent on the intense search activity of males (Ceballos & Kiørboe, 2011). At such low temperatures, the distance actively covered by males is several times shorter than the displacement described under the influence of gravity over the same period of time (Fig. 6C). Therefore, the males ordinarily lay at the bottom of the experimental vessel. Only at temperatures higher than 12ºC are the males capable of swimming along the water column at a speed about 10-fold higher than that of the females. Based on these experimental pieces of evidence, the end of November/mid-December, when the temperature exceeds 10ºC–12ºC, turns out to be the most probable time of insemination of females before their wintering, followed by a massive death of the males (Svetlichny *et al.,* 2018).

In contrast to males, females, which also become torpid at 6ºC–7ºC in autumn, are capable of acclimating to such a temperature in winter. The level of activity in winter females, i.e. the number and frequency of routine leaps, is not significantly lower than that in summer-autumn individuals at 20ºC (Fig. 7), ensuring their survival during the prolonged winter. According to Svetlichny *et al.* (2016), females kept at 8ºC during 2.5 months began to lay viable eggs over several days after their transfer to warm water. The lower critical temperature for laying eggs to the ovisac was determined to be 12ºC while the maximum egg production rate was registered at 20ºC, both in the field and under laboratory conditions (Svetlichny *et al.,* 2018).

Temperature dependence of energy metabolism in females

The main part of the copepods' energy budget is typically represented by R_t, which can exceed R_b by 4–7 times (Buskey, 1998; Petipa, 1966; Pavlova & Minkina, 1987; Svetlichny & Umanskaya, 1991). However, in prolonged cruising species, R_t is usually only 2–3 times higher than R_b (Svetlichny *et al.,* 2012; Svetlichny *et al.,* 2017). In our experiments, R_t in summer-autumn females at high temperatures and in winter females over the entire range of studied temperatures, is 2.3 times higher than R_b. This indicates a high motor activity of *O. davisae* females despite their high density (60–80 ind. mL^{-1}). Apparently, the absence of a crowding effect during short exposures (without a physiologically significant decrease in oxygen concentration) is typical for estuarine copepods. For example, no effect of crowding was reported on the respiration rate of *Calanipeda aquaedulcis* and *Arctodiaptomus salinus* at densities up to 27 ind. mL^{-1} and 10 ind. mL^{-1}, respectively (Svetlichny *et al.,* 2012), whose V_b is approximately 30 times greater than that of *O. davisae*. There was no significant respiratory response upon acute stress due to a high culture density (up to 10,000 ind. L^{-1} for 12 hours) reported in adult *A. tonsa* (Nilsson *et al.,* 2017) as well. Short-term crowding (up to 80 ind. mL^{-1}) did not affect the motor activity parameters of *O. davisae* (Svetlichny *et al.,* 2016), nor did large crowding conditions (up to 100 ind. per 15 mL^{-1}) affect its respiration rate (Hiromi, 1994).

According to Hiromi (1994), the respiration rate of *O. davisae* females is on average 0.0073 µl O_2 ind^{-1} h^{-1} at a density of 5–6 ind mL^{-1}, which turned out to be 1.6 times more than the previous assessments (Hiromi *et al.,* 1988) for separate individuals using the gradient diver respirometer system. The peculiarity of both these studies is that the respiration rate was not dependent on temperature despite a very wide range of its change (5ºC–30ºC). In our experiments, R_t in both summer-autumn and winter females at 20ºC turned out to be about 0.004–0.005 µg O_2 ind^{-1} h^{-1} or 0.0052–0.0066 µl O_2 ind^{-1} h^{-1}, which is lower than the values obtained by Hiromi (1994). The difference in R_t is attributable to the lower body mass of Black Sea females, whose dry weight (~ 0.19 µg) is 2-fold lower than that of females from the Tokyo Bay (0.43 µg) (Hiromi, 1994).

In contrast to the results of Hiromi (1994), in the present and previous (Svetlichny *et al.,* 2016) experiments, R_t, R_b and R_s in winter females, as well as R_b in summer-autumn females in the temperature ranges of 8ºC–28ºC and 9ºC–32ºC, respectively, are in proportion to the temperature in accordance with the temperature coefficient $Q_{10} \approx 2$ (Fig. 8). A close temperature dependence of the respiratory rate was found in the cyclopoid copepod *Oithona similis* (Castellani *et al.,* 2005) and can be considered as a typical eurythermic respiratory response for many copepods (see Ikeda *et al.,* 2007; Lee *et al.,* 2001).

In summer-autumn females, a similar type of temperature dependence is observed in the temperature range of tolerance of 14ºC–32 ºC. When the temperature dropped to 10ºC, R_t decreased to the level of R_b, due to the dramatic multiple drop in R_s (Fig. 8 B), confirmed by a sharp decrease in the motor activity in our behavioural experiments (see paragraph 4.2). A similar accelerated decrease in the respiration rate with a decrease in temperature from 28ºC to 16°C was observed in *O. davisae* nauplii reared at 20°C (Almeda *et al.,* 2011). The fact that the R_b of summer-autumn females has the same temperature dependence as that of wintering ones indicates that seasonal variations in R_t occur due to adaptive changes in R_s. Moreover, the results obtained (Fig. 8) show that, at all the studied temperatures, the R_b of summer-autumn females on average exceeds by 35% that of overwintering copepods.

Since *O. davisae* females were taken directly from the sea for the experiments, it can be assumed that this difference is due to the large influence of SDA (specific dynamic action of food) in summer-autumn females, which grew at higher temperatures and under better feeding conditions. The SDA of copepods can vary from 10% of R_t in individuals in a state of maximum activity to 85% in inactive animals (Kiørboe *et al.,* 1985; Svetlichny & Hubareva, 2005; Thor, 2002; Thor, 2003). It might be argued that *O. davisae* females in the Black Sea are capable of surviving prolonged low temperatures of about 8ºC due to the physiological shift from the stenothermic type of the relationship between the metabolism and temperature during the warm period to the eurythermic type in winter, which is peculiar to the species with high metabolic plasticity (Conover, 1956).

Conclusion

The evolutionary advantage ensured by specific physiologic, metabolic and behavioural traits provides non-indigenous species invaluable tools to adapt to wide abiotic windows, and thus to different environments. The present study sheds light on the specific tolerance to haline and temperature variations in *O. davisae* males and females, as well as on their behaviour and (for females only) respiration rates. The picture delineated by our new findings complements the previous knowledge on this species, confirming *O. davisae* as one of the most successful alien estuarine planktonic copepods in the temperate regions of the World Ocean. The integrated approach implemented in this study could be employed to investigate the fitness of other species and perform comparative studies to further understand which may be the most prominent traits sustaining the success of non-indigenous species.

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