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Effect of salinity variation and pesticide exposure on an estuarine harpacticoid copepod, *Microarthridion littorale* (Poppe), in the southeastern US ☆

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Abstract

The harpacticoid copepod *Microarthridion littorale* (Poppe) was tested for interaction effects between salinity change and acute pesticide exposure on the survival and genotypic composition of a South Carolina population. Previous data suggested a significant link between a combined exposure to chlorpyrifos (CHPY) and dichloro-diphenyl-trichloroethane (DDT) and mitochondrial haplotype in the cytochrome *b* apoenzyme for this euryhaline species when exposed at 12-ppt salinity seawater. Our tests demonstrate a significant non-linear survival response for *M. littorale* to short-term immersion (24 h) in 3-, 12- and 35-ppt seawater, with copepods transferred to 12-ppt seawater having the lowest survival. There was significant statistical interaction between salinity and pesticide exposure for the dependent variable "survival." However, changes in genetic composition of survivors were not significant, and they were complicated by extremely low survival in the pesticide/

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3 ppt and pesticide/36 ppt treatments. As noted for many studies of harpacticoids, males faired worse than females in all treatments, with none surviving pesticide exposure at 45 μg/l CHPY and 6 μg/l DDT.

Keywords: Copepods; Genetics; Multiple stressors; Pesticide; Salinity; Toxicity

1. Introduction

The estuarine environment is a dynamic habitat that presents a constant physiological challenge to its inhabitants. Animals that predominate in estuaries must face physical extremes in the form of periodic desiccation and large fluctuations in temperature and salinity. Nowhere is this more true than in the meiofaunal community inhabiting the littoral zone of salt marshes in the southeastern US. Tidal cycles and periodic rains can change the physical environment, especially salinity, over a matter of minutes (Morris, 1995). The stress of this change has been measured in only a few copepod species in terms of survivorship and biochemical changes, and rarely in the context of toxicant/salinity stress interaction.

The dynamic physical environment is often compounded by anthropogenic inputs from point and non-point contaminant loadings. While many studies have addressed the relative effects of these contaminants on estuarine species, rarely have these effects been measured under varying physical stress (i.e., salinity). For copepods, only the pelagic species *Eurytemora affinis* (Poppe) has been tested for the impact of contaminants at various salinities (Hall et al., 1994, 1995a,b).

Recent work by Schizas et al. (2001) demonstrated a potential genetic link between resistance to pesticide toxicity and mitochondrial haplotype in the benthic harpacticoid copepod *Microarthridion littorale* (Poppe). In the previous and present study, mitochondrial haplotype serves as a measure of population genetic diversity by which changes induced by pesticide exposure might be gauged. Extreme selection (~ 13% survival) from exposures to a mixture of chlorpyrifos (CHPY) and dichloro-diphenyl-trichloroethane (DDT) pesticides produced a genetic bias that significantly increased the frequency of one of three haplotype groups of cytochrome *b* apoenzyme (cyt*b*) among the surviving copepods (Schizas et al., 2001). A similar bias in haplotype frequency (Schizas et al., 2002) has been recorded in *M. littorale* populations from field sediments in South Carolina contaminated with elevated pesticide, polyaromatic hydrocarbons, and metal contaminants (Hyland et al., 1996, 1998).

In this study, we set out to duplicate the design of Schizas et al. (2001) using *M. littorale* from the same field population but with the potentially confounding factor of osmotic stress (i.e., an abrupt salinity change) introduced. Thus, two stresses that likely affect on the survival of this species in its euryhaline estuarine environment were assessed in combination. This permitted a reevaluation of conclusions of Schizas et al. (2001) under a more complex and potentially environmentally relevant factorial model of salinity stress and pesticide exposure.

2. Materials and methods

2.1. Origin of materials, toxicological testing and salinity treatments

M. littorale were collected from Buck Hall Recreational Reserve on the Intracoastal Waterway in the Francis Marion National Forest, South Carolina, USA (33°2′ 46″N, 79°31′ 47″W). Adult copepods were concentrated by sieving the upper 5-mm of mud hand-skimmed from the edge of a *Spartina alterniflora* community to the low low-tide line to retain material that pass through a 500-μm sieve but are retained on a 125-μm sieve. During past sampling and tests (Schizas et al., 2001), salinity in this area was 12 ppt, however, salinity was 35 ppt during this sampling period (5 June 1999). Sieved mud with animals was immediately transported to our laboratory in Columbia, SC. Animals were extracted from mud under conditions described in Schizas et al. (2001). An initial 12-h trial demonstrated no mortality from direct transfer of copepods to any salinity from 1 to 35 ppt; several copepods even survived in distilled water immersion for this period. The subsequent main experiment used 600 adult copepods (450 females and 150 males) that were assigned randomly per each 300-mm diameter glass culture dish in 3×2 experimental design (four replicates each), totaling 14,400 individuals. This sex ratio (3:1) approximates that found in the Buck Hall area for this species. Copepods were tested at 3-, 12-, and 35-ppt salinity. All salinity transfers were direct without an acclimation period. While the 3-ppt salinity was chosen as a low extreme, the medium salinity was chosen to duplicate the previous exposure (Schizas et al., 2001), and the high salinity duplicated the collection salinity for the test population. Within each salinity, four replicates were spiked at a nominal LC_{90} of combined CHPY (45 ng/ml) and DDT (6 ng/ml). Whereas these are high relative to field concentrations, they are the same as the concentrations and protocols as described in Schizas et al. (2001). The remaining toxicant-free treatments were spiked with an acetone carrier control (<0.5 ml/l). All treatments were held in the dark at 20 °C for 24 h. At completion, survivors were counted and collected under a stereo dissection microscope. Survivors were defined as those individuals with normal, unimpaired swimming ability. All survivors were preserved in 95% ethanol and stored at 4 °C for DNA extraction.

2.2. DNA amplification and sequencing

DNA from individual copepods was extracted according to the procedure of Schizas et al. (1997). Small aliquots of extracted nucleic acids (typically 2–3 μl) were used as template for polymerase chain reaction (PCR) amplification (Saiki et al., 1988). Cyt*b* amplifications used the following conditions: 50 mM KCl, 10 mM Tris–HCl, pH 8.3 (Perkin-Elmer Cetus), 200 μM dNTP (Pharmacia), 5 pmol forward and reverse primer, and 1 unit *Taq* DNA polymerase (Promega). Amplifications used primers 151F (5′-TGTGGRGCNACYGTWATYACTAA-3′) and 270R (5′-AANAGGAARTAY-CAYTCNGGYTG-3′) (Merritt et al., 1998). A "hotstart" was facilitated using non-barrier wax beads containing the $MgCl_2$ (Lumitekk, Salt Lake City, UT, USA), at 3.0 mM final concentration. Template DNA and negative controls were initially denatured at 94 °C for 3 min followed by 10 cycles of 94 °C for 30 s (denaturation), 47 °C for 30 s (annealing) and

72 °C for 45 s (extension). These initial 10 cycles were followed immediately by 30 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 45 s. A small aliquot (5 μl) of each amplification was screened on a 1.5% agarose gel containing ethidium bromide for amplification products for visualization under UV light.

Past research (Schizas et al., 2001) found three diverse cyt*b* haplotype lineages (*li*I, *li*II, and *li*III) in *M. littorale* from Buck Hall Recreational Reserve occurred in nearly equal frequencies. Thirteen microliters of PCR product was digested with 1 unit of the restriction endonuclease *Dpn*II to produce diagnostic fragment patterns that identify each lineage. A total of 1275 of the survivors from all treatments were typed in this manner for this study with at least 60 individuals typed for each replicate, unless fewer individuals survived exposure.

2.3. Statistical analyses

We investigated two variables in response to salinity change and pesticide exposure: survivorship and percent change in haplotype frequency of the survivors. Most statistical analyses were performed using SPSS rel. 10.0.0 (1999, SPSS, Chicago, IL, USA).

A two-way analysis of variance (ANOVA) with both factors fixed was performed to assess the level of significance for change in survivorship in response to salinity and pesticide exposure. Levene's test of equality of error variances (Levene, 1960) was implemented to test assumption of equal variances of the ANOVA. Tukey's comparison test was used to test for significant differences in survivorship within the different salinity levels. Even though ANOVA is robust to deviations from normality (Scheffé, 1959, Chap. 10), we tested our data for deviation from normality using a Shapiro–Wilkes test implemented in Dataplot™ (Information Technology Laboratory, NIST, USA).

To test for significant changes in each haplotype frequency between treatment conditions, a second ANOVA was performed, using the same model as above, but using a repeated measures design where haplotypes were the repeated measures. Change in haplotype frequency was calculated as percent difference in haplotype frequency between survivors and the field frequencies, scaled to field frequencies. A Box's test of equality of covariance matrices implemented in SPSS was carried out to address whether covariance matrices of the dependent variables were equal across groups.

3. Results

Survivorship patterns for *M. littorale* were not as expected, given past results and our initial 12-h test of salinity exposure (Fig. 1). In salinity-only treatments, *M. littorale* survived worst in the 12-ppt, salinity-only treatments and best in 35-ppt salinity. Technician error led the loss of two of the replicates for the 35-ppt treatment, but survival averages of the remaining two replicates were 98.9% and 99.6% for females and 84.0% and 90.0% for males. In the 3- and 12-ppt salinity-only treatments, males faired similarly in trend and exhibited lower overall survival than females.

In salinity/pesticide treatments, a different survival pattern emerged. Females exposed to the pesticides at 12 ppt survived best at 21.4 ± 5.5% (mean ± S.E.). Males faired much

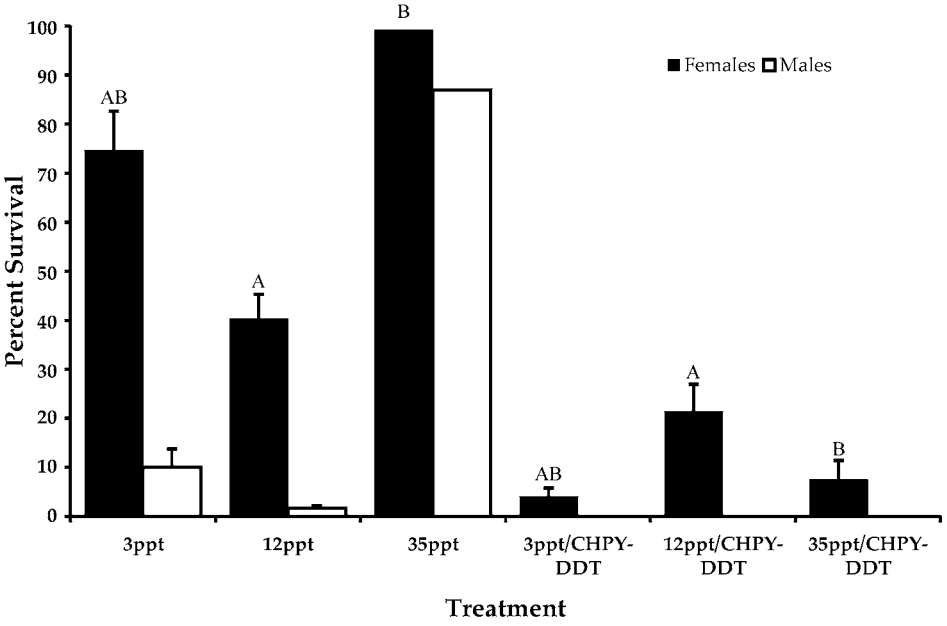

Fig. 1. Survival of exposure to salinity stress and combined salinity and pesticide stress by *M. littorale*. Means for all treatments are shown (± S.E.). Each replicate represents the percent survivorship of 450:150 females:males, with an original total of four replicates per treatment. Shared letters above bars demonstrate the post hoc non-significant differences in treatment means for salinity as noted from Tukey's comparison test on females only. Note the 35-ppt salinity-only treatment means result from only two replicates.

worse than females having no surviving individuals exposed to any treatment combination of salinity/pesticide. At the 3- and 35-ppt pesticide treatments, females survived at 4.05 ± 1.7% and 7.50 ± 3.9%, respectively.

The significance of these patterns was tested by a two-way ANOVA on survivorship of females (Table 1). In all statistical analyses, we only considered females because most of the males died. Levene's test of equality of error variances demonstrated that the error variance for survival was equal across treatments and replicates ($F = 1.58$, $df1 = 5$, $df2 = 16$; $P = 0.222$). Replicate treatments were not significantly different from normal within treatment using a Shapiro Wilk normality test ($\alpha = 0.05$; Shapiro and Wilk 1965). The two-way ANOVA for the linear model was highly significant ($P < 0.001$; all factors)

Table 1
Survivorship by treatment of *M. littorale* for analysis of variance (ANOVA) with results by variance component and *F* table probabilities for females only

Source	Type III sums of squares	*df*	MS	*F*	Significance level (*P*)
Salinity	89,717	2	44,858	21.036	< 0.001
Pesticide	530,200	1	530,200	248.634	< 0.001
Salinity × pesticide	171,325	2	85,662	40.171	< 0.001
Error	34,119	16	2132		

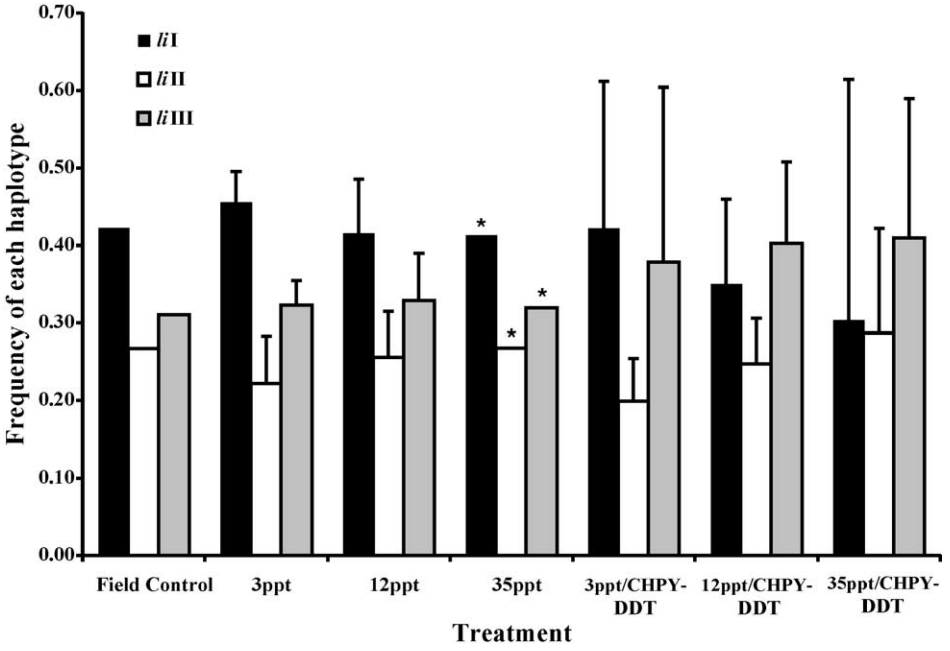

Fig. 2. Haplotype group composition of each treatment group. Mean frequencies for each type within treatments are shown (± S.E.). Frequencies are based on at least 60 individuals per replicate, unless fewer survivors occurred. Asterisks (*) indicate treatments composed of only two replicates each.

for each main factor (Salinity or Pesticide) and for their interaction effects on survivorship. Tukey's comparison test on salinity means found no significant difference between means of the 3- and 12-ppt, or 3- and 35-ppt treatment ($\alpha = 0.05$, Fig. 1), using a harmonic mean of group sizes to adjust for unequal groups.

In sharp contrast to Schizas et al. (2001), there were no significant changes in haplotype group composition with pesticide or salinity ($\alpha = 0.05$) relative to field frequencies. However, survival was low (< 5%) in two of four replicates for each of the 3- and 35-ppt salinity/pesticide treatments. Variance among replicates tended to be larger (Fig. 2) among salinity/pesticide treatments than among salinity-only treatments, however, these variances were not significantly higher by Box's Test of equality of covariance matrices (Box's $M = 118.8$, $F = 0.777$, $df1 = 24$, $df2 = 173$, $P = 0.762$).

4. Discussion

There is clearly an interaction between salinity and pesticide toxicity for *M. littorale*, even with exposures as short as 24 h. We were surprised by the result that the poorest survival for salinity exposure alone was a common salinity (12 ppt) in which *M. littorale* is found, the medium salinity tested here, and the experimental salinity of Schizas et al. (2001). The habitat of *M. littorale* is characteristically the brackish water zone (Lang,

1948) where it is often the predominant species (Coull and Dudley, 1985). While abrupt transfer to 12-ppt salinity from 35 ppt is stressful, these copepods probably experience this level of stress multiple times over a season and at 6-h tidal cycles or less (Morris, 1995). There seems to be some level of long-term adaptation to 12-ppt conditions as the combined effect in our study of 12-ppt salinity and pesticide exposure produced higher survivorship (>20%) than that reported ($11.22 \pm 1.17\%$) for *M. littorale* exposed in Schizas et al. (2001).

The less extreme effect at the 3-ppt salinity-only treatment may result from a physiological response to extremely low salinities. While larger crustaceans have been shown to avoid low salinity plumes (Jury et al., 1994), this is not an option for poorly mobile estuarine meiofauna. Physiological response of benthic harpacticoid copepods to salinity changes has not been studied in general, however, mucus secretions were noted in lower salinities in Swedish mesopsammic (i.e., interstitial) copepods (Jansson, 1968). Also, a planktonic harpacticoid copepod, *Euterpina acutifrons* (Dana), secretes small strands of mucus from the hind and ventral part of the abdomen in low salinity (Moreira, 1975). Secretion of mucus under salinity stress in marine animals is well documented (Kinne, 1964), and serves to greatly reduce the permeability of the integument (Potts and Parry, 1964). Secondarily, *E. acutifrons* ceases activity altogether when plunged into extreme salinity changes (Moreira, 1975), which might contribute to the reduction of short-term energetic costs by effecting a torporous state. Longer than 24-h exposures to low salinity for *M. littorale* may result in death, as it did for *E. acutifrons* which survived for 24 h when transferred directly from 35- to 5-ppt seawater. In post hoc observations of *M. littorale* behavior transferred to 2.5- from 35-ppt salinity, males were less active than females. However, no obvious mucus strands were noted during a period of exposure to distilled water for 2 h. As compared to the general male response, female copepod robustness to contaminant exposure has been documented previously (e.g., Dalla Venezia et al., 1981; Strawbridge et al., 1992; DiPinto et al., 1993). A similar pattern is also seen in our data. In each case, females appear to be less sensitive to higher exposure concentrations than males. This result could be explained by females either accumulating contaminants less quickly, sequestering toxicants into non-labile lipid pools, or eliminating contaminants from their tissues faster. In the few studies where this has been examined, ^{14}C-labelled polychlorinated byphenyls (PCBs) were absorbed initially at similar rates for male and female copepods, but females of *Acartia tonsa* were shown to significantly reduce levels of these contaminants after short time periods as compared to males (McManus et al., 1983). In their study, the lipid-bound PCBs were shown to be sequestered preferentially into the females egg mass (McManus et al., 1983), which has also been suggested as a possible mechanism for *M. littorale* for PCBs (DiPinto et al., 1993). However, differences in survival between sexes based on salinity exposure cannot be related to differences arising from lipid partitioning. In our study, exposures were concentrated, acute and constant during the test; therefore, differential elimination of pesticides after exposure was likely not a concern for our data. Clearly, males must be physiologically different as to make them more susceptible than females to these stresses, but more definitive tests are needed to identify the mechanism of this difference.

Changes in salinity and pesticide do not correlate with changes in mitochondrial type in this study, as opposed to results from Schizas et al. (2001). Individuals plunged into 12-ppt

salinity and exposed to the same nominal concentration of CHPY/DDT survived slightly better than previously, but there was little difference in haplotype frequencies between field control, salinity-only or salinity/pesticide treatments (Fig. 2). There was a non-significant tendency for increase in variance associated with the means of salinity/pesticide treatments over salinity-only treatments. In the previous study, there was increased survival of one haplotype group relative to the other two for all pesticide treatments (Schizas et al., 2001). The results of Schizas et al. (2001) indicate a linkage between the cytoplasmic genetic marker, cyt*b*, and genetic attributes selected for in the nuclear genome. The present results, however, are more consistent with variation due to random genetic drift of the mitochondrial gene frequency (i.e., increase in variance of means) in the separate replicates, although our study was not designed to test this hypothesis. Clearly however, the addition of salinity stress confounds the previously described relationship between haplotype and pesticide stress alone (Schizas et al., 2001).

Survival patterns of *M. littorale* in salinity/pesticide treatments was similar to that observed for *E. affinis* exposed to cadmium at different salinities (Hall et al., 1995a). Although LC_{50} measurements occurred over 96-h exposures at different salinities, the highest LC_{50} occurred at 15-ppt seawater in *E. affinis*. For both species, this pattern is suggestive of long-term acclimatization or even physiological/genetic adaptation to estuarine salinities. However, this is not the case for all contaminant exposures as survival for *E. affinis* exposed to atrazine at different salinities yielded a linear decrease in LC_{50} with salinity (Hall et al., 1995b). Even considering the high mortality in our study, our original estimates of LC_{90} for *M. littorale* predicted less survival than was eventually observed ($\sim$ 20%). Physiological responses that combat osmotic stress (similar to those reported in Moreira, 1975) may reduce the survival of salinity stressed copepods exposed to pesticides.

The results from this study therefore reflect a cautionary tale for toxicologists. First, molecular responses based on genetic variation are a complex of direct and epistatic effects between the recombining genome and a highly variable environment. Obviously, a significant genetic result for *M. littorale* exposed to pesticides at intermediate salinity (Schizas et al., 2001) does not extrapolate to a complex changing osmoregulatory landscape combined with contaminant exposure. Second, and more importantly, this changing landscape and its strongly interactive effects on pesticide tolerance potentially has major implications in management strategies of estuarine habitats for commercially important species, yet it is under studied in the toxicological testing of most estuarine organisms. Future research in these areas will have to focus on these complicating factors to reach more realistic and predictive contaminant-effect models for environmental management.

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