

Osmoregulatory and demographic responses to salinity of the exotic cladoceran *Daphnia exilis*

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Daphnia exilis is a halophylic species that was recently found in Chile, distant from its natural range. In this study, we analyze the osmoregulatory and life-history responses to salinity exhibited by *Daphnia exilis*, as a first step towards understanding the ecology of this exotic species whose invasion potential has been related to its ability to tolerate saline environments. A set of laboratory experiments were carried out, in which we exposed organisms to a salinity gradient, and measured internal and environmental osmolality, assessed the effect of acclimation time and measured life-history traits that were used to perform a demographic retrospective analysis. Our results showed that (i) *D. exilis* exhibited a hyper-osmoconformer response, (ii) acclimation time did not exert effects on the osmoregulatory response, (iii) salinity up to 6 g L⁻¹ did not alter the population growth rate, (iv) at 8 g L⁻¹ population growth rate declined, mainly due to a delayed age at maturity and reduced fertility. Based on this information, we suggest that the responses to salinity exhibited by the exotic *D. exilis* confer an advantage over its natural enemies and may facilitate colonization through exploiting disturbed places as chemical refuges.

KEYWORDS: osmolality; LTRE; zooplankton; invasion; *Daphnia exilis*

INTRODUCTION

Salinization of lakes and ponds constitutes an acute form of environmental perturbation (Mack *et al.*, 2000), recognized as a serious environmental problem globally (Williams, 1987). In freshwaters, an increase in salt levels has been shown to affect zooplankton abundance (Shallemberg *et al.*, 2003; Amsinck *et al.*, 2005; Sarma *et al.*, 2006) and diversity (Frey, 1993; Jeppesen *et al.*, 1994; Shallemberg *et al.*, 2003). The mechanistic basis of these detrimental effects is primarily related to the physiological stress resulting from limited osmoregulatory

capabilities of the exposed organisms (Aladin, 1991; Frey, 1993), which can affect higher level processes such as feeding rate, body growth, reproduction and survival (Achuthankutty *et al.*, 2000). Consequently, knowing the physiological and life-history responses of residents and also of potential invaders to osmotic stress improves our ability to understand the underlying mechanisms of biodiversity shifts driven by current environmental disturbances.

Daphnia exilis has been described as a halophilic species (Brendonck and De Meester, 2003), with a range

of salt tolerance between 0.07 and 6.8 g L⁻¹ (Hebert and Finston, 1993). It is usually found in temporary and shallow ponds (Hairston *et al.*, 1999), and its natural distributional range covers the southwestern USA and northeastern Mexico. However, this species was later found more than 1000 km outside of its natural range, in Onondaga Lake north of New York (Hairston *et al.*, 1999). This site had been contaminated and salinized as a result of industrial activity. Due to this, Hairston *et al.* (Hairston *et al.*, 1999) suggested that the invasive ability of *D. exilis* could be facilitated by an increase in salinity.

In the past few years, *D. exilis* has also been found in Chile, inhabiting an artificial lake (Huechún) and a set of nearby ponds chemically disturbed to different degrees by local mining activities. We sampled this population of *D. exilis* over three consecutive years (2007–2009). No previous records on the occurrence of this species exist, considering both studies on fresh and brackish waters systems over the entire country (see reviews in Ruiz and Bahamode, 1989; Oyanedel *et al.*, 2008; Márquez-García *et al.*, 2009).

In this contribution, we studied the osmoregulatory and life-history responses to salinity exhibited by the exotic cladoceran *Daphnia exilis* from Chile. The organisms' tolerance to perturbed (i.e. salinized) environments confers them opportunities for exploiting predator-free habitats, thus increasing population growth and invasion potential in those areas. Consequently, this study represents a first step towards understanding the ecology of an exotic species of a southern hemisphere freshwater habitat whose invasion potential has been related to its capabilities of tolerating saline environments. In addition, we present new empirical information towards understanding the functional significance of conformer/regulator strategies of aquatic organisms facing stressful environments. The objectives of this work were: (i) to determine the osmoregulatory response exhibited by *D. exilis* over a salinity gradient, (ii) to assess the effects of acclimation time on its osmoregulatory response and (iii) to assess the effects of salinity on life-history traits of this exotic cladoceran.

METHOD

Experimental organisms

The experimental organisms used in this study were isolated from a clone of *D. exilis*, recently collected from Huechún reservoir (33°4'0"S; 70°47'60"W) located 45 km north of Santiago, Chile.

Osmoregulatory response of *D. exilis* to salinity

Acclimation

The internal ion concentration of *Daphnia*, as a function of environmental salinity, was tested under two different acclimation levels: 10 generations (i.e. long-term acclimation allowing for maternal effects) and 6 h (short-term acclimation). The acclimation levels were crossed with five salinity levels: 0, 2, 4, 6 and 8 g of salt per liter of reconstituted hard water (American Society for Testing and Materials, 1980). The salt solution was prepared with commercial sea salt (SERA Premium, Heinseberg, Germany).

For the long-term acclimation level, we used 120 females per salinity level born from the second or third brood from sisters from a single-line culture acclimated during 10 generations to each of the salt levels. The experimental organisms were grown in reconstituted water (American Society for Testing and Materials, 1980), at a density of 80 ind. L⁻¹ during their first 5 days of life, and 40 ind. L⁻¹ thereafter. For the short-term acclimation level, we used 120 females grown in ASTM water without added salt. These *Daphnia* were then transferred to the five salinity levels 6 h prior to hemolymph extraction. During the execution of all procedures, temperature was kept at 20 ± 1°C, with a photoperiod of 14:10 L:D, and pH was adjusted to 7.9 ± 0.1. The organisms were fed daily with the green alga *Chlorella vulgaris*, at 10⁶ cells mL⁻¹, and enriched with 2.5 mL L⁻¹ of nutritional supplement (Hayashi *et al.*, 2008), composed of algal extract (Phyllum by ANASAC, Lampa, Chile). The medium was renewed every 48 h.

Hemolymph extraction and osmolality measurements

We extracted hemolymph from a set of adult females, which had just released their first clutch. For each salinity level and acclimation treatment, a measurement of internal osmolality was made on a sample of ≈10 μL obtained from 120 organisms. Prior to hemolymph extraction, each organism was quickly dried externally on a piece of filter paper. Hemolymph from each animal was removed by piercing the carapace near the heart using a microcapillary, connected to a micro-screwed syringe. Each sample was immediately frozen until measurement. Osmolality of both hemolymph and external media was measured using a 5520 Westcor vapor pressure osmometer.

Data analysis

The osmoregulatory response of *Daphnia* to salinity (i.e. conformer, regulator and mixed) was assessed through

the statistical relationship between internal and environmental osmolality. For this purpose, we fitted four alternative models: (a) linear: $y = k_1x + k_2$, (b) hyperbolic: $y = k_1 + (k_2 - k_1)/(1 + (x/k_3))$, (c) three-parameter sigmoid: $y = k_1/(1 + \exp((k_2 - x)/k_3))$ and (d) four-parameter sigmoid: $y = k_1 + (k_2/1 + (x/k_3)^{k_4})$, where x is the osmolality of the medium, y is the internal osmolality and k are fitting parameters. These models were chosen due to their simplicity, and because they include a broad range of expected osmolality responses. Strict conformers are expected to show a linear response with positive slope. Strict regulators are expected to show their internal osmolality independent of environmental osmolality. A mixed response should show a curve including increasing and horizontal zones, a shape captured by either the hyperbolic or the sigmoid functions. Hyperosmotic and hyposmotic responses can be determined by inspecting the curvature of the response. Model selection was carried out by means of the corrected Akaike's information criterion (AICc). The individual AICc values were rescaled to $\Delta\text{AICc} = \text{AICc} - \text{AICc}_{\min}$, where AICc_{\min} is the minimum among AICc values calculated for the different models. This transformation forces the best model to have $\Delta\text{AICc} = 0$, with the rest having positive values. Models with $\Delta\text{AICc} \leq 2$ are considered to have substantial support (Burnham and Anderson, 2004).

Demographic response of *D. exilis* to salinity

For this experiment, organisms were individually maintained in beakers with 40 mL of filtered lake water (GF75 ADVANTEC, Tokyo, Japan). Temperature, pH, photoperiod and food conditions were the same as described above. The experimental organisms were obtained from the fourth brood of three parthenogenetic sisters. To avoid pseudoreplication (Hurlbert, 1984), these newborns were randomly allocated to treatments with different nominal salt concentrations: 0, 2, 4, 6, 8 and 10 g L⁻¹, with six replicates each. These newborns were individually grown in beakers with 40 mL of medium, which was renewed daily, until rearing their third brood. Each 24 h, survival and reproduction were recorder for each animal. The experiment was finished after each individual released its third brood (Porter et al., 1983; Vanni, 1986).

From our daily records of age-specific survival and fertility, we conducted a life table response experiment (LTRE) analysis (Caswell, 2001) in order to identify which demographic rates are responsible for the observed effect of salinity on the population growth rate. First, we constructed a parameterized Leslie

Matrix for each treatment, using the formulae proposed by Caswell (Caswell, 2001) for birth-flow populations.

Following Levin et al. (Levin et al., 1996), the full Leslie Matrix was reduced to a two-stage model with juveniles and adults as state variables (Fig. 1). This parameterization allows assessing the effects of time to maturity on the observed changes in population growth rate. Time to maturity often exerts major influences on population growth rates of *Daphnia* (Vanni and Lampert, 1992). In addition, this two-stage parameterization aggregates the many age-specific survival and fertility values into a small set of parameters, allowing a more straightforward interpretation of effects. Here, juveniles survive with probability P_J to reach maturity, spending α time units (days) in the process, thus $P_J = \sigma_1^\alpha$, where σ_1 is the characteristic juvenile survival from one time unit to the next and is directly taken from the corresponding sub-diagonal elements of the Leslie Matrix. Adults survive with probability σ_2 during one projection interval (i.e. 1 day).

Parameter α is the average number of days from birth to the day on which first reproduction occurred, it was calculated from the age class at maturity m as $\alpha = m - 1$. We obtained the age class at maturity m from the individual life tables, as the rounded arithmetic mean of the first age classes with non-zero maternity (Ramos-Jiliberto and Aránguiz-Acuña, 2007). Finally, adult survivorship σ_2 and fertility F were calculated according to Levin et al. (Levin et al., 1996) as $\sigma_2 = \sum_{i=m}^{\infty} w_i S_i / \sum_{i=m}^{\infty} w_i$ and $F = \sum_{i=m}^{\infty} w_i F_i / \sum_{i=m}^{\infty} w_i$, where S_i and F_i are survival and fertility of age class i from the Leslie Matrix, and w_i are elements of the stable stage distribution vector, obtained as the right eigenvector associated to the dominant eigenvalue.

The effect of each salt concentration on population growth rate was measured relative to the control animals (0 g L⁻¹) from which we obtained the reference projection matrix $A^{(r)}$. The total effect of each salt concentration on λ is decomposed into contributions

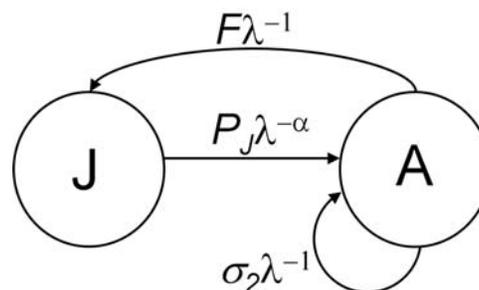


Fig. 1. Two-stage life cycle graph: (J) juveniles and (A) adults, with transition rates defined by time to maturity α , probability P_J of surviving to maturity, adult survival probability σ_2 and fertility F

from the four defined vital rates to the observed difference between the λ value of the treatment $\lambda^{(k)}$ and the reference $\lambda^{(r)}$. Each contribution is composed by the observed change in the vital rate parameter, and the sensitivity of λ to changes in the parameter,

$$\lambda^{(k)} \approx \lambda^{(r)} + \sum_i \left(a_i^{(k)} - a_i^{(r)} \right) \frac{\partial \lambda}{\partial a_i} \Big|_{\mathbf{A}/2}$$

where a_i are the vital rate parameters (i : 1–4, for σ_1 , σ_2 , α and F) at treatment k and reference r , and sensitivities are evaluated as the mean of both parameter sets ($\mathbf{A}/2$). We used a bootstrap resampling procedure to calculate 95% confidence intervals for λ and the vital rate parameters, with a resampling size of 3000.

RESULTS

Osmoregulatory response

The results of the model selection procedure by AICc clearly favored the linear model, independent of the acclimation regime of the organisms (Table I).

The values of the fitted parameters of the linear model, and their corresponding 95% confidence intervals are shown in the legend of Fig. 2. There were no differences in the constant or slope parameters between short- and long-acclimation levels. The slope did not depart from unity, and the intercept is significantly higher than zero.

These results indicate that the exotic cladoceran *D. exilis* exhibited an osmoregulatory response that corresponds to hyper-osmoconformer organisms (Fig. 2). On the other hand, acclimation time did not exert any significant effect on the response.

Demographic response

The values of the population growth rate (λ) did not show significant differences among treatments, with the exception of the treatment of 8 g L⁻¹, where λ was the lowest, although indeed larger than one (Fig. 3). It is important to note that in this experiment *Daphnia* did not release males.

Our LTRE results revealed that in the 8 g L⁻¹ treatment, fertility F and time to maturity α contributed negatively to population growth, relative to the control. In the treatment of 2 g L⁻¹, the contributions of adult survival and fertility were positive and negative, respectively, and thus they cancelled out. In treatments 4 and 6 g L⁻¹, the contributions of the parameters were not different from zero (Fig. 4).

DISCUSSION

In this study, we analyzed some osmoregulatory and life-history responses of the exotic cladoceran *Daphnia exilis*, faced with a salinity gradient. Our results show that (i) this clone exhibits a hyper-osmoconformer physiological response, (ii) acclimation time did not exert discernible effects on the osmoregulatory response, (iii) salinity up to 6 g L⁻¹ did not alter the population growth rate, (iv) at 8 g L⁻¹ population growth rate declined, mainly due to a delayed age at maturity and reduced fertility.

The osmoconformer response found in *D. exilis* has evolved in most marine crustaceans and in brackish-water species as a mean to minimize ion and water diffusive movements along with the associated energetic cost (Pêqueux, 1995). Although osmoregulation allows exploiting a variety of habitats, the costs of this strategy are relatively high (Lowe *et al.*, 2005). Thus osmoconformers should have, in general, lower energetic demands than their osmosregulator counterparts. The same kind

Table I: Results of the model selection procedure for the osmoregulatory response of *D. exilis* in a salinity gradient

Acclimation	Model	n	R^2	F	P	AICc	$\Delta AICc$
6 h	Linear	2	0.90	72.20	< 0.001	27.57	0.00
	Hyperbolic	3	0.97	223.75	< 0.001	32.71	5.14
	Sigmoid	3	0.99	633.99	< 0.001	31.69	4.12
	Sigmoid	4	1.00	2848.40	< 0.001	41.20	13.63
10 generations	Linear	2	0.93	113.28	< 0.001	26.80	0.00
	Hyperbolic	3	0.93	113.28	< 0.001	33.00	6.20
	Sigmoid	3	0.94	124.61	< 0.001	32.91	6.11
	Sigmoid	4	0.96	181.65	< 0.001	43.55	16.75

Animals were acclimated for 6 h and 10 generations. Four models with number (n) of parameters were evaluated. The best models, according to the corrected Akaike's Information Criterion (AICc), are shown in bold.

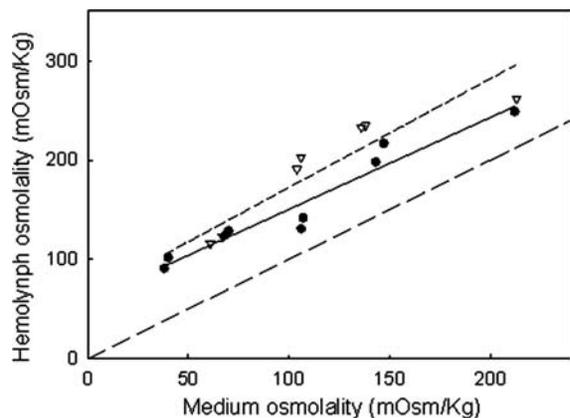


Fig. 2. Osmolality of *Daphnia* acclimated during 10 generations (black circles, best fit shown by continuous line) and 6 h (white triangles, best fit shown by dashed line) as a function of environmental osmolality. Fitted parameters and their 95% confidence intervals for long-time acclimation were 57.92 (32.30–83.54) and 0.93 (0.71–1.15) for intercept and slope, respectively. For short-time acclimation, the values were 63.04 (26.03–100.06) and 1.09 (0.77–1.42).

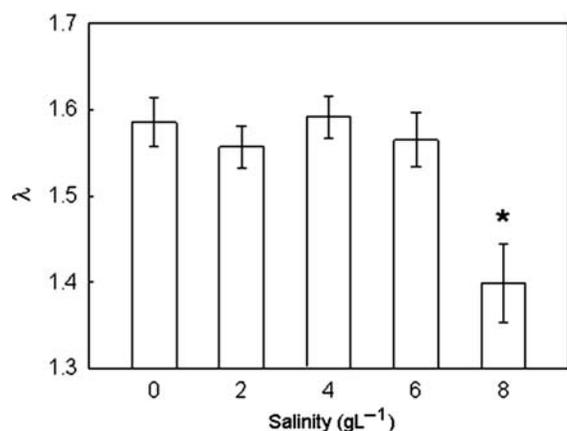


Fig. 3. Population growth rate λ of *Daphnia* under different salt concentrations. Error bars represent 95% confidence intervals. A significant decrease in λ is shown by an asterisk.

of osmoconformer response has been observed by Fritsche (Fritsche, 1916) in *D. magna* at salinities above 5 g L^{-1} , and in *D. pulex* living in brackish ponds (Weider and Hebert, 1987). In addition, we found that *D. exilis* exhibited a hyperosmotic response, i.e. the crustacean maintains a positive and constant osmolality difference with the environment. In this case, *Daphnia* showed a difference of $\sim 60 \text{ mOsm kg}^{-1}$, which is slightly higher than typical values for hyperosmotic crustaceans (ca. $10\text{--}40 \text{ mOsm kg}^{-1}$, Charmantier *et al.*, 2008). Hyperosmotic responses have been reported previously for *D. pulex* (Weider and Hebert, 1987), and have been postulated as a mechanism that facilitates ecdysis (Charmantier *et al.*, 2001).

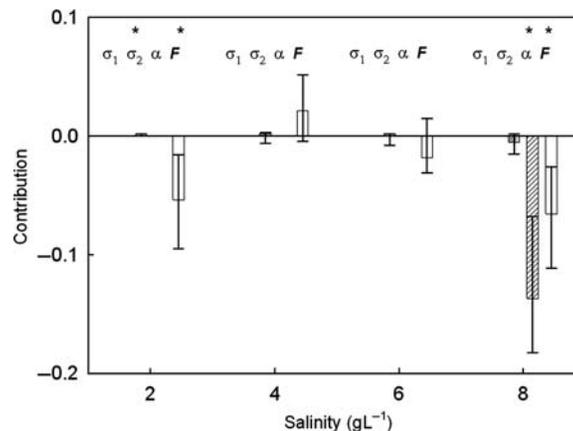


Fig. 4. Contribution of population parameters: juvenile survivorship (σ_1), adult survivorship (σ_2), time to maturity (α) and fertility (F) to changes in λ , for different salt concentrations relative to the control. Error bars correspond to 95% confidence intervals. Asterisks show significant contributions.

In this study, both short- and long-acclimated organisms displayed the same osmoregulatory response to the salinity gradient. This reflects rapid changes in hemolymph osmotic concentration during salinity acclimation. Accordingly, Burton and Feldman (Burton and Feldman, 1982) found that the copepod *Tigriopus californicus* showed detectable accumulation of free amino acids within 3 h. We suspect that this rapid osmotic adjustment, together with the relatively wide tolerance range exhibited by *D. exilis*, may facilitate colonization of contrasting environments (Teschner, 1995) whenever other ecological constraints are less important.

At the demographic level, our results reveal that the detrimental effects of salinity at 8 g L^{-1} on both fertility and development of *Daphnia* translated into a decreased, although still positive, rate of population growth. Although we cannot discard a negative effect of salinity on the resource, we assume that this effect is not large since *Daphnia* were fed daily. In addition, similar life-history shifts caused by increased salinity have been found in other cladocerans (Arnér and Koivisto, 1993; Martínez-Jerónimo and Martínez-Jerónimo, 2007; Santangelo *et al.*, 2008). These demographic effects derive from energetic demands, at higher environmental salinities, for maintaining an osmolality gradient between body organs and the external medium.

It is relevant to emphasize that this clone of *D. exilis*, a species recently found in Chile, survived and reproduced at salinities as high as 8 g L^{-1} , which extends the tolerance limit reported for this species in other latitudes (Hebert and Finston, 1993). Freshwater microinvertebrates exhibit, in general, low tolerances to salinity, lethal effects being observed at concentrations below

2 g L⁻¹ (Jeppesen *et al.*, 1994; James *et al.*, 2003). Small differences in salinity tolerance could lead to important differences in population growth of competing zooplankters (Lowe *et al.*, 2007). Therefore, our results support that the tolerance to salinity exhibited by the exotic *D. exilis* confers an advantage over potential competitors.

In addition to resource competition, whose outcome is largely determined by relative food thresholds for zero growth (Gliwicz, 1990), a major biotic factor often limiting the establishment of a species is predation (Reusch, 1998; Miller *et al.*, 2002). Particularly, planktivorous fish exert a strong control on zooplankters with large body size (Brooks and Dodson, 1965; Zaret, 1980; Lampert, 1987). Consequently, *D. exilis* should be especially vulnerable to visual predators given its relatively large size (1.8–4.5 mm length). The high-salt tolerance found in *D. exilis* also confers an advantage in relation to predation losses, since most freshwater fish are stenohaline and do not tolerate high-salt concentrations (Evans, 1993), especially during their early life stages (James *et al.*, 2003) where they could constitute the most acute source of mortality (Romare *et al.*, 1999). In habitats with reduced stress conditions, biotic interactions can be more important than physico-chemical forces in structuring communities (Herbst, 2001). In contrast, in habitats with physiologically stressful conditions such as increased salinity, the organisms that perform better under these conditions find a refuge against intense predation and competition (Wright and Shapiro, 1990; Herbst, 2001; Santangelo *et al.*, 2008). In this way, *D. exilis* appears to be able to exploit available chemical refuges (saline water bodies) as establishment sites and sources of dispersion.

Regarding the scarce ecological knowledge of this species, together with the current global trend of freshwater salinization (Rahel and Olden, 2008), this work offers new and relevant information about the ecology of an exotic species and represents a first step towards the assessment of its potential of invasion of southern hemisphere freshwaters. Future research should focus on understanding the interacting effects of salinity and other environmental stressors (Charmantier and Charmantier-Daures, 2001; Newman, 2001) on the physiology of organisms and their consequences at higher levels within the ecological hierarchy.

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