Regional and fine-scale local adaptation in salinity tolerance in *Daphnia* inhabiting contrasting clusters of inland saline waters

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Abstract

Freshwater salinisation is an important threat to biodiversity, ecosystem functioning, and the provision of ecosystem services. Therefore, understanding the capacity of species to adapt to salinity gradients is crucial. Clusters of naturally saline habitats represent ideal test cases to study the extent and scale of local adaptation to salinisation. We studied local genetic adaptation of the water flea *Daphnia magna*, a key component of pond food webs, to salinity in two contrasting landscapes - a dense cluster of sodic bomb crater ponds and a larger-scale cluster of soda pans. We show regional differentiation in salinity tolerance reflecting the higher salinity levels of soda pans versus bomb crater ponds. We found local adaptation to differences in salinity levels at the scale of tens of metres among bomb crater pond populations but not among geographically more distant soda pan populations. The population-level salinity tolerance range was reduced in more saline bomb crater ponds through an upward shift of the minimum salt tolerance observed across clones and a consequent gradual loss of less tolerant clones in a nested pattern. Our results show genetic adaptation to salinity gradients at different spatial scales and fine-tuned local adaptation in neighbouring habitat patches in a natural landscape.

Key words: local and regional adaptation, salinity tolerance, nestedness, zooplankton, sodic waters, pondscape

INTRODUCTION

Salinity is a key abiotic gradient in aquatic systems. Inland waters cover a wide range in salinity levels [1], which have a strong structuring role on biodiversity, community composition, and ecosystem functioning [2–4]. Naturally saline waters are very common in large areas of the world, especially in dry climatic regions [1,5] and in areas that are dominated by mineral-rich soils, such as soda pans and lakes in Central Europe [6]. Many of these naturally saline waters have a high importance for conservation, by hosting unique salt-adapted communities and providing feeding grounds for large flocks of waterbirds [5,7,8]. Recently, there has been an increased focus on saline lakes in the context of anthropogenic freshwater salinisation, a process driven by mining effluents, road deicing, unmanaged irrigation and climate change, and emerging as a global threat to freshwater resources [9–11]. Understanding to what extent species can adapt to increasing salinities and how this relates to landscape features such as geographic distances among patches and the steepness of the salinity gradients thus becomes increasingly important in our efforts to predict responses to global climate change.

Salinisation leads to strong changes in community composition through species sorting. Communities of inland saline water bodies often are a nested subset of freshwater communities [3,12,13], whereas species replacement becomes more prominent at higher salinities [2,13,14]. Parallel to environmental sorting structuring metacommunities in the landscape, changes in salinity can also lead to strong selection pressures impacting the evolution of local populations and thereby affecting metapopulation structure [15,16]. There is evidence for local adaptation to salinity gradients in multiple taxonomic groups such as fish, amphibians, and insects [17–19]. In one example, it has been shown that the anuran amphibian *Hyla cinera*, which is increasingly occurring in anthropogenically salinising coastal wetlands, has evolved an increased salinity tolerance in coastal compared to inland freshwater populations, showing a higher fecundity and increased hatching success and tadpole survival upon salt-exposure [18].

It is important to understand at what spatial scale genetic adaptation occurs in nature. Theory predicts that the occurrence of local adaptation depends on the selection-migration balance [20–23]. It can therefore be expected that the likelihood of local adaptation is higher when selection gradients are stronger and when gene flow is not so

high as to homogenise genetic variation across habitats. The latter suggests that local adaptation may depend on geographic distances. Yet, there are several mechanisms that interfere with this general inference and that allow patterns of local adaptation at very small spatial scales [24].

Clusters of naturally saline aquatic habitats provide excellent model systems to explore how, to what extent and at which spatial scale freshwater species can adapt to salinity gradients. The Pannonian Plain in Central Europe is characterised by sodic waters that strongly vary in salinity in space and time [3,25]. The typical soda pans dry out every year and thus exhibit a wide range in salinities, from sometimes freshwater at the start of the growing season to hypersaline levels up to >50 g L^{-1} at the end of the growing season [6]. Other sodic habitats in the region, such as bomb crater ponds, are less extreme in their salinity, with not all ponds drying up every year [26].

Daphnia magna is a freshwater zooplankton species that typically occurs in small ponds and shallow lakes. Because it has a relatively broad salinity tolerance compared to many other cladoceran zooplankton species [3,27,28], it thrives in sodic waters, also because they are generally fishless and turbid, which reduces the vulnerability of this large-bodied cladoceran to predation [26,29]. The widespread occurrence and high abundances of *D. magna* in sodic waters [3] and the fact that these habitats can widely differ in salinity in time and space offers a unique model system to study the degree of local adaptation and to reveal at which spatial scale it occurs. *Daphnia* is a key component of many inland food webs by linking primary production to higher trophic levels [30,31], and has been shown experimentally to have the capacity to adapt to salinisation [32,33]. One of the best studied examples involves obligate parthenogenetic lineages of *D. pulex*, which were shown to be adapted to salinity gradients by strong clonal sorting, with clear differences in salinity tolerance between clones originating from low- and high-salinity habitats [34,35].

Here, using a quantitative genetics common garden approach, we test for the salinity tolerance of 126 clones from 21 populations occurring in the same ecoregion. We use *D. magna* populations from both soda pans and sodic bomb crater ponds as model systems to quantify the degree and spatial scale of local genetic adaptation to differences in salinity level. Given the higher salinity levels of soda pans compared to bomb crater ponds, we expect regional differences, with the *D. magna* populations isolated from the soda pans

showing a higher salinity tolerance compared to the populations isolated from the bomb crater ponds. Within the soda pans, we do not expect local adaptation, not only because they show strong intra-year variation in salinity, but also because they are both important waterbird habitats and prone to strong winds, both of which can facilitate dispersal [36,37]. In the bomb crater pond system, even though they are much closer to each other in space than the soda pans, we do expect local adaptation. This is because they systematically differ in salinity, are more sheltered from wind, and not intensively frequented by birds. We also aimed to explore how local habitat salinities determine clonal variation in salinity tolerance of populations inhabiting the bomb crater ponds or soda pans. We test the hypothesis that, similar to patterns seen in species occurrences along salinity gradients, salinity tolerances of the populations inhabiting habitats characterised by relatively high salinities are subsets of the salinity tolerances of populations inhabiting habitats characterised by lower salinities. This pattern would reflect that genotypes characterised by lower salinity tolerances are weeded out in the high salinity habitats.

MATERIAL AND METHODS

Study systems

Soda pans are shallow, saline, and fishless water bodies, which generally dry out in summer. Within Europe, their occurrence is restricted to the Pannonian Plain; the lowlands of Hungary, eastern Austria, and northern Serbia. The pans are alkaline, and their salinity exhibits a wide gradient from almost freshwater to hypersaline conditions [6]. The predominant environmental gradient, salinity, is a strong selection factor, and only a limited set of highly tolerant species account for the largest part of zooplankton biomass [3]. This includes *D. magna*, which is the most widespread species in these habitats [29]. The Seewinkel region in the North-western part of the Pannonian Plain is part of a UNESCO World Heritage Site. It hosts a cluster of 34 soda pans, formed in a confined region of approximately 200 km^2 (Fig. 1; [3]). They are spatially discrete ecosystems without natural in- or outflow; the nearest distance to another pan ranges from 0.1 km to 18 km [6]. The surface area of the pans varies from 0.2 to 97.3 ha. The pans have a high surface to depth ratio and are very exposed to wind. The region experiences very constant unidirectional

winds, which result in a directional spatial imprint on zooplankton metacommunity structure [36].

In the Kiskunság region of Central Hungary, there is a dense cluster of more than 100 bomb crater ponds situated within an area of ~1 km^2, created by mistargeted bombing during World War II (Fig. 1). They hold sodic water and exhibit a strong gradient in salinity (1.0–5.6 g L^{-1}, [26]). Their diameter varies between 3 and 12 m. The average distance (calculated based on pond centre points) between bomb craters is 300 m and ranges from 9 to 860 m, which in some cases results in ponds to be only a mere 2-3 m apart (effective distance). *D. magna* is one of the dominant species in these ponds, being present in 66.7% of the ponds and across a wide salinity gradient [26].

Sampling and culturing of *D. magna*

During the period 10-13 April 2018, a total of 21 *D. magna* populations were sampled; 14 bomb crater pond (47°7.403'N 19°8.187'E, Fig. 1) and seven soda pan populations (47°46'51.31"N, 16°52'5.39"E, Fig. 1). During sampling we also measured conductivity (Fig. 1, Table S1, Supplementary Information) and other environmental variables (pH, water depth, total suspended solids and chlorophyll *a* concentrations) following the methods described in [3] and [26] (Table S1, Supplementary Information). We converted conductivity to salinity by applying a multiplying factor of 0.774 established for soda waters [3,38]. At the time of sampling, pond salinities ranged from 0.69 to 3.56 g L^{-1} in bomb crater ponds and from 1.36 to 7.07 g L^{-1} in soda pans (Table S1, Supplementary Information).

Between 8 and 20 *D. magna* individuals were isolated from each population and cultured as clonal lineages. Of these, six clonal lineages from each population were randomly picked out to be used in the common garden experiments, resulting in 21 populations x 6 lineages = 126 lineages. All clonal lineages were expected to be unique clones based on a genetic analysis using 13 microsatellite markers carried out on multiple soda pan and bomb crater populations that were sampled in April 2014, where we observed that all individuals differed in multi-locus genotype ([39]; Horváth Z. pers. comm.). Based on this, we expect that there were none or only very few genotypes that were represented by two or more individuals. A parthenogenetically produced offspring of each clone was used to inoculate 6 independent monoclonal cultures (n = 126 x 6) consisting of one *D. magna* individual in a 210-

mL jar containing 180 mL of dechlorinated tap water and kept under standardised laboratory conditions (climatized walk-in room 20°C ± 1°C; 14:10h Light:Dark photoperiod, 50% medium renewal three times a week) and fed daily by the unicellular batch-cultured green algae *Acutodesmus obliquus* (10^5 cells mL^{-1}, ~ 1 mg C L^{-1}). All lineages were cultured for at least two generations under these standardised conditions to reduce interference from (grand)maternal effects. At the start of each new generation, the culture was re-inoculated with two randomly picked second clutch offspring juveniles reared until sub-adult stage, after which rearing continued with one individual. At the start of the third generation, 15 newborn second clutch juveniles (<24h old) were inoculated in 500-mL jars filled with dechlorinated tap water, transferred to a 20°C temperature-controlled water bath and kept under the same culture conditions and feeding regime as described above.

Salinity tolerance experiments

In an effort to cover the natural range of salinities in the studied landscapes, we established 8 different salinity treatments containing 0, 3, 4, 5, 6, 7, 8, 9 g L^{-1} $NaHCO_3$. Salt solutions were prepared daily for use in the experiment. Three replicate cohorts of 10 <24h old juveniles (2nd to 4th clutch) of animals grown under common-garden conditions were exposed in 210 ml jars containing 180 mL of a pre-set $NaHCO_3$ and immobilisation was assessed after 48h as a proxy for mortality. Animals were considered dead when they did not show any muscular response after stirring the jar gently [40]. This design resulted in 21 populations x 6 clones x 8 salinity treatments x 3 replicates = 3024 experimental observations at 48h. Animals were not fed during the trial [40]. To generate independent data for each replicate, the 126 lineages were cultured in such a manner that animals used for replicate observations were separated in time by at least two generations.

Statistical analyses

Mortality upon salt exposures and evolution of salinity tolerance

All statistical tests were carried out using R version 4.3.1 [41]. We aimed to test for the impact of increased salinity levels on mortality and the signal of evolution in salinity tolerance among the clones isolated from bomb crater ponds and soda pans. Given the geographical distance

and overall differences in salinity concentration between the two systems, we used a hierarchical approach, testing for the effect of region (bomb craters vs soda pans), populations within region, and clones within populations. We built hierarchical linear models for both raw data on mortality across all salinity treatments and for EC_{50} values (estimation methodology detailed in Supplementary Information, section Material and Methods).

Differences in salinity tolerance of *D. magna* cohorts across laboratory salt exposure levels (0-9 g L^{-1}) were statistically tested using a generalised linear mixed-effect model (GLMM, package 'lme4', [42] using the number of dead and alive animals in each experimental trial as a binomial dependent variable, fitted via the insertion of a binomial error distribution with logit link function. Pond type (bomb crater vs soda pan), laboratory salt concentration (continuous) and their interaction were included as explanatory variables. 'Population' (nested in 'pond type') and 'clone' (nested in 'population') were included as random effects. The model was tested and corrected for overdispersion ('blmeco' package, [43]; overdispersion function by [44]). The 'car' package [45] was used to estimate type II Wald chi-square statistics and p-values. Random effect 'clone' was investigated further via a model-comparison (full model, model without the random effect 'clone') to test for overall within-population variation in salt tolerance, indicative of overall evolutionary potential for salinity tolerance in populations. Half maximal effective concentration (EC_{50}) offers an integrated way to assess tolerance to a chemical stressor by estimating a dose response function integrating data across all tested concentrations. We therefore also tested for adaptation to salinity among habitat types and among populations within each of the two habitat types applying linear mixed-effect models (package 'lme4') regressing EC_{50} values (estimation detailed in Supplementary Information, section Material and Methods) against field salinity (continuous, g L^{-1}) and pond type (categorical, soda pan vs bomb crater). 'Population' was included as a random effect (nested in pond type). In order to have more reliable estimates of clone-specific EC_{50}, we used all replicate data of a given clone (n = 3 per clone) to calculate one integrated value for EC_{50}. As a result, no clonal random factor was added to the model as there was only one value per clone. A restricted maximum likelihood estimation (REML) method was used and degrees of freedom for fixed effects were corrected using the Kenward-Roger approximation [46]. P-values were computed using the 'car' package [45]. Model assumptions (normality, heterogeneity of variances across pond types)

were visually checked (normality: plotting histograms of model residuals, residuals versus fitted values, and normal Q-Q plots) and formally tested (normality: Shapiro-Wilk test, heterogeneity of variances: Levene's test with pond type as a grouping factor). No deviations with regard to model assumptions were found. Given the uneven spread along the field salinity gradient of bomb crater and soda pans, the above mentioned model was run with a log-transformed field salinity as well, and yielded qualitatively the same results (Supplementary Information, section Results, Table S2). In addition, linear mixed-effect regression models on EC_{50} values were run separately on the bomb crater and soda pan datasets, with field salinity (continuous) as explanatory variable and 'population' included as a random effect, yielding similar qualitative output (Supplementary Information, section Results, Table S3).

Variation in salinity tolerance within populations

To explore whether variation in salinity tolerance within *D. magna* populations was a function of salinity of their original habitats and whether this reflects a weeding out of genotypes with lower salinity tolerance, we ran linear regression models. As response variables, we used the population level minimum EC_{50} (i.e. the EC_{50} value of the clone of a given population with the lowest salinity tolerance), the maximum EC_{50}, (i.e. the EC_{50} value of the clone of a given population with the highest salinity tolerance) and the range in EC_{50} values for a population (difference between EC_{50} values of the clones with the highest and lowest salinity tolerances). All three models included salinity (continuous), pond type (bomb crater vs soda pan) and their interaction as explanatory variables. Model assumptions were visually checked and formally tested, and were met in all models.

As we have multiple mortality data per clone (three replicate values per clone per exposure concentration), we can obtain a rough estimate of heritability of the trait and thus evolutionary potential using a clonal repeatability approach (quantifying the relative importance of among- and within-clone variation in salinity tolerance for each population. The calculated heritabilities are rough estimates because of the low number of replicate observations per clone and the low number of clones per population, but they provide a first indication of evolutionary potential. Given the observation that most variation in mortality was present between 5 and 7 g L^{-1} (below 5 g L^{-1} full survival, above 7 g L^{-1} quasi-full mortality,

see Fig. 2a), heritability in each population was calculated based on average clonal mortalities across salt exposure concentrations 5 to 7 g L^{-1} $NaHCO_3$. Heritability as given by $h^2 = V_g / V_g+V_e$ was estimated for each of the 14 bomb crater pond and 7 soda pan populations separately following a clonal repeatability analysis and maximum-likelihood method [47,48]; we calculated among clonal variation (V_g + V_e) and within clonal variation (V_g) based on mean square model output values of among clonal variation (V_g + V_e) and an error term (V_e). We carried out a regression analysis on estimated heritabilities calculated for each population with salinity observed in their habitat (continuous), pond type (bomb crater vs soda pan) and their interaction as explanatory variables. Model assumptions were visually checked and formally tested, and were met in all models.

There are in essence two ways in which populations can adapt to higher salinities: they can show a reduced occurrence or frequencies of genotypes with low salinity tolerance, or they can show a general shift in genotypes towards higher salinity tolerances. The former reflects a weeding out of sensitive genotypes and results in a 'nested' pattern where the range in sensitivity of genotypes in high salinity ponds is a subset of the range of sensitivity in low salinity ponds. The latter reflects a turnover type of process, resulting in a general upward shift in sensitivities of genotypes without necessarily reducing the range in sensitivities. Given that we observed a strong correlation between minimum EC_{50} values and pond salinity in the bomb crater populations while no such correlation was observed for maximum EC_{50} values (see further), we tested for a nestedness pattern in salinity tolerance along the natural salinity gradient across habitats. Nestedness is typically tested in community data and use occurrence data on taxa. In our analysis, we cannot use genotype ID, because every individual likely belongs to a different genotype. To develop a parallel analysis, we therefore binned the genotypes into functional identities based on their EC_{50} values. We applied a manual binning to the full gradient of EC_{50} values in bomb craters, creating 6 classes of EC_{50} values with equal ranges (ID1: 3.73 - 4.11, ID2: 4.11 - 4.49, ID3: 4.49 - 4.87, ID4: 4.87 - 5.24, ID5:5.24 - 5.62, ID6: 5.62 - 6 g/L). These categories were treated as functional IDs for genotypes exhibiting a gradient of EC_{50} across the ponds. We first tested whether their number in a local population decreases along the natural salinity gradient with a linear model. We then tested for the general presence of any nestedness pattern in the genotype composition of the ponds with the functions 'nestedchecker" and 'oecosimu' in the 'vegan' package [49], using C-score

statistics based on the null model method 'r0' with 2000 simulations. A nestedness plot was created using the 'nestednodf' function in "vegan". Finally, we also tested whether the level of nestedness of individual ponds corresponds to the natural salinity gradient with another linear model. For this model, we manually corrected the order of ponds projected on the nestedness plot to include ties in the analysis, given that in our case, only presence-absence data was available, therefore the order of two ponds with the presence of the same functional identities is random on the nestedness plot. If genotype thinning is the mechanism consistent with increasing salinity, a positive relationship is expected between the order of ponds according to their nestedness and salinity, with the first and most diverse ponds being the least saline, and the ponds with higher salinity showing a reduced occurrence of functional identities representing lower salinity tolerances.

RESULTS

Mortality and evolution of salinity tolerance (EC_{50})

Mortality increased significantly with exposure to higher salinities, with a steep increase between 5 and 6 g L^{-1} (b = 2.820 ± 0.087 on log-odds scale, $p < 0.001$, chi-square: 1346.12; Fig. 2a). The curve slopes differed for *D. magna* populations from soda pans vs bomb crater ponds, with a flatter slope for animals from soda pans compared to bomb craters (pond type x experimental salt concentration: $p < 0.001$, chi-square = 20.22; Fig. 2a). This is paralleled by a higher range of salinities measured in the field for soda pans compared to bomb craters (Fig. 1 and Fig. 2C). Random effect 'clone' (nested within 'population') was significant ($p < 0.001$, chi-square = 20.893) indicating that there was an overall effect of clonal variation within populations on survival upon salt exposures.

Average median effective concentration (EC_{50}) values for salinity tolerance of *D. magna* were significantly higher for soda pan than for bomb crater pond populations (b = 1.256 ± 0.224, $p < 0.001$; Table 1, Fig. 2b), consistent with the higher average mortalities of bomb crater pond populations observed at intermediate experimental salt concentrations (Fig. 2a). Soda pan and bomb crater pond populations differed in how average EC_{50} values of populations varied as a function of pond salinity observed in the field (pond type x salinity; b = −0.236 ± 0.08; $p = 0.011$; Table 1, Fig. 3). Separate analysis on bomb crater pond and soda

pan populations confirmed that while average EC_{50} values of bomb crater pond populations increased significantly with pond salinity (b = 0.210 ± 0.056, p = 0.009; Table S3 Supplementary Information, Fig. 3), no such signal of local adaptation was found in soda pan populations (b = −0.026 ± 0.056, p = 0.659; Table S3 Supplementary Information, Fig. 3). Random variation attributed to population-level variation among both bomb crater ponds and soda pans was not significant in any of the analyses (Table 1 and Table S2, S3, Supplementary Information).

Variation in salinity tolerance within populations

Linear regression on the population-level range in clonal EC_{50} values revealed a marginally significant effect of salinity x pond type, with the range in EC_{50} values showing a decreasing trend with increasing salinity in the bomb crater ponds but not in the soda pans (b_{BC}= –0.12, pond salinity x pond type: $F_{1/17}$ = 4.346, p = 0.052; Fig. S1, Table S4 in Supplementary Information). The EC_{50} values of the clones with the highest salinity tolerance within each population showed no relationship with pond salinity (salinity: $F_{1/17}$ = 0.097, p = 0.759; Table 2, Fig. 4a). Within-population clonal maximum EC_{50} values were significantly higher in soda pans compared to bomb craters (b_{SP} = +0.697, pond type: $F_{1/17}$ = 21.482, p < 0.001; Table 2, Fig. 4a). The EC_{50} values of the clones with the lowest salinity tolerance within each population show a significant increase with increasing pond salinity in bomb crater ponds but not in soda pans (b_{BC} = 0.40, pond salinity x pond type: $F_{1/17}$ = 13.906, p < 0.001; Table 2, Fig. 4b). Heritabilities, estimated based on a repeatability analysis of clonal replicates for mortality across the range 5-7 g L^{-1} $NaHCO_3$, show a trend for a negative association with salinity in bomb crater ponds but not in soda pans (b_{BC} = –0.18, pond salinity x pond type: F= 3.813, p = 0.067; Table S4, Fig. S2 in Supplementary Information). However, we found low significances for heritabilities based on the clonal repeatability analyses for each population separately (bomb crater populations: three populations with 0.8 < p < 0.1 and one with p < 0.001; soda pan populations: one with 0.8 < p < 0.1 and one with p = 0.022).

Local diversity in salinity tolerance (measured as the number of genotypes belonging to six equal sub-ranges of the EC_{50} gradient of bomb crater populations) showed a marginally significant negative trend with pond salinity in the bomb craters (R^2 = 0.229, p = 0.083; Fig. 5a). A significant nestedness pattern was detected in these populations (C-score

= 7.4, $p < 0.01$; Fig. 5b), which showed a marginally significant trend corresponding to the salinity gradient ($R^2 = 0.225$, $p = 0.088$; Fig. 5c).

DISCUSSION

Our results clearly show a strong and significant pattern of both regional genetic differentiation between the two study systems, as well as local genetic differentiation in salinity tolerance in the bomb crater pond but not in the soda pan system. Both of these patterns are in line with predictions of adaptive evolution. First, the higher average salinity tolerance of soda pan populations is associated with the generally higher regional salinity levels of soda pans compared to the bomb crater ponds. Second, the salinity tolerance of bomb crater pond populations is positively correlated with the salinities measured in their local habitats. The pattern observed in the bomb crater pond system represents a striking case of local genetic adaptation at very small spatial scales, as these ponds are located only a few to a few hundred metres separated from each other. These results also show that the degree to which local genetic adaptation is observed is not necessarily associated with geographic distance [24], as the soda pans are geographically much more distant to each other than the bomb crater ponds. Finally, we also show that the range in clonal values for salinity tolerance in bomb crater ponds tends to be higher in populations with low salinities. This is driven by the minimum clonal value of salinity tolerance within a population significantly increasing with pond salinity.

Patterns of local genetic adaptation have been reported for a wide range of ecosystems and at wide spatial scales [24,50]. Earlier studies have provided ample evidence for the water flea *Daphnia* to have the capacity to genetically adapt to a wide range of abiotic and biotic stressors (e.g. fish predation pressure: [51,52]; algal toxins: [53,54]; parasites: [55]; warming: [56,57]; urbanization: [58,59]; pollutants: [60,61]. Earlier studies, mostly focusing on contrasting two populations inhabiting ponds or lakes with different salinities, have also shown that salinity tolerance levels of *Daphnia* clones and populations originating from habitats with contrasting salinities reflect the salinity of their habitats [62,63]. Studies on obligately parthenogenetic *D. pulex* showed clonal sorting along salinity gradients in ponds in the Churchill region of northeastern Manitoba, Canada [34,35].

Experimental evolution revealed that local adaptation of *Daphnia* to a moderate increase in salinity can occur within 2.5 months, representing an estimated 5-10 generations of parthenogenetic reproduction, reflecting fast clonal sorting during the parthenogenetic phase of reproduction and the potential to show significant adaptation within one growing season [32]. Our study quantified local genetic differentiation in salinity tolerance of 21 *D. magna* populations in relation to habitat salinity in natural landscapes of both closely-spaced bomb crater ponds and geographically more distant soda pans. Given the extent and pace of freshwater salinisation, these results are important as they indicate that *Daphnia* populations may adapt at different spatial scales including at the very local scale of ponds being separated by just a few metres. Given their important role as grazers and prey in the sodic waters we studied [26,29,64,65], it is likely that this level of genetic adaptation will have important ecosystem implications [66].

In line with our initial expectation, the pattern of regional genetic differentiation that we observe between the soda pans and the bomb crater ponds reflects the differences in salinity ranges between the two types of habitats. Soda pans are very shallow and relatively large in surface area compared to the bomb craters, resulting in much higher surface to volume ratios. As a result, most soda pans dry out yearly [67,68], whereas only a subset of bomb craters dry up yearly and a large share only dry up in exceptionally dry years (C.F. Vad, pers. obs.). The result is that overall salinity levels in the soda pans are higher than in the bomb craters and especially show higher peaks (see also Fig. 2 and Table S1). Our results indicate that the *Daphnia* populations studied have regionally adapted to these habitat-specific differences in salinity dynamics.

We observe a pattern of local genetic adaptation in the bomb craters but not in the soda pans. While local genetic differentiation in the bomb craters occurs at a very small spatial scale of a few metres to at most a few hundreds of metres, we do not observe local genetic adaptation among soda pans, even though distances between soda pans range up to almost 20 km. There are multiple potential explanations for this counter-intuitive difference. First, because of the higher surface-to-volume ratio of the soda pans compared to the bomb crater ponds, there is a higher amount of within- and among-year differences in salinity levels within individual soda pans, and thus a higher ratio of within compared to among habitat variation in salinity levels than in the bomb craters. This reduces the degree of

differential selection while increasing the overall strength of selection for high salinity tolerances. Second, while geographic distances among soda pans are larger than among bomb crater ponds, it is not certain that this translates into more gene flow among bomb craters than among soda pans. Their large surface areas, the fact that they have a dry phase exposing dried dormant eggs to wind every year, and the fact that they attract large amounts of migrating waterbirds [8,37] and are exposed to strong directional winds [36] all promote dispersal of dormant stages of *Daphnia* among soda pans. In contrast, the bomb crater ponds are sheltered from wind due to their lower surface-to-volume ratio further enhanced by reedbelt in many of them and are too small to attract large numbers of waterbirds. Furthermore, they are also physically more isolated due to their elevated rim created during the explosion of the original bombs (Fig. S3, Discussion section, Supplementary Information). This is expected to reduce their connectivity through dispersal even under close geographic distances, which is also a prevalent pattern in the structure of their zooplankton metacommunities [69].

In addition to the observed EC_{50} population means that strongly indicate local adaptation in the bomb crater system, our data show substantial overall inter-clonal variation in mortality upon laboratory salt exposure within populations of both bomb craters and soda pans. In case of the bomb crater ponds, the additional analyses on salinity tolerance ranges, minima and maxima reveal a lack of genotypes with low salinity tolerance in the more saline bomb crater ponds, while genotypes with maximum salinity tolerance show no relationship with salinity. Consequently, there is a tendency for a reduction of the population-level salinity tolerance range in more saline compared to less saline bomb crater ponds (Fig. S1). This implies that selection against clones with low salinity tolerances in the high salinity ponds is stronger than selection against clones with high salinity tolerances in low salinity ponds. The presence of genotypes with high salinity tolerance in ponds with rather low salinities might reflect occasional success of genotypes with high salinities in extreme years. The broad range in salinity tolerances we observe in low salinity ponds has important consequences, as it is expected to facilitate rapid adaptation to higher salinity stress also in the low salinity ponds. These findings were further supported by our observation that estimated heritabilities (based on clonal repeatability analyses of mortality between 5-7 g L^{-1} of laboratory salt concentrations) tended to decline with increasing pond

salinity in the bomb crater pond but not in the soda pan populations (Fig. S2, Supplementary Information).

Our observation of a shift in the minimum tolerance to salinity with increasing salinity stress in local habitats inspired us to carry out a nestedness analysis. Nestedness analysis is a common approach in community ecology [70,71,71] to test whether responses to an environmental gradient is driven by a mere removal of species or replacement by other species. As we do not work with an obligately parthenogenetic species and most or all individuals in our study are genetically unique, we cannot apply the method using genotype identity. Rather, to test our hypothesis of thinning out low salinity tolerance genotypes in high saline habitats, we binned genotypes in functional identities defined by their salinity tolerance. The nestedness analysis we performed confirmed the pattern suggested by the significant relationship between minimum salinity tolerance and observed salinity of the habitat. Our analysis indeed shows a striking nested pattern, with a tendency of the presence of a more diverse set of salinity tolerances at lower salinities, with tolerance levels thinning to clones of higher tolerances (identified by higher IDs on the nestedness plot; Fig. 5b) in more saline bomb crater ponds. This result parallels the observation in community ecology that nestedness is a general pattern along salinity gradients [3,12,13]. Such types of analyses, originated in community ecology, here bring valuable insights into the mechanisms of local adaptation. The pattern observed for heritabilities and for the association between nestedness rank and observed salinities in the habitats are only indicative trends as they are marginally non-significant. But they are further supporting the significant association between minimum salinity tolerance of clones in populations and observed salinities in the field. For our heritability estimates, the observation that only a few estimates are significant (clonal repeatability analyses per population) is not a surprise, as we focused in our study on studying a large number of populations at the expense of having many clones per population (n=6) and a large number of replicates (n=3), both of which would have increased our capacity to estimate heritabilities with lower confidence intervals.

Salinity is a major abiotic stress factor for freshwater biota strongly impacting community composition and diversity [2,13,14]. Salinisation is an important component of global change and there are several drivers of increasing salinity in inland systems [9–11]. Our results show that the large-bodied cladoceran *D. magna* is capable of locally adapting to

differences in natural salinity level and may thus also potentially adapt to contemporary and future freshwater anthropogenic salinisation. Our results extend earlier work on evolution of salinity tolerance in *Daphnia* by quantifying adaptation to natural salinity gradients in two sets of contrasting habitats, showing how regional and local genetic adaptation integrate to shape patterns of salinity tolerance. We also show that local adaptation is achieved mainly by a weeding out of genotypes with low salinity tolerances rather than by a shift in both minimum and maximum clonal tolerances within populations. This nested pattern results in a broad range of salinity tolerances of clones in low salinity habitats, while only a high salinity tolerance subset of these functional genotypes is present in the high salinity populations. These results suggest that most populations harbour genotypic variation in salinity tolerance and thus show evolutionary potential to quickly adapt to changes in salinity, and that increased salinity in bomb crater ponds results in local adaptation, with low tolerant genotypes being weeded out, resulting in a tendency for a reduction in population-level heritability.

Given the important role large-bodied *Daphnia* play in top-down control of algae and other ecological processes [31,72] and given their dominance in the study systems [3,26,29], it is our prediction that regional and local genetic adaptation may have important implications for the ecology of the study systems. Quantifying these effects would require integrated field transplant experiments using animals grown under common garden conditions. Our results suggest that this impact of local adaptation on ecosystem processes will likely be especially strong in the bomb crater landscape, even though the bomb craters are geographically very close to each other.

Acknowledgements

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Tables

Table 1 – Anova model output of linear mixed-effect model on salinity tolerance (measured as EC_{50} upon exposure to $NaHCO_3$) in response to pond salinity (g/L), pond type (bomb crater pond vs soda pan) and their interaction, including a random effect for population (nested in pond type). Significant results ($p < 0.05$) are indicated in bold.

		EC_{50}	
fixed	F	df(dfres)	p
pond type (P)	48.600	1(17)	**< 0.001**
pond salinity (S)	1.049	1(17)	0.32
S x P	7.599	1(17)	**0.013**
random		χ2	p
population		0.0001	0.996

Table 2 – Anova model output of linear models on within-population clonal level minimum and maximum EC_{50} (upon exposure to $NaHCO_3$) in response to pond type (bomb crater vs soda pan) and pond salinity (g/L). Significant results ($p < 0.05$) are indicated in bold.

	minimum EC_{50}			**maximum EC_{50}**		
	F	df(dfres)	p	F	df(dfres)	p
pond type (P)	16.6989	1(17)	**0.007**	21.482	1(17)	**<0.001**
pond salinity (S)	6.168	1(17)	**0.0237**	0.097	1(17)	0.759
S x P	13.906	1(17)	**0.002**	0.004	1(17)	0.984

Figures

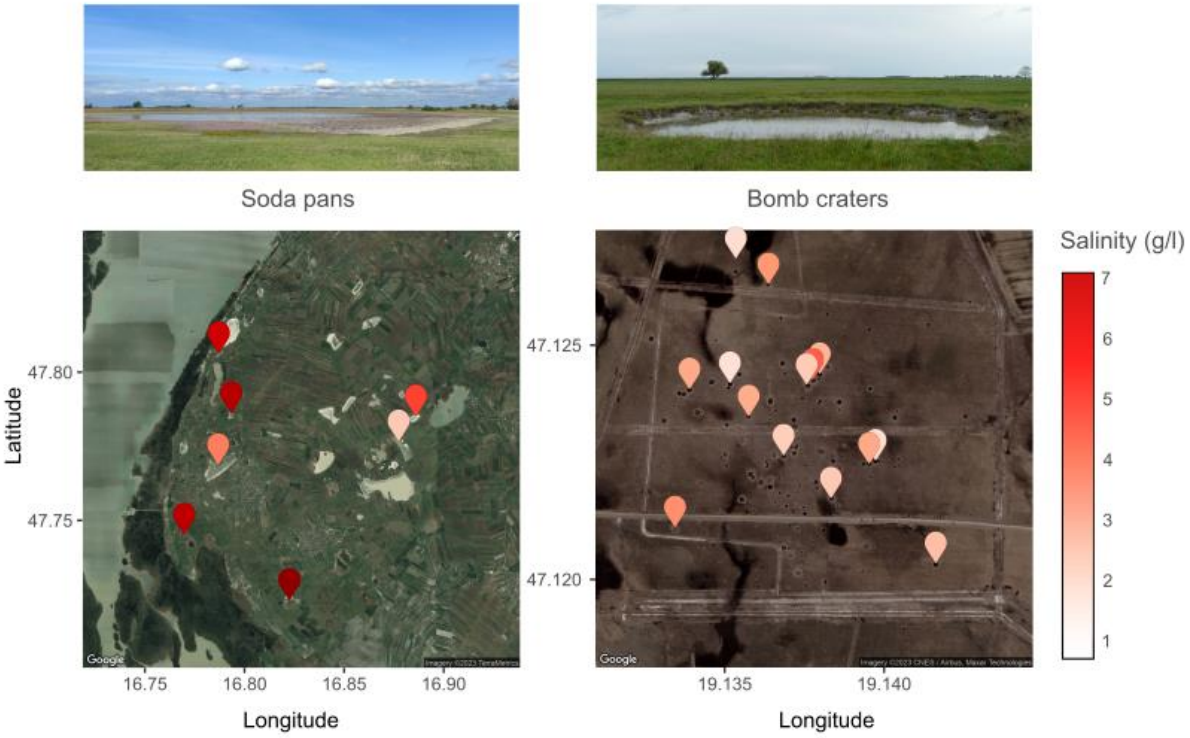

Fig. 1 – Sampling locations with colour coding visualising salinity values of the studied soda pans in Austria (left) and bomb crater ponds in Hungary (right). Detailed information on local habitat characteristics is given in Table S1, Supplementary Information).

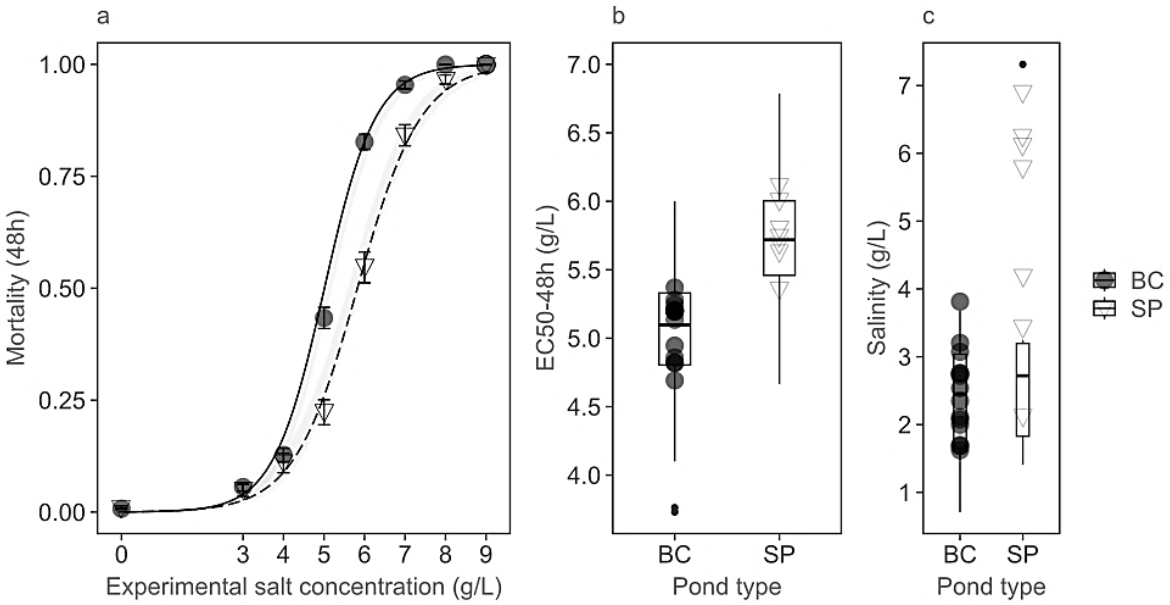

Fig. 2 – a) 48-h mortality (%, mean ± 1SE) of *Daphnia magna* individuals from populations originating from bomb crater ponds (N = 14, BC, filled circles, full line) and soda pans (N = 7, SP, open triangles, dashed line), exposed to the experimental salinity gradient (experimental salt concentrations: 0-9 g/L of $NaHCO_3$). Trend lines represent fitted generalized linear mixed-effect models (grey bands indicate 95% confidence intervals). b) Salinity tolerance (mean EC_{50} upon 48-h exposures to the experimental gradient of $NaHCO_3$ ± 1SE) of *D. magna* populations originating from bomb crater ponds (BC, N = 14) and soda pans (SP, N = 7). Black and white symbols indicate population mean EC_{50} values for both systems respectively. c) Bomb crater and soda pan pond salinity range (boxplots), and average salinity (overlayed black and white symbols, measured at multiple time points across spring and summer for both soda pans and bomb crater ponds illustrating variation across the season; sampling information detailed in Supplementary Information, Table S1).

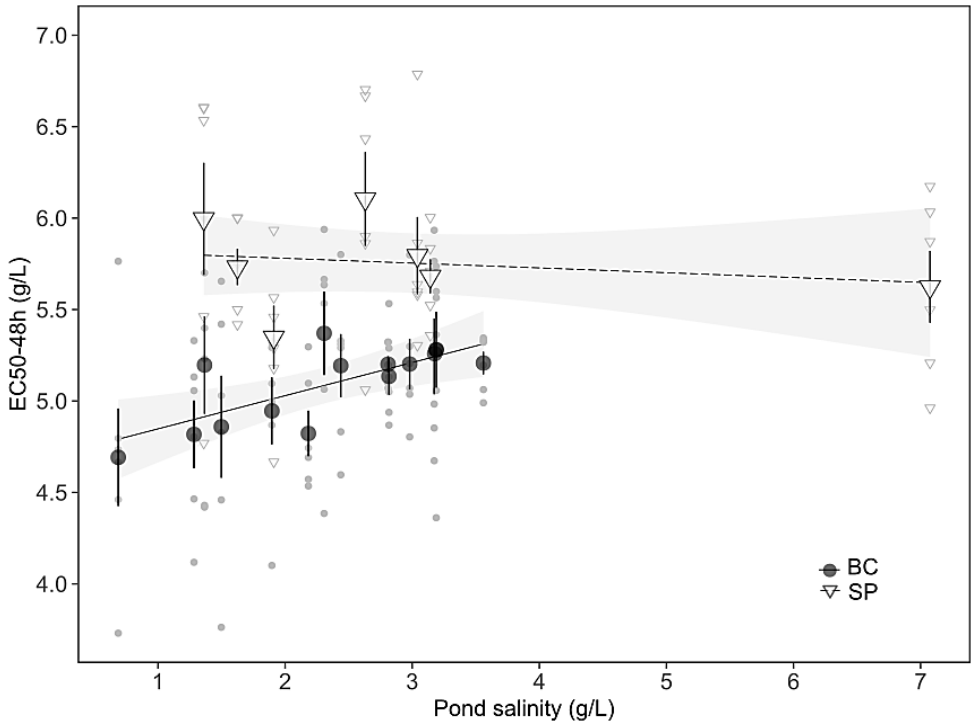

Fig. 3 – Salinity tolerance (mean EC_{50} upon 48-h exposures to $NaHCO_3$ ± 1SE) of *Daphnia magna* populations originating from bomb crater ponds (BC, N = 14, filled circles, full line) and soda pans (SP, N = 7, open triangles, dashed line) as a function of pond salinity as observed in the field (g/L). For each population, EC_{50} was estimated based on 6 clonal lineages triplicated across all eight experimental salt concentrations (see material and methods) (total N = 21 pops x 8 experimental salt concentrations x 6 clonal lineages x 3 replicates = 3024 experimental units). Clonal (grey small symbols) and population means (black and white large symbols) are depicted in triangles (bomb craters) and diamonds (soda pans). Grey bands indicate linear model confidence intervals.

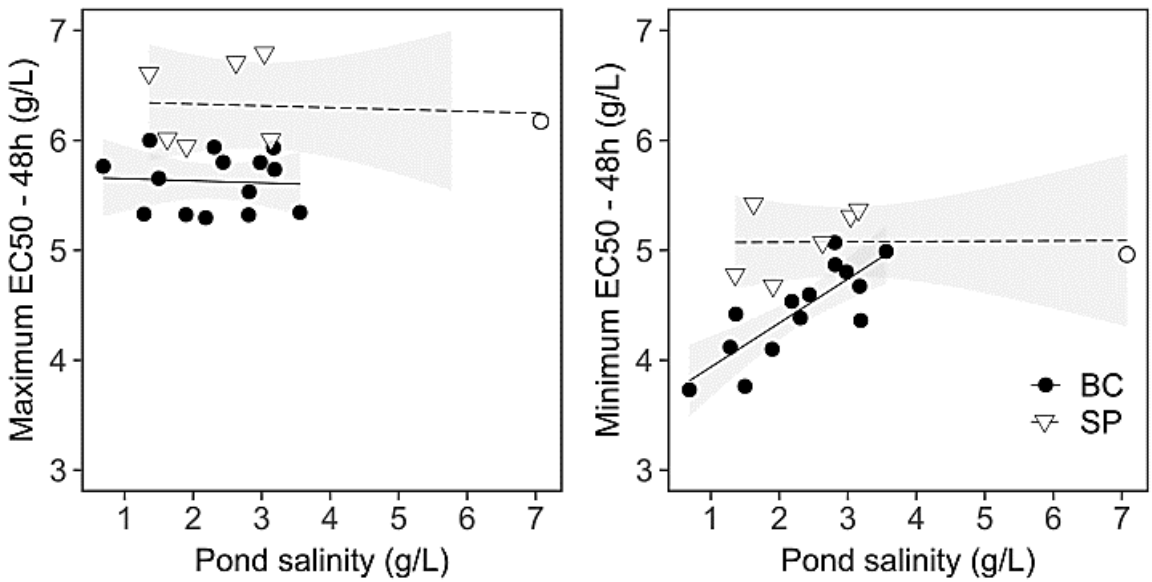

Fig. 4 – Observed maximum and minimum EC_{50} based on 48-h exposures to the experimental gradient of 8 salinities of $NaHCO_3$ with a) maximum tolerance within each population, as the EC_{50} value of the clone with the highest tolerance of the given population, and b) minimum tolerance within each population, as the EC_{50} value of the clone with the lowest tolerance of the given population, for all studied bomb crater ponds (BC, black circles, full line) and soda pans (SP, open triangles, dashed line), in response to their pond salinity. Grey bands indicate linear model confidence intervals.

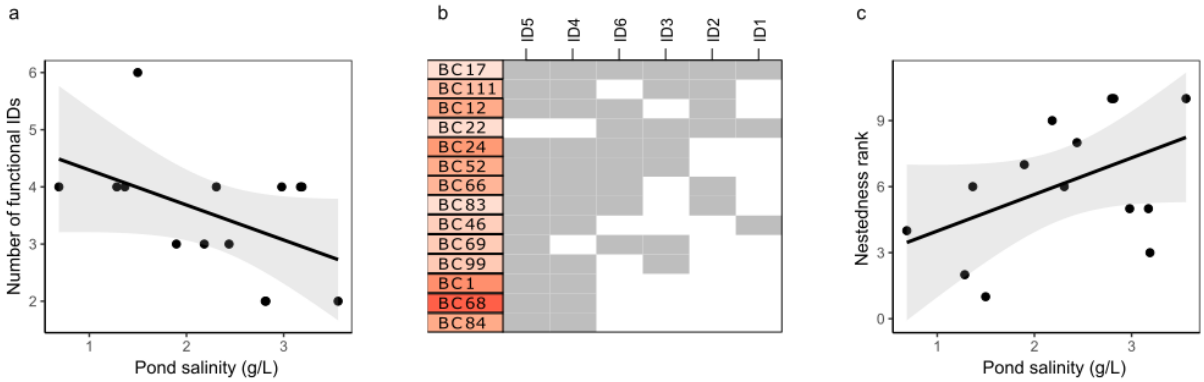

Fig. 5 – a) The observed number of functional identities (IDs; genotypes belonging to six classes of EC_{50} salinity tolerance values with equal ranges) along pond salinity in the 14 bomb crater ponds. Grey zones indicate linear model confidence intervals. b) Nestedness plot based on the six functional IDs (ID1 - ID6, ranging from high salinity sensitivity to high salinity tolerance) in the 14 bomb crater populations, with grey shading showing the presence of functional IDs in a given population; ponds are given colour codes visualising pond salinities (using the same colour gradient as in Fig. 1). c) Relationship between the nestedness rank of populations (lowest rank being the population with the most complete representation of functional IDs) and pond salinity in the bomb crater ponds. Grey bands indicate linear model confidence intervals.