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PRIMARY RESEARCH PAPER

Combined effects of two abiotic stressors (salinity and temperature) on a laboratory-simulated population of *Daphnia longispina*

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Abstract Sea level rise is expected to continue apace, with a concomitant rise in temperature on the globe's surface. Natural populations genetic pool compromised by increased salinity would contribute to decrease resilience under future temperature changes. Therefore, this work aimed to understand the long-term effects of salinization on the genetic diversity of a cladoceran-simulated laboratory population under different temperature regimes. For that, six clonal lineages of the cladoceran *Daphnia longispina* were chosen based on their reported differential lethal sensitivity (LC_x) to salinity. The chronic experiment was initiated when each individual clonal lineage reached the population's carrying capacity, and then were exposed to the $LC_{70,48\ h}$ for the most tolerant clonal lineage of *D. longispina* (corresponding to 5.91 mS/cm) to 17°C, 20°C and 23°C for at least 30 days. Salinity affected *D. longispina* survival and reproduction, with the disappearance of salt-tolerant earlier than salt-sensitive lineages after chronic exposures. Different sensitivity ranks were observed for clonal lineages when comparing short-term and chronic survival, most probably due to acclimation-driven population recovery. Non-optimal tested temperatures (17°C and 23°C) enhanced negative effects of salinity through loss of the most sensitive clonal lineages, suggesting a potential future synergistic effect between both abiotic factors.

Keywords Seawater intrusion · Loss of clonal lineages · Clonal lineage · Chronic exposure · Acclimation

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Introduction

Given the constantly changing environmental scenarios present and predicted by the Sixth Assessment Report of the intergovernmental panel on climate change (IPCC, 2021), abiotic factors such as salinity and temperature have gained prominence as they strongly affect the resilience and distribution of aquatic species. Predicted recurrent episodes of salinization in coastal freshwater systems are expected

through storm surge-induced seawater overtopping, especially during the winter months, and groundwater intrusion, especially during prolonged summer droughts (IPCC, 2021). On the other hand, forecasts on temperature rise were preliminarily established in a long-term temperature strategy to not exceed 1.5°C (worst-case scenario, it was set at 2°C; Paris Climate Agreement, Framework Convention on Climate Change), but current assessments have provided evidence with medium confidence that it could reach 2.5°C (or more) by the end of the century (IPCC, 2021).

Both stressors, solely, have been extensively studied over a wide array of species and ecological groups. Effects related to osmotic stress go from those expressed at the individual level, such as decreased feeding or growth rates, impairment of reproduction, up to the community and ecosystem level through community structure and abundances fluctuations due to interspecific relationship changes and/or suppression of most sensitive ecological receptors (e.g., Albecker et al., 2017; Jeremias et al., 2018; Venâncio et al., 2018; Ofoegbu et al., 2019; Venâncio et al., 2019a, 2019b; Venâncio et al., 2022; Vignatti et al., 2022). Likewise, temperature has also been a subject of research. Temperature is of special relevance in ectotherm organisms, as it dictates many features of their life cycle, by speeding up or slowing down molting cycles, growth, or reproduction, as already empirically validated by previous research works (e.g., Goldstein et al., 2017; Hoefnagel et al., 2018; Müller et al., 2018; White et al., 2020; Vignatti et al., 2022). Nonetheless, few have studied the combined effects of both stressors on zooplankton and those pointed to a similar outcome of potential synergistic effect (e.g., Kaya et al., 2010; Loureiro et al., 2015; Garreta-Lara et al., 2018). This potential synergism may be related with dissimilar (or even opposite) coping mechanisms triggered by the different abiotic stressors. For instance, Garreta-Lara et al. (2018) found out that under salt exposure, *Daphnia magna* Straus, 1820 carbon- and amino acid synthesis-related metabolic pathways were downregulated (meaning that less sugars, lipids, and minerals were storage), whereas under thermal stress, metabolic rates were stimulated dispending this way more energy (Garreta-Lara et al., 2018).

When including population genetic variability to the combination of both stressors (temperature and salinity) that field of research is even more limited. Overall, population genetic variability has been severely neglected as most laboratory tests are based on single clonal lineages (often with *Daphnia* sp.). Evaluating such effects using several clonal lineages (as a proxy for population genetic diversity) may reveal far-reaching effects than those assumed so far using single clonal lineages since the pattern of response exhibited by one single clonal lineage may not accurately reflect the diversity of responses of all genotypes that constitute a population (De Coninck et al., 2013; Venâncio et al., 2018; Chain et al., 2019). For instance, during long-term maintenance to low levels of salinity induced with sodium chloride (NaCl, ≈0.73 mS/cm), Venâncio et al. (2018) reported four different reproductive patterns of response with just six clonal lineages of *Daphnia longispina* (Loureiro et al., 2012). Also, Asselman et al. (2015) found very different patterns of DNA methylation when exposing two different *D. magna* genotypes to a similar level of NaCl (5 g/L). Directional selection and/or bottleneck of the population's genetic pool, may compromise its resilience which can, in the worst-case, lead to the extinction of the population locally (Ribeiro and Lopes, 2013; Venâncio et al., 2016). Estimating the probability of long-term survival, plus what percentage of the population would still be viable under these abiotic factors would be of value in understanding how resilient a population might be. In addition, an empirical understanding of extinction processes is necessary to advance the theory and predict the extinction of the population resulting from climate change-induced salinization and/or increased temperature (Fordham, 2015; Wiens, 2016). Overall, this study aims at providing knowledge on how cladocerans' population dynamics may vary under abiotic-challenging scenarios induced by salinity and temperature.

Given the above, two hypotheses were tested: (i) a chronic exposure to salinity at other than the optimal temperature would lead to a faster extinction of local zooplankton populations (simulated in the laboratory using different clonal lineages of daphnids) compared to solely chronic exposure to salinity (at their optimal temperature); and (ii) in the laboratory-simulated population, the most salt-sensitive clonal lineages would be the first ones to disappear regardless of temperature, and therefore the permanence of the more

salt-tolerant clonal lineages would sustain the resilience of populations in salt-impacted locations.

Materials and methods

Artificial sea water

A stock solution of artificial seawater (to be used to increase the conductivity of the standard daphnia medium, and therefore simulating seawater entrance) was prepared by dissolving 33 g of artificial sea salt (Ocean Fish, Prodac International, Cittadella, Italy) in 1 l of ultra-pure water (Milli-Q Academic system; Millipore, MA, USA). This stock solution was subsequently diluted (with the culture medium) to obtain the desired salinity level to perform the experiments of 5.91 mS/cm (please see "Chronic assays" section for more information on the calculation of this value). Artificial seawater was chosen for this study rather than natural seawater or commonly used salt (sodium chloride, NaCl) because: (i) natural seawater as the risk of contamination with other pollutants and thus it would be difficult to discriminate the toxicity due to increased salinity or to other contaminants, and because (ii) despite sodium (Na^+) and chloride (Cl^-) are major ions in seawater, there are many other minor ions which can be chronically toxic and are neglected using solely NaCl solutions.

Culture of *Daphnia longispina*

Daphnia longispina was selected as a model species due to its highly sensitivity to salinity stress in the context of climate changes (Leitão et al., 2013; Venâncio et al., 2018) and ubiquitous presence in freshwater ecosystems (Kim et al., 2018). In addition, as cladocerans, they reproduce principally through parthenogenesis, strategy that allows to maintain identical clones for several generations under controlled laboratory conditions.

To conduct this study, six clonal lineages clonal lineages of *D. longispina* were used. They differ in lethal sensitivity to natural seawater, were selected to perform this study: E84, E89, E99, N35, N37, and N91 (for further information on origin and isolation, and categorization of these clonal lineages please see Lopes et al,. 2004; Martins et al., 2007, 2009; Leitão et al., 2013; Venâncio et al., 2018). The lethal conductivities causing 50 and 70% of mortality (LC_{50} and LC_{70}) were retrieved from Venâncio et al. (2018) and are depicted at Table S1.

Individual cultures of each clonal lineage were maintained at 20 ± 1°C and 16:8 h L:D photoperiod, in ASTM hard water (American Society for Testing and Materials; OECD, 2004) and standard organic extract Marinure 25 from the algae *Ascophyllum nodosum* (Pann Britannica Industries, Waltham Abbey, UK) (Baird et al., 1989). Organisms were fed everyday with the green algae *Raphidocelis subcapitata* (Korshikov) Nygaard et al. at a concentration of 1.5×10^5 cells/ml, and medium was renewed every other day. Cultures were renewed with neonates born from 3rd or 4th broods.

Chronic assays

The chronic assays were conducted by exposing each clonal lineage to the determined salinity level of 5.9 mS/cm, under 3 temperatures: (i) 17 ± 1°C to mimic a scenario of seawater flooding during winter; (ii) 20 ± 1°C as control, since this is the optimal rearing temperature for the cladocerans; (iii) and 23 ± 1°C to simulate seawater subterraneous intrusion during summer. The choice of the range of temperatures was based on the Sixth IPCC Assessment Report (2021) where estimations on temperature oscillations were around 2.5°C. The setting of a 3°C difference was considered as a worst-case scenario.

As a note, the terminology used here (and onwards) as "chronic exposure" is intended to simplify the reading in relation to the type of test carried out and does not identify with that established by the OECD standard guideline for daphnia reproduction of 21 days. Likewise, the terminology used as short-term (corresponding to a period of 96 h within the full duration of the chronic assay) it is only used with the purpose to simplify reading and understanding the present work. A schematic workflow of the main steps of experimental design are represented in Fig. 1.

For each temperature and each clonal lineage of *D. longispina*, 10 neonates less than 24 h-old and from third to fifth broods were introduced per replicate. Each replicate consisted of a 1 l glass vessel containing 800 ml of ASTM. Six replicates, in ASTM solely, were set per clonal lineage and per temperature, which were reared under a 16:8 h L:D photoperiod until each population reached their carrying capacity (23 d for

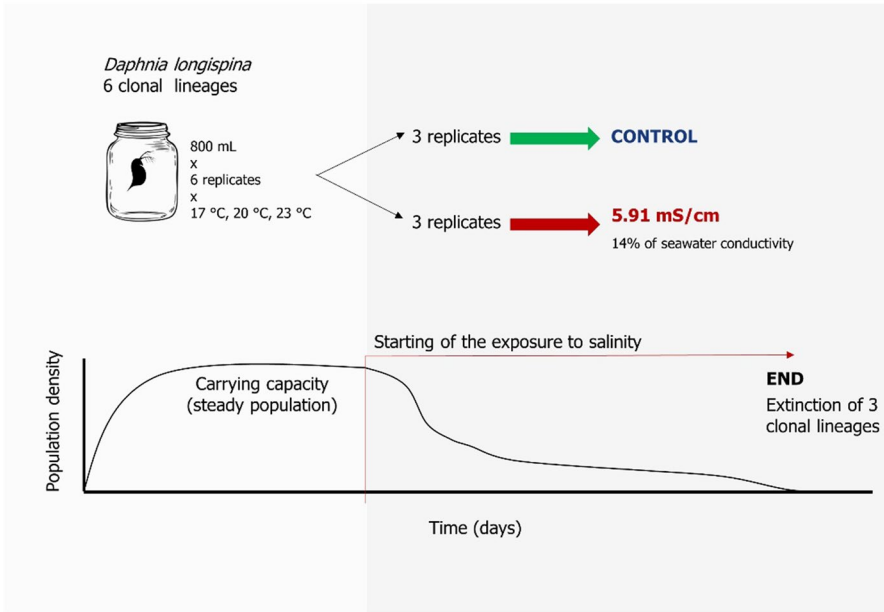

Fig. 1 Schematic workflow of the chronic assays carried out with the six *Daphnia longispina* clonal lineages

daphnias exposed at 17°C, 21 d for daphnias exposed at 20°C, and 19 d for daphnias exposed at 23°C) and were fed with the green algae *R. subcapitata* (1.5×10^5 cells/ml) every day, with culture medium being renewed every other day. The population density was monitored daily to check when its carrying capacity was reached (the carrying capacity was determined from the counts made daily and considering that this point had been reached when for a period of 3 or 4 days the counts varied less than 10%) (Fig. 1). The daily counting's were performed in three 100 ml subsamples for each vessel, and then the average value extrapolated to the total volume of 800 ml. At the carrying capacity point, and for every temperature, three replicates from each clonal lineage were kept exposed to ASTM and the other three were exposed to the concentration of artificial seawater that caused 70% mortality ($LC_{70,48\ h}$) to the most tolerant clonal lineage (clonal lineage N35 with a $LC_{70,48\ h}$ of 5.91 mS/cm; Table S1) and to the respective negative control (ASTM hardwater with a conductivity of ≈ 0.52 mS/cm) (Fig. 1). Same photoperiod conditions, feeding, and renewing practices were maintained as in laboratory cultures throughout the experiment. Immobilization of organisms (here considered as mortality; organisms remaining immobile during 15 s after gentle prodding) were check every 24 h until half of clonal lineages (i.e., three) totally disappear (further detailed in the results section; corresponded to 47 d at 17°C, 41 d at 20°C, and 31 d at 23°C), time at which the experiment ended. The exposure time causing 50 and 90% of mortality (LT_{50} and LT_{90}, respectively) in each clonal lineage was computed at 96 h of exposure and at the end of the experiment. The observations were made by performing also three 100 ml subsampling of each vessel.

Data analysis

The data on the LC_{50s} was collected from the literature (Venâncio et al., 2018). Short-term exposure (corresponding to the 96 h exposure period of the chronic experiment) and chronic (full duration of each experiment) exposure duration causing 50% and 90% of mortality (LT_{50} and LT_{90}, respectively) were computed with exponential, logistic, and Gompertz models using STATISTICA 7.0 (StatSoft, Hamburg, Germany), being chosen the model leading to the smallest relative spread:

$$Relative\ spread = \frac{Upper\ 95\%\ confidence\ limit - Lower\ 95\%\ confidence\ limit}{LT_{50}\ or\ LT_{90}}$$

Spearman's correlations were computed hypothesizing that there was a correspondence between the LC_{50} or LC_{70} and the LT_{50}, i.e., clonal lineages with higher lethal tolerance, would be also the ones dying later. For LT_{90} such correlation was not made as these values are close to population extirpation.

Among-clonal differences in controls population density at the end of each experiment was checked to evaluate possible tolerance-associated fitness costs, under no salt stress, with one-way ANOVA. As well as, for each species, the survival time under seawater exposure of the three most sensitive clones was compared with a two-way ANOVA.

The short- and chronic LT_{50} values were compared to identify any recovery ability along salt exposure time with one-way ANOVA. The recovery (or no recovery ability) to salt exposure was estimated (for each of the three temperatures, independently) by $Recovery = \frac{LT_{50}\ on\ the\ long-term}{LT_{50}\ on\ the\ short-term}$, considering that if the ratio was ≈1, there is no recovery from salt exposure as the long- and short-term LT_{50} are similar, if < 1, there is a higher probability for lineages to disappear suddenly under salt exposure, and > 1, there is a higher probability for lineages to recover from salt exposure in the long-term.

The LT_{90} is considered the best estimation to have an understanding about probable extinction of populations, in this case among lineages, where almost 90% of the population dies off and have the minimum possibility to recover back (Wiens, 2016). To identify possible significant differences of extinction risk, a one-way ANOVA was performed with the LT_{90} values obtained for each set of six clonal lineages, under different temperature regimes. Data sets were tested for normality with the Kolmogorov–Smirnov test and for homoscedasticity with the Bartlett test. Tukey's and Dunnett´s multicomparison tests were used after ANOVA to identify within-factor among-level differences. The probability of extinction was estimated by $Probability\ of\ extinction = \frac{LT_{90}@20°C}{LT_{90}@17°C or 23°C}$, considering that if the ratio was ≈1, there is no difference in the probability of extinction after salt exposure at neither temperature, if < 1, there is a higher probability of lineages to be extinct under salt exposure at their optimal temperature of 20°C, and > 1, there is a higher probability for lineages to be extinct at other temperature than their optimal (17 or 23°C).

Results

Correspondence between the LC_{50} or LC_{70} and the LT_{50}

The computed LC_{50} or LC_{70} and respective confidence limits at 95% for all clonal lineages of *D. longispina* are presented at Table S1. No correlation was found between LC_{50} or LC_{70} and the short- or chronic LT_{50} obtained for each of the six clonal lineages of *D. longispina*. The Spearman correlations' values were between − 0.71 and 0.37 ($0.14 \le p \le 0.92$).

Densities under controlled conditions at three temperatures

The final densities of the six clonal lineages of *D. longispina* exposed under control conditions (with no salinity stress) were compared within each temperature (17, 20 and 23°C), (Fig. 2). All data sets passed normality and homocedasticity tests.

No significant differences were found for the six clonal lineages final densities between at any of the three temperatures (Fig. S1a–c; Tukey's, $i \ge 0.54$).

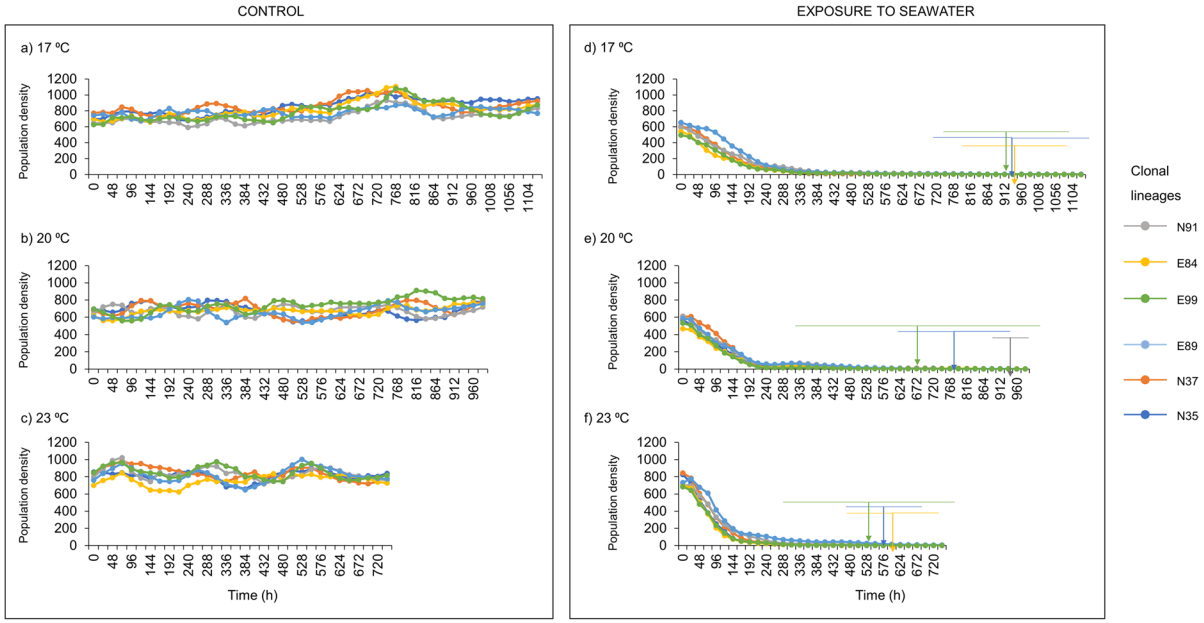

Fig. 2 Population density of six clonal lineages of *Daphnia longispina*, presented as pooled running averages, exposed to control conditions (panel on the left) and exposed to seawater (panel on the right) at three distinct temperatures (17°C, 20°C and 23°C). Seawater exposure corresponded to a conductivity level of 5.91 mS/cm. Three replicates were assembled per lineage and control or treatment. Vertical arrows indicate the average time of extinction ($n=3$) for each one of the three clonal lineages to be extinct faster, time at which the assay was ended. The horizontal lines aligned with the vertical arrows indicate the standard deviation ($n=3$)

Densities under seawater exposure at three distinct temperatures

Overall, some fluctuations along time were observed in the experimental controls for all *D. longispina* clonal lineages at all temperatures (Fig. 2a–c). Under seawater exposure, increasing temperature seems to have a synergistic effect. The time taken to disappear at least three clonal lineages (time at which the assay was ended) decreased with temperature increase (Fig. 2d–f). At 17°C, the assay was ended after 1128 h of exposure to a salinity level of 5.91 mS/cm

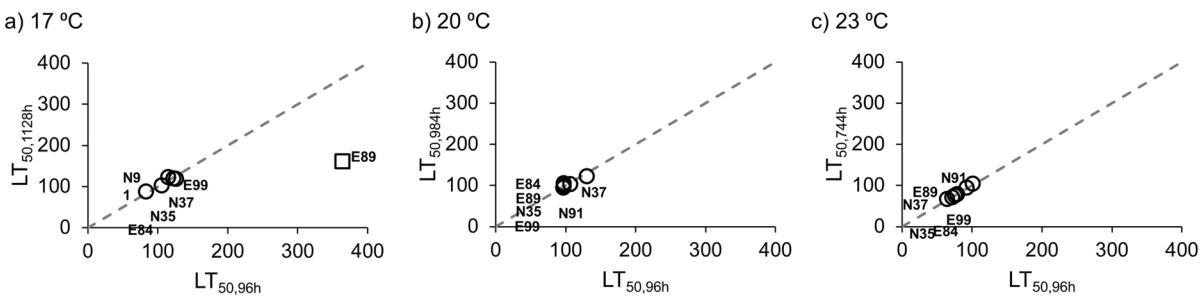

Fig. 3 Relation between the mean lethal time (LT_{50}, in hours) after short- and chronic exposure of six clonal lineages of the freshwater cladoceran *Daphnia longispina* to artificial seawater (5.91 mS/cm), at three distinct temperatures (17°C, 20°C, and 23°C). Clonal lineages are as follows: N91 (most sensitive), E84, E99, E89, N37, and N35 (most tolerant). The gray dashed diagonal line indicates no recovery. Clonal lineages marked with circles are considered not different from the no recovery line; whilst those clonal lineages apart from the line are considered to disappear suddenly (if positioned on the right of the line) or to recover (if positioned on the left of the line)

(Fig. 2d), while at 20 and 23°C the assay was ended after 984 h and 744 h, respectively, at the same salinity level (Fig. 2e, f). From the three clonal lineages to disappear faster, time to extinction of E99 was significantly reduced from 17 to 23°C (928 ± 169 h to 528 ± 216 h; Tukey's, $P = 0.04$). Moreover, clonal lineages E99 (intermediate tolerance to salinity based on the LC_{50}; Table S1) and N35 (clonal lineage presenting the highest tolerance based on the LC_{50}; Table S1) were always the first and second lineages to disappear, respectively, independently of temperature. The clonal lineage E84 was the third to disappear at 17°C and 23°C, whilst at 20°C the third lineage to disappear was N91.

Recovery ability on short- and long-time scale

The ability to recover was analyzed by plotting the LT_{50}'s obtained at the short-term exposure against those obtained on the chronic assay. Results are depicted in Fig. 3 and details on the LT_{50}'S are presented at Table S1. Overall, no recovery ability was observed at any tested temperature for *D. longispina* (Fig. 3a–c). The LT_{50} obtained at the chronic assay were very similar to the LT_{50} obtained on the short-term exposure period (96 h), with data points distributed along the dashed diagonal line (Fig. 3a–c). Clonal lineage E89 was the only one to stand out at 17°C (Fig. 3a), with a short-term LT_{50} of 364 h and a chronic LT_{50} of 161 h, indicating that if exposed for a long period of time to salinity it might disappear suddenly.

Probability of extinction in different lineages under different temperature regimes

The LT_{90} is the best estimate to have an understanding about probable extinction among lineages where almost 90% of the population died off and have the minimum possibility to recover back.

Overall, the LT_{90} values obtained for *D. longispina* decreased with increasing temperature, suggesting here as well, that salinity and increasing temperature may have a synergistic effect. The LT_{90} values ranged from 234 to 305 h at 17°C, whilst ranging from 151 to 250 at 23°C (Table S3). Also, the LT_{90} values did not showed statistical differences between 17 and 20°C in four out of the six *D. longispina* clonal lineages (Tukey's post hoc, $P > 0.05$ for N35, N91, E84, and E89), but LT_{90}@23°C were significantly lower than LT_{90}@20°C, except for clonal lineage E89 (Table S3). The time took for the extinction of the three first clonal lineages was on average of 212 h at the control temperature of 20°C (clonal lineages E99, N37, and N35); considering the same time frame, four clonal lineages were considered extinct at the temperature of 23°C (clonal lineages N35, E84, E99, and N37; Table S3), indicating a narrower within lineages variability at higher temperatures.

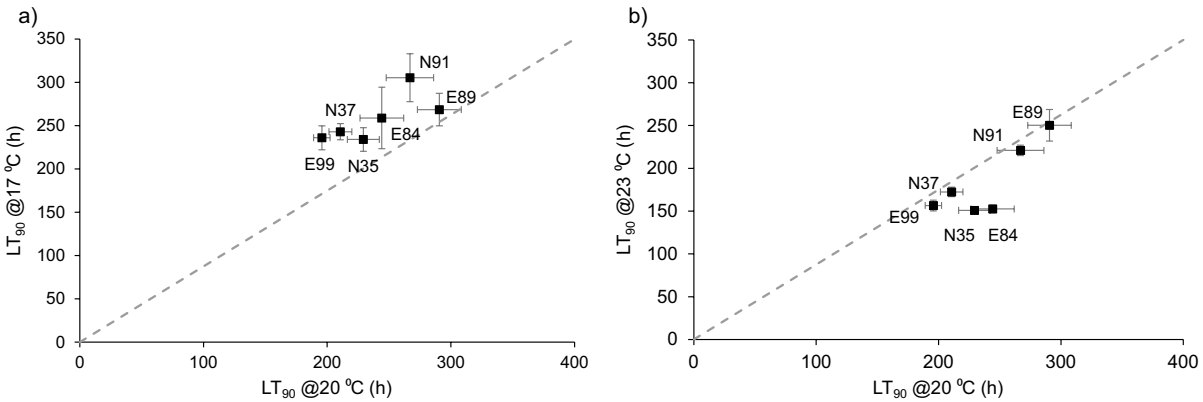

Fig. 4 Lethal times until the disappearance of 90% of the population (LT_{90} in hours) computed for six clonal lineages of *Daphnia longispina* after exposure to seawater treatment (5.91 mS/cm) at three distinct temperatures. The dashed diagonal line indicates no difference in the LT_{90} between temperatures. If positioned at the left of the line, the LT_{90}@17°C/LT_{90}@23°C is higher than the LT_{90}@20°C; if positioned on the left of the line, the LT_{90}@20°C is higher than the LT_{90}@17°C/LT_{90}@23°C. Error bars indicate the confidence limits at 95%. The LT_{90}@20°C is the control temperature

Plotting of the LT_{90}@20°C against the LT_{90}@17°C /LT_{90}@23°C, the crossing of the respective confidence limits over a diagonal line allows to distinguish statistically significant differences between temperatures (Fig. 4). The confidence limits of the LT_{90}@17°C of all *D. longispina* clonal lineages (except E89) did not cross the diagonal line, indicating to be different from the LT_{90}@20°C (Fig. 4a). Moreover, all data points were positioned on the left of the diagonal, indicating that overall, the LT_{90}@17°C were higher than to those computed for 20°C (Fig. 4a). Comparing the LT_{90}@23°C with LT_{90}@20°C, only clonal lineages N35 and E84 were clearly distanced from the diagonal line, and positioned on the right of the diagonal line indicating that the LT_{90}@23°C were lower than the LT_{90}@20°C (Fig. 4b).

Discussion

This study aimed at provide further insights on the combined effects of increased salinity and temperature on the populational diversity of zooplanktonic species. For that purpose, six clonal lineages of *D. longispina* were chronically exposed to a pre-determined salinity level under three different temperature regimes. The $LC_{50,48\ h}$ for six clonal lineages of *D. longispina* used for this study ranged from 2.68 to 4.99 mS/cm (Venâncio et al., 2018) and are in accordance with others previous values reported in the literature giving account on short-term and chronic tolerance of the same species to NaCl (e.g., Gonçalves et al., 2007; El-Gamal et al., 2014).

Prior to studying the combined effects of salinity and temperature, it is relevant to understand the influence of temperature alone (no salt added). Temperature alone did not seem to have a role with respect to the final densities of *D. longispina* clonal lineages, since no statistical differences were found in this parameter among clonal lineages in each tested temperature. Though, it must be said that lineages were selected according to their differential tolerance to salt and not temperature, and more, this range of temperature seems to be within the optimal reported for other daphnids (e.g., Bruijning et al., 2018) and in agreement also with other study reporting an almost complete acclimation process in around a week in daphnias exposed to temperatures ranging from 11 to 25°C (e.g., Müller et al., 2018).

Understanding the combined effects of temperature and salinity can be used to predict effects of extreme events induced by salinization that can occur in summer and winter conditions (extreme temperatures) on the genetic diversity of zooplankton populations and provide useful information likely to be fitted in more suitable regulatory actions. Overall, increasing temperatures lead to decreasing median lethal times and decreased extinction lethal times, which suggest a synergistic effect between both abiotic factors. During seawater exposure of 5.91 mS/cm, the time taken to disappear at least half of *D. longispina* clonal lineages (time at which the assay was assumed ended), was reduced in over one third when increasing the temperature from 17 to 23°C (from 1128 to 744 h, respectively). Based on previous studies, one might assume that, first, increasing temperatures, despite being reflected in shorter time to maturity and possible higher reproduction frequency, reduces the lifespan of cladocerans (e.g., Adamcuzk & Mieczan, 2019; Vignatti et al., 2022) possibly due to indirect effects of oxygen solubility that challenged daphnids to deal with increased anoxic environments, whilst challenged with increased osmotic pressure. A potential mismatch between oxygen supply and energy demand/expenditure might, thus, be reflected in their (shorter) chronic tolerance to salinity. Actually, Paul et al. (2004) has referred that long-term acclimation to temperature has a minimal role in posterior thermal tolerance, and that differences of acclimation to distinct temperatures may vanish as soon as 24 h later. So, based on that, it is hypothesized that once diverting constantly energy to deal with unfavorable temperatures, less is channeled for osmoregulatory purposes, which is reflected in lower survival rates and shorter life span at higher temperatures, as observed in other daphnia species such as *Daphnia menucoensis* Paggi, 1996 (Vignatti et al., 2022). According to the results obtained with *D. longispina*, salinization events occurring at high temperatures in summer may have a greater potential to lead to the disappearance of some clonal strains than winter marine floods.

The capacity for chronic recovery of the clonal lineages would present a "win–win" situation, and thus one of the initial assumptions (based on previously reported lethal concentrations) was that the most salt-sensitive clonal lineages would be the first ones to disappear under salt exposure regardless of temperature, and therefore the permanence of the more

salt-tolerant clonal lineages would sustain the resilience of populations in salt-impacted locations. The resilience of the population would be ensured whilst maintaining its genetic pool (and therefore its capacity to respond to future scenarios of environmental variability). Yet, overall, for *D. longispina* no recovery ability was observed at any tested temperature (i.e., LT_{50} computed on short-term exposures were very similar to those computed for chronic exposures). Results obtained with *D. longispina* may in part be explained by the salinity level here chosen (5.91 mS/cm), since previous works have reported the ability of other daphnids to evolve tolerance to intermediate levels of salinity (2.57 mS/cm) within just 5 to 10 generations (2.5 months), whilst showing sharp declines in survival at higher salinity levels (3.76 mS/cm) (Coldsnow et al., 2017). Aside results of Coldsnow et al. (2017) with *Daphnia pulex* Leydig, 1860, other works on zooplankton species [*Simocephalus vetulus* O.F. Müller, 1776 (Loureiro et al., 2012)] have demonstrated long-term evolved halotolerance within natural populations (Loureiro et al., 2012). On a finer scale (attending to the fact that mechanisms underlying salt exposure on the short- and on the long-term are yet very blur, even more when adding different clonal lineages to simulate a real population to the equation), it is to consider the lack of agreement in the ranking of the lineages in their lethal and sublethal tolerance namely to metals and salt (e.g., Lopes et al., 2004; Venâncio et al., 2018), due to the fact that they most probably are ruled by different mechanisms.. In general, lethal mechanisms are dependent on one or few mechanisms, whereas sublethal, long-term responses are dependent on many and general mechanisms. For instance, reproduction under long-term stress is related to, for instance, physiology, feeding/filtering/ingestion, lipid/sugar metabolism. The later ones are thus linked to fitness and performance thus having low heritability, since they will be under continuous selection (Lopes et al., 2004). Therefore, different clonal lineages that may display a higher short-term tolerance may not be the most resilient under long-term exposure, and vice-versa. More recently, sub-lethal mechanisms such as epigenetic traits (DNA methylation) have been brough to light as one the potential processes responsible for evolved tolerance to salt in daphnia populations, and are also known to differ within genotypes, as corroborated by the results of Asselman et al. (2015) with two *D. magna* lineages. Likewise, it is also hypothesized that such mechanisms may be transferred along more generations in the most sensitive clonal lineages than in more tolerant lineages, thus partially explaining the long-term resilience of the most sensitivity clonal lineages rather than the most tolerant.

Temperature increments maybe rather gradual, unlike salinization events that might be sudden for instance if scenarios of sudden storming are pictured. The simulation of extinction scenarios as here presented by computation of the LT_{90} might be a good surrogate to assess how vulnerable a determined lineage is to, in this case, abiotic stressor combination. Yet, research often neglects sudden changes or abrupt regime shifts, and thus the probability of extinction. Results indicated that increased temperatures scenarios (from 17 to 20°C) may significantly speed up time to extinction in 5 out of the 6 of *D. longispina* clonal lineages. Thus, it is likely that, for zooplankton, loss of resilience under extreme salinization scenarios may be boosted by temperature. These findings are somehow supported by the results of Garreta-Lara et al. (2018) when studying the metabolome of *D. magna* under salt (control and 5 g NaCl/L) and thermal (20°C—control, and 25°C) stress. The authors found out that the metabolic pathways underlying the tolerance to both stressors are quite dissimilar. When under thermal stress, only 15% of the metabolites were de-regulated and were mostly related with energy supply demands, whilst under salt exposure about of 70% of the metabolites were down-regulated and related to deficient carbon metabolism, amino acid synthesis, protein digestion, and mineral adsorption (Garreta-Lara et al., 2018).

Using clonal lineages and parthenogenetic species allows to set aside maternal and environmental effects throughout the study, thus the differences observed across populations were due to their genetically determined variations and population density fluctuations were the response of population solely to triggered selective pressure, in here salinity. Despite the strategies displayed by cladocerans (cryptobiosis—production of dormant structures) to resist and avoid stressful and assure maintenance of the populations (e.g., Nielsen et al., 2012; Radzikowski, 2013), low levels of salinity may endanger the viability of such resource, and based on the results above, expectedly to worsen

at extreme temperatures. For instance, the richness and abundance of zooplankton egg banks were sharply reduced at salinity levels > 1.56 mS/cm (Nielsen et al., 2003; Brock et al., 2005).

The genetic structure and diversity are important factors in invertebrate populations which have limited dispersal capabilities (Fasola et al., 2015). This study confirmed that temperature itself may be, in the long-term, a selective pressure to zooplanktonic species, and in combination with salinity might worsen and/or speed the loss rate of clonal lineages. As genetic diversity is a crucial factor for population's resilience its reduction may further influence fitness, environmental plasticity, co-tolerance and tradeoff mechanisms of the population and ultimately lead to the extinction of species (Chen et al., 2012; Ribeiro and Lopes, 2013; Fasola et al., 2015).

Conclusion

Climate change-induced salinization events are evident in freshwater ecosystems and may synergically be intensified by oscillating temperatures. The results of the chronic laboratory experiments with six clonal lineages of *D. longispina* (selected based on their differential sensitivity to salinity) combining a salinization scenario with different temperature regimes indicated that scenarios of extreme temperatures such as summer droughts (23°C) and winter sea flooding (17°C) can further enhance the loss of genetic variability of zooplankton, thus, shaking the cornerstone of population resilience. Moreover, clonal lineages presenting higher or intermediate tolerance to salinity where the first ones to disappear and thus, it is anticipated that the loss of genetic variability within the population might be higher than expected and increase substantially the susceptibility of the population to future stressors and/or environmental changes (Ribeiro and Lopes, 2013; Venâncio et al., 2016). These are groundbreaking results since results from ecotoxicological assays combining abiotic factors rely almost always on a single lineage and, therefore, this work has a pivotal role in understanding/predicting populations local extinction and/or resilience under climate change scenarios.

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Data availability All data will be made available by the authors upon request.

Declarations

Conflict of interest The authors declare they have no competing interests.

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