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Effect of environmental stress on clonal structure of *Eucypris virens* (Crustacea, Ostracoda)

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Abstract Environmental stress imposes strong natural selection on clonal populations, promoting evolutionary change in clonal structure. Environmental stress may also lead to reduction in population size, which together with clonal selection may reduce genotypic diversity of the local populations. We examined how clonal structure in wild-collected samples of two parthenogenetic populations of the freshwater ostracod *Eucypris virens* responded to hypersalinity and starvation, and the combination of the two stressors. We applied the stress treatments in a factorial design for one generation. When 60% of the individuals per experimental unit had died, post-experimental clonal structure was compared to that of the start of the experiment, which reflected the field conditions. We used five polymorphic allozyme loci as genotype markers. All stress treatments reduced survival

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compared to the control treatment. In the population "Rivalazzetto", we observed a reduction of clonal richness in the control treatment, with the initially dominant clone maintaining dominance. This may have resulted from interclonal competition and clone-specific survival under the different laboratory conditions. Clonal richness remained high in the salinity treatment while it was reduced in the combined stress and starvation treatments. In the population "Fornovo", clonal richness reduced in all treatments including control, while the salinity and combined stress treatment reduced clonal evenness. The clone dominating at the start of the experiment increased in frequency in all treatments, but the change in clonal structure during the experiment was more pronounced in this population. These results suggest that in some conditions an intermediate level of environmental stress may lessen the decline in genetic diversity by strong inter-clonal competition. Moreover, the variation in clonal structure among the stress treatments and distinct genetic backgrounds indicates that more general predictions of stress effects on clonal structure may be difficult.

Keywords Genotypic diversity · Adaptation · Environmental change · Parthenogenesis · Clonal richness · Clonal evenness

Introduction

Genotypically diverse multiclonal populations of obligatory and cyclical parthenogenetic species are good models for examining the processes maintaining genotypic diversity and coexistence of clones. Heterogeneous environment (Vrijenhoek 1978, 1979), pressure by coevolving parasites (Jokela et al. 2003), temporal and spatial stochasticity (Lynch 1987; Ellner and Hairston 1994; Hedrick 1995) and ecological divergence (Ayre 1995) have been suggested to be important for the persistence of multiple genotypes. As genotypically diverse populations of parthenogens are common in ecologically and phylogenetically disparate groups, it may be that several common processes are involved in the maintenance of clonal diversity.

Stress often leads to directional selection (Hoffmann and Parsons 1997) and it may reduce genetic diversity in a population as adaptive gene variants sweep to fixation. In parthenogenetic organisms selection by stress is expected to sort clones by tolerance, promoting extinction of the lineages less fit to local conditions (Hoffmann and Daborn 2007). Environmental stress may however interact with competitive ability and modify intra- and interspecific interactions between organisms. A direct effect of stress may be the switch in allocation of energy from reproduction and competitive interactions to survival (Parsons 1996a). Spatial and temporal variation in the level and sources of stress are part of the environmental heterogeneity that may facilitate long-term maintenance of genotypic diversity (Hoffmann and Parsons 1997; Weeks and Hoffmann 1998; Nevo 2001). Therefore, understanding the processes promoting genetic dynamics in stress-exposed populations may help to understand and predict population responses to environmental change.

In this study we examined how environmental stress affects clonal genotypic diversity in two asexual populations of the freshwater ostracod *Eucypris virens*. The species is mainly found in ephemeral ponds, a habitat subject to severe environmental variation, requiring specific adaptations to withstand extreme changes in hydrology, water chemistry and ecological interactions. Earlier studies reported a high genetic diversity in this species even within local ponds (Rossi et al. 1998; Schön et al. 2000). Recent analyses show

evidence of multiple origins of asexuality from genetically distinct and very distant sexual lineages. These sexual lineages are phylogenetically distant enough to be considered as a cryptic species complex. Furthermore, most ponds have been colonized more than once by more or less related asexual lineages (Adolfsson et al. in press; Bode et al. in press). These genotypically diverse populations (1–68 multilocus genotype per pond, Adolfsson et al. in press; 2–13 MLG per pond, Martins et al. in prep.) are well suited for studies of environmentally induced changes in genotypic frequencies. Local environmental conditions may determine the fitness of clonal lineages in which case the frequency of dominant clones may be expected to change when the environment changes (e.g. Carvalho 1987; Weeks and Hoffmann 1998; Etterson 2004). Shifts in clonal composition depend on the initial diversity and ecological requirements of local clones (see Parsons 1996b). Abiotic environmental stress and competition may affect the dynamics of population genetic structure. Yet, little information is available on the importance of interaction between biotic and abiotic factors.

We investigated the change in population genetic structure in two populations of *E. virens* during a factorial application of two stress treatments in the laboratory. We hypothesised three possible effects on the clonal structure: (1) in the control conditions (no stress treatment) we expected an exclusion of the less fit clones; (2) initial clonal diversity is maintained by a reduction of interclonal competition for local resources such as food and space; (3) stress may affect clonal fitness leading to rank shifts in fitness of the clonal lineages.

Materials and methods

Model organism

Eucypris virens (Jurine 1820) is a freshwater ostracod species complex. It occurs mainly in winter and spring in ephemeral ponds (Meisch 2000). *Eucypris virens* has life history adaptations to survive periods of drought or freezing. The life-cycle includes subitaneous (immediately hatching) and diapausing eggs (desiccation-resistant). *Eucypris virens* can survive short drought periods embedded in the mud (O. Schmit, unpublished data), a feature common in freshwater ostracods (e.g. Barclay 1966; Delorme and Donald 1969). Delay in juvenile development rate in harsh conditions may also occur in freshwater ostracods (e.g. Mezquita et al. 1999). Both diploid sexual and diploid and triploid apomictic parthenogenetic lineages occur in the species complex (Adolfsson et al. in press). Parthenogenetic lineages can be found throughout Europe, North Africa, North America and Palearctic Asia (Meisch 2000; Semenova 2005). Populations with males are known only from the circum-Mediterranean area and central Asia (Meisch 2000), where males and sexual females may co-exist with clonal lineages (Schön et al. 2000). Parthenogenetic populations of *E. virens* often consist of many clones (Rossi et al. 1998; Schön et al. 2000). As typical to *E. virens* populations, the material in our experiments consisted of coexisting triploid and diploid parthenogens (Appendix A and B) belonging to different (phylogenetically distant) mitochondrial clades (data not shown). Examining ploidy level, we verified that neither diploid nor triploid clones were superior in survival under any stress treatment. Similarly, clonal clade of origin did not have a direct effect on clonal survival. Also the overall maintenance of clonal diversity under stress was independent of ploidy level or clade.

Collection of animals and experimental design

We collected adults and subadults (last two juvenile stages) from two sites in the region of Emilia-Romagna, Italy (Table 1). The sites are characterized as hydrologically isolated temporary ponds, the preferred habitat of *E. virens,* and are located in areas with distinct human impact ("Fornovo"—road side; "Rivalazzetto"—agricultural area). The animals were collected with a pipette or a hand net (250 μm) and transported in filtered water of the original pond to the University of Parma, Italy within 2 h after collection. We randomly divided the collected animals per population into 14 batches, each of 100 individuals. Two batches were immediately deep frozen (−80°C) to assess clonal structure of the source populations. The remaining batches were randomly assigned to one of the four experimental treatments: control (Contr), starvation (Starv), hypersalinity (Sal; salinity 2 g l^{-1}), and the combination of starvation and salinity (Sal + Starv). *Eucypris virens* inhabits temporary ponds which typically show marked fluctuations in salinity as ions get diluted (rainfall) or concentrated (evaporation). In the framework of the SexAsex project, snapshot records of salinity levels were made in 126 ponds inhabited by *E. virens* and distributed all over Europe. Only in about 5% of the ponds ($n = 7$) did the salinity exceed 2 mg/l, suggesting that higher levels pose a stress on the survival of *E. virens*. The species typically feeds on periphyton and dead organic material (Schmit et al. 2007). The abundance of these food items varies seasonally (periphyton usually gains abundance throughout the season, pers. observ.) or stochastically (e.g. leaf litter, cut grass, fallen fruits, etc.), and is likely to be periodically limiting survival of *E. virens* in the wild. Filtered pond water was used as culture medium; spinach was provided ad libitum (except in the Starv treatment). Salinity level was attained by the addition of marine salt (brand: Sosalt). Each treatment was replicated in three aquaria (volume: 1 l), kept in acclimatized conditions (16°C, 8 h light photoperiod). Water and food were renewed every 3 days. Eggs deposited throughout the experiment were removed with a pipette, to avoid juveniles hatching in the cultures. Survival was checked daily. Mortality was confirmed by examining movement of the individuals; passive individuals were examined under a binocular microscope (40× magnification) for absence of valve and appendage movement. Monitoring was continued until the number of survivors did not exceed 40 individuals for a given aquarium. That day, survivors were frozen at −80°C.

Table 1 List of sampled *E. virens* populations with information on the locality, date of sampling, dominant land use, hydroperiod, main origin of water and pond morphometry at the time of collection

Pond name	Fornovo	Rivalazzetto
Code	FOR	RVL
Coordinates	N44°40′42″ E10°05′34″	N44°49′07″ E10°08′20″
Sampling date	2008/02/21	2008/02/22
Land use	Urban (road side)	Agriculture area
Hydroperiod	November–April	October–May
Water origin	Rain	Rain
Dimension (m)	20 × 15	25 × 0.5
Maximum depth (m)	0.30	0.30

Genotyping

We used cellulose acetate gel electrophoresis to genotype the individuals. Five polymorphic loci were used as genetic markers: AAT (aspartate amino transferase) (E.C. 2.6.1.1), AO (aldehyde oxidase) (E.C. 1.2.3.1), GPI (glucose-6-phosphate isomerase) (E.C. 5.3.1.9), MPI (mannose-6-phosphate isomerase) (E.C. 5.3.1.8), and PGM (phosphoglucomutase) (E.C. 2.7.5.1). Each individual was screened for all polymorphic loci and was classified according to its multilocus genotype (MLG). For more details of the protocol see Adolfsson et al. (in press).

Data analyses

Survival curves were calculated by averaging the fraction of survivors over the three replicates as a function of time. These averaged curves were compared between the treatments and the control using Gehan's generalized Wilcoxon test. The variation across replicates was accounted for in a supplementary ANOVA test with pairwise post-hoc comparisons using Tukey HSD test. These tests are based on a parameter that summarizes the survival curves (i.e. T_{50}: the number of days to reach 50% mortality). The assumptions of normality and homogeneity of variances were tested with the Kolmogorov–Smirnov test and the Bartlett's test, respectively. All tests were calculated with the software STATISTICA vs. 6.0 (StatSoft Inc 2007).

Changes in clonal structure were analysed by calculating bootstrapped evenness and richness indices for treatment groups using MATLAB (vs. 7.0.4). Before bootstrapping replicates of a given treatment group were pooled to increase the sample size. Evenness of clonal structure was described using the E5 evenness index ($E5 = \left(1/\Sigma\left(p_i^2\right) - 1\right)/\left(\left(\exp\left(-p_i * \mathrm{Ln}(p_i)'\right)\right) - 1\right)$, p_i = frequency of ith genotypes; Ludwig and Reynolds 1988) and clonal richness using Stoddart's G_0 index ($G_0 = 1/\Sigma(p_i^2)$, p_i = frequency of ith genotypes; Stoddard and Taylor 1988). We assessed statistical significance of changes in the genetic structure of the treatment groups by calculating the difference between the index value in the start and at the end of the experiment for each treatment group. We then compared this value to a distribution of differences in index values that were calculated between 1,000 random samples of similar size that were drawn with replacement from a pooled sample containing all genotyped individuals. This randomized distribution of differences in the index values sets the expectation for null hypothesis "H_0: genetic structure in the treatment groups does not differ from the start" and avoids bias due to differences in sample sizes. If the true difference between the index values was outside of the 2.5 or 97.5% percentiles of the random distribution, it was declared as statistically significant at $P < 0.05$. We conducted a similar test to compare the control treatment to stress treatments at the end of the experiment.

Results

In both populations the stress treatments significantly reduced the survival (Fig. 1; Table 2; ANOVA on T_{50} test comparing survival curves in stress treatments and control: $P < 0.005$). In the absence of an imposed stress, the animals collected in population RVL survived on average 2 weeks longer than those from FOR (Control: $T_{50,\mathrm{FOR}}$: 22.7 ± 2.4 days; $T_{50,\mathrm{RVL}}$: 36.7 ± 2.7 days). In population FOR, the time until 50% of the

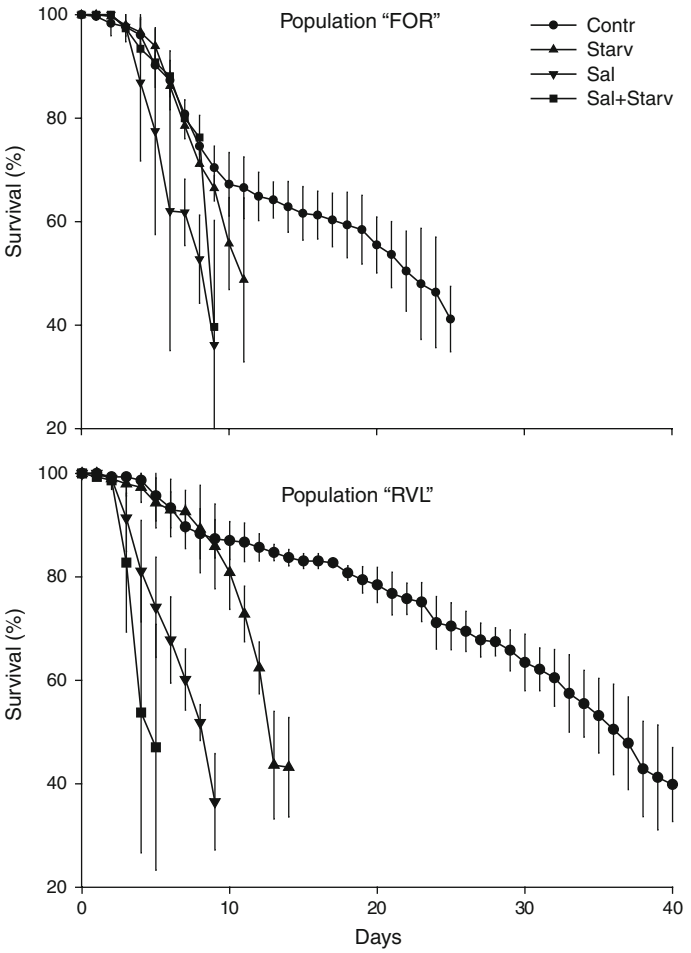

Fig. 1 Survival curves for *E. virens* individuals of population FOR and RVL in different treatments (*Contr* control, *Sal* high salinity, *Starv* starvation). The *lines* show averaged values over replicates; the *error bars* indicate the variation among replicates (2× SE)

Table 2 *F*-values and significance levels (*P*-values) of Gehan's Wilcoxon test verifying differences between mean survival time curves of the treatments versus the control condition per population

	RVL		FOR	
	F-value	*P*-value	*F*-value	*P*-value
Starv vs. contr	6.08	<0.0001	2.73	0.0063
Sal vs. contr	6.67	<0.0001	4.46	<0.0001
Sal + starv vs. contr	7.11	<0.0001	2.88	0.0040

Sal high salinity, *starv* starvation, *contr* control

individuals died was statistically independent of the type of stress, and varied from 7.7 days (hypersalinity) to 11.3 days (starvation; Tukey HSD: $P > 0.05$). RVL animals were more tolerant to food deprivation ($T_{50,starv}$: 13.3 ± 0.7 days) than to hypersalinity

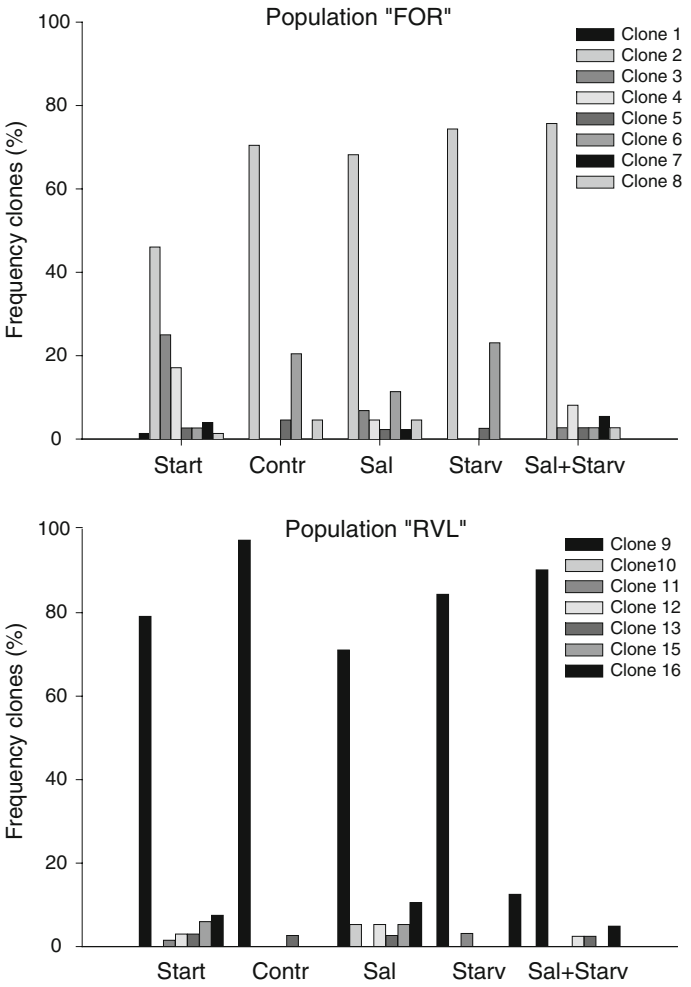

Fig. 2 Clonal frequency of *E. virens* from populations FOR and RVL at the start of the experiment ("Start") and after 60% mortality in the different treatments (*Sal* high salinity, *Starv* starvation, *Contr* control). Clonal frequencies (post-experiment) are calculated after combining the data for all three replicate aquaria (sample sizes per clone per aquaria are too low to estimate clonal frequency reliably)

(T_{50sal}: 8.7 ± 0.7 days; $T_{50starv+sal}$: 5.3 ± 2.7 days; Tukey HSD: $P_{starv\ vs.\ sal} = 0.038$, $P_{starv\ vs.\ starv+sal} = 0.002$).

The 240 and 216 individuals genotyped in the populations FOR and RVL, respectively, consisted of eight (FOR) and seven (RVL) distinct multilocus genotypes (MLG) (Appendix A). The MLG were defined based on all five loci used (Appendix B); all markers were polymorphic at both populations. None of the MLG clones (from here on "clone") was shared between populations (Fig. 2).

In population FOR, clone number 2 (>40%) was the dominant clone throughout the experiment. The other clones changed in frequency in response to stress treatments. Clones 3 and 4 reduced in frequency in the salinity treatment and in "Sal + Starv" and were not observed in the "Starv" and "Contr" treatments (Fig. 2). Clone 6, on the other hand,

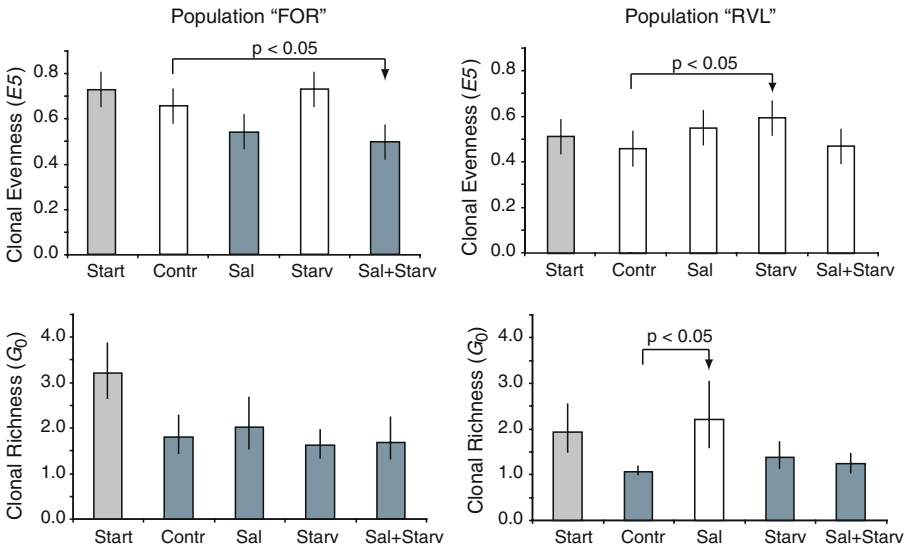

Fig. 3 Clonal evenness and clonal richness of *E. virens* individuals from populations FOR and RVL at the start of the experiment ("Start") and after 60% mortality in the different treatments (*Sal* high salinity, *Starv* starvation, *Contr* control). *Bars* depict median index values of 1,000 bootstrap samples of the multilocus genotypes (*error bars* indicate 2.5 and 97.5% percentiles). These values were compared to a distribution calculated between 1,000 random samples of identical size that were drawn with replacement from a pooled sample containing all genotyped individuals. *Dark grey bars* indicate treatments which are significantly different from the start conditions (median is outside the 2.5–97.5% percentile area, $P < 0.05$); *white bars* indicate treatments which are not significantly different. A similar test was carried out to compare the control treatment to stress treatments. *Horizontal lines* indicate which treatment is significantly different from the control conditions

increased in frequency in all experimental conditions when compared to the initial frequency, except in "Sal + Starv". Clonal richness was reduced in all treatment combinations including the "Contr"; evenness was reduced in the salinity and combined stress treatments (Fig. 3).

In the population RVL, at "Start" condition, ca. 80% of the animals were of the same genotype (Clone 9). Frequency of the dominant clone increased at all treatment combinations except in treatment "Sal", the only treatment where clonal richness was maintained. Clonal evenness was significantly higher in the "Starv" treatment than under control conditions, and remained relatively low in the other treatments.

Discussion

The competitive exclusion principle states that competition for a limiting resource should lead to dominance of the strongest competitor resulting in loss of diversity (Gause 1934). We hypothesized that stress might alleviate the effects of competition, or even change the fitness ranking of the clones after we moved the clones from the heterogeneous natural environment to the more homogenous laboratory aquaria. Indeed, clonal richness significantly decreased in the control treatments of both populations compared to the "start" conditions. Changes in clone dominance were not observed, but in general, a higher

number of clones persisted under the stress treatments. In the population FOR a shift in rank of some other common clones was observed. Frequency changes of rare clones also occurred, but these are most likely due to stochastic events. Lastly, both populations responded similarly to some stress factors (e.g. "Starv + Sal" on richness), while some factors only affected one population or the other (e.g. salinity did not affect richness of RVL population), indicating that the response to stress may depend on the genetic composition of the population. Site differences might also reflect a demographic effect between the source populations. FOR animals may have been on average older than RVL animals as suggested by their shorter survival in the control treatment (ca. 2 weeks difference). Yet, it is unlikely that this age difference could have major implications for the between site differences to stress effect patterns, given the relatively long lifespan of *E. virens* (3–4 months; Baltanás 1994; Meisch 2000).

Studies dealing with stress (e.g. pollutant) and density dependent effects have long recognized the possible reduction of competition under stress as an indirect effect of density decrease (e.g. Forbes et al. 2001). Models designed for sessile aquatic animals and plants (Connell 1975) and applied to settlement in rocky intertidal communities predicted that interspecific competition for space can be unimportant when densities of the competitors are reduced by a mortality increase as an effect of physical or biotic stresses (Connell 1985). Experimental studies on various systems have confirmed these predictions (e.g. Moe et al. 2002; Gui and Grant 2008). Our argument takes a different direction. We hypothesised that stress emphasizes the importance of abiotic factors for fitness at the expense of ecological interactions, for example reducing the importance of competitive ability for fitness (Parsons 1996a, b). Maintenance of clonal diversity would then result from relaxation of interclonal competition. We observed that in one of our study populations the control treatment shifted towards monoclonality, while in the other the clonal diversity remained moderate when compared to stress treatments. Taking together the variation in clonal structure observed among the stress treatments in the two genetic backgrounds, we conclude that more general predictions of stress effects on clonal structure in *E. virens* will be difficult.

In parthenogenetic populations, interclonal variation in stress tolerance is expected. Under rapid changes in environmental conditions, shifts in clonal composition are likely (Hoffmann and Daborn 2007). Recent studies indicate that *E. virens* populations are genetically diverse with many local clones coexisting within populations (Rossi et al. 1998; Adolfsson et al. submitted). Furthermore, laboratory experiments on *E. virens* suggest that clonal lineages differ in their ecological requirements (Otero et al. 1998; J.V. unpublished data). Therefore, we expected that the clones that dominate in the field would not necessarily be the dominant clones in the laboratory. Contrary to our expectation, we did not observe decline of the dominant clone in either of our study populations, although rank of some other common clones changed. One explanation for this would be wide variance in the fitness of clones, independent of the environment. In other words, the dominating clones would also have the broadest environmental tolerance. An alternative explanation for the persistent dominance of the common clones across variable stress treatments could be demographic heterogeneity of the populations. For example, due to hatching asynchrony the clones of the population may represent different demographic cohorts which might differ in sensitivity to environmental parameters. While hatching asynchrony is documented in ostracods (Rossi and Menozzi 1990; Martins et al. 2008), and there is some evidence of demographic sensitivity, for example, juveniles being different in tolerance than adults (Mezquita et al. 1999; J.V. unpublished data), it is an unlikely explanation for this case. In population RVL the dominant clone consisted mainly of last stage juveniles at

the start of the experiment, but in the population FOR this was not the case. Furthermore, not only the members of the most common clone, but also the other genotypes in RVL were mainly juveniles when the experiment was started. In FOR the individuals of the dominant clone were a demographic mix, and in general the population was dominated by adults.

Eucypris virens is mainly found in ephemeral environments, where temporal/spatial stochastic variation in environmental conditions may allow local adaptation and specialization to different habitats (Ayre 1995). In general, fluctuating selection is predicted to promote genetic variability (Hedrick 1986; Ellner and Hairston 1994; Etterson 2004). Weeks and Hoffmann (1998) verified that environmental heterogeneity influenced clonal frequencies and that the large fitness differences among clones suggest maintenance of genetic diversity through environmental heterogeneity. Kis-Papo et al. (2003) verified that an increase in stress conditions paralleled with an increase in genomic diversity. In the self-pollinating wild emmer wheat unexpectedly high within and between population diversity was found in relation to habitat heterogeneity in the studied geographical area (Peleg et al. 2008). Temporal stochasticity may also favour evolution of dormant stages and diapausing eggs may serve as "diversity reservoirs" (Ellner and Hairston 1994; Hedrick 1995). It is known that induction and termination of dormancy affects clonal structure, population demography and clonal succession (Gyllström and Hansson 2004). It is also know that in ostracods environmental parameters may drive the hatching patterns, which may also be clone specific (Rossi and Menozzi 1990; Rossi et al. 1996; Otero et al. 1998) leading to some degree of ecological specialization (e.g. Rossi and Menozzi 1990). On the other hand, harsh environmental conditions may also reduce the strength of ecological interactions. For example, by reducing competition among individuals as an effect of reduced population density or as a direct effect of stress with the divergence of the energy from biotic interactions and production to survival (Parsons 1996a), more room for coexistence of tolerant genotypes is possible. Environmental stress has been indicated to both promote and reduce genetic diversity (e.g. Nevo 2001; Dvornyk and Nevo 2003). This leads us to the question of how local genetic diversity responds to warming climate (Hoffmann and Parsons 1997). In *E. virens*, despite its vast geographical distribution, the optimum temperature lies around the 15°C, with higher temperatures leading to increasing juvenile mortality and lower fecundity (Otero 1998). High genetic diversity with some highly tolerant lineages may occur within this cryptic species, but to what extend this will lead to the presence of few highly tolerant genotypes with reduction of the overall genetic diversity remains an open question.

Our results show the complexity of possible responses to environmental stress. The two populations we used in the experiment expressed different responses to stress treatments. Multiple stressors may have direct negative effects on populations, reduce clonal richness and evenness, but also reduce competition among the individuals, which may promote persistence of genotypes that would be otherwise lost. Environmental stress, whether of natural or anthropogenic origin, is an important selective force behind evolutionary processes (Hoffmann and Parsons 1997; Nevo 2001). Adaptation, once thought of as a slow, gradual process, can be rapid and dynamic (Kinnison and Hendry 2001; Reznick and Ghalambor 2001). Understanding the connection between genetic diversity and ecological factors is essential for effective conservation and population management efforts responding to changing environments. Local population structure, stochastic effects and interclonal interactions play an important role in determining the dynamics following exposure to stress.

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