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Differential Survival of Three Mitochondrial Lineages of a Marine Benthic Copepod Exposed to a Pesticide Mixture

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In South Carolina estuaries, the harpacticoid copepod *Microarthridion littorale* (Poppe 1881) consists of three distinct mitochondrial lineages (*lit*I, *lit*II, and *lit*III), whose distributions may be partially explained by the presence of toxic contaminants in the sampled habitats. The frequencies of *lit*II and *lit*III are greatly diminished and sometimes absent in South Carolina contaminated tidal creeks where *lit*I is omnipresent. In this study, representatives of these lineages or haplotype groups were collected from sediments of an estuarine creek containing low to undetectable levels of toxicants and then exposed to a toxic ($\sim LC_{90}$) aqueous mixture containing an organophosphate (chlorpyrifos) and organochlorine pesticide (DDT, mixed isomers). A comparison was conducted for the frequency of each of the three haplotypes among the survivors of the exposed animals relative to that among the survivors of the control group. The haplotype group with the highest frequency in contaminated SC estuaries (*lit*I) was statistically higher in frequency in survivors of the pesticide-exposed group than in the control group. The two rarer groups (*lit*II and *lit*III) were less abundant among the survivors of the pesticide-exposed group than the control group. The frequencies of *lit*I, *lit*II, and *lit*III did not change significantly among the survivors of the control group. The differential survival of the three haplotype groups in the pesticide mixture may be one of the reasons that some haplotype groups are more likely to be found in clean or contaminated tidal creeks on the South Carolina coast.

Introduction

Toxicants can exert rapid and strong selective pressures on populations (*1*, *2*) that can ultimately lead to broad genetic changes. Organisms living in habitats where toxicants represent a significant and chronic environmental pressure often survive under these conditions through tolerance (*3*) or genetic adaptation. If genotypes in a population subjected to contaminant stress vary in resistance or tolerance to stress, then the differences in relative fitness among genotypes will be expressed eventually as population-level genomic changes. Therefore, potential mechanisms of natural selection can be studied experimentally by using environmental toxicants as controlled stressors.

Even though impacts on marine ecosystems are important, studies on the genetic effects of pollution on marine organisms are not common (*4*). Genetic adaptation and/or genetic change in the presence of contaminant stressors has been documented in various taxa such as isopods (*5*), barnacles (*6*), copepods (*7*), mussels (*8*), gastropods and shrimps (*9*), and oligochaetes (*10*). However, these studies have rarely combined both laboratory experiments and field surveys to confirm observed genetic patterns of potentially impacted species (see refs *9*−*11* for exception). If genetic patterns are to be used as a tool in ecotoxicology for monitoring and assessment of population-level environmental impacts, genetic patterns should be validated by appropriate laboratory experiments whenever possible (*12*). One of the most sensitive groups of marine animals to toxicants is the meiofauna (small benthic metazoans that pass through a 0.500-mm sieve but are retained on a 0.063-mm sieve). Over 250 studies have shown meiobenthos to be sensitive indicators of anthropogenically stressed environments and typically more sensitive than macrobenthos (*13*).

The meiofaunal harpacticoid copepod *Microarthridion littorale* is well-suited for contaminant-related genetic studies because it is found abundantly in estuaries of the southeast Atlantic and Gulf coasts, and it is amenable to laboratory culture and experimental manipulations (*14*−*16*). *M. littorale* has a short life cycle (egg to egg in 30 days at 25 °C; *17*), comprises 18% of estuarine sediment-dwelling fauna on average in North Inlet, SC (*18*), and is an important food source for commercially important fish and shrimp (*19*). *M. littorale* is an obligatory benthic dweller inhabiting the surficial oxic zone of sediments where toxicants are most bioavailable and where it may be continuously exposed to sediment-associated contaminants through bioturbation and ingestion (*13*). *M. littorale* has exhibited reduced survival and reproductive output in single- and mixed-contaminant toxicity tests (*15*, *16*, *20*, *21*), and it is known to bioaccumulate pollutants (*22*, *23*). The biogeography in the southeast United States and Gulf of Mexico (*24*) and population genetic structure of *M. littorale* within South Carolina and Georgia (*25*) have been estimated by molecular markers. Genetic analyses based on variations in cytochrome apoenzyme *b* uncovered three mitochondrial haplotype groups within *M. littorale* (*25*). Within South Carolina, the three haplotype groups exist sympatrically in many tidal creeks, with the level of creek contamination partially correlated with the observed frequency distribution of the SC groups (*25*).

Because many urbanized tidal creeks harbor a milieu of anthropogenic contaminants, we decided to investigate the role of toxicants as genetic selection agents by exposing a genetically balanced, a priori pristine population of *M. littorale* to two toxicants with very different modes of action but commonly found in South Carolina coastal sediments—chlorpyrifos (CHPY) and DDT. CHPY is a potent acetylcholinesterase inhibitor used to control a variety of pests, and it can be toxic to aquatic invertebrates and fishes (*26*, *27*). CHPY has been used extensively for insect control in coastal regions of the southeastern United States on turf grasses and in termite/fire ant control. Concentrations have been encountered in the field up to 255 ng/g wet sediment in

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Buzzard's Bay, MA (*28*). CHPY is characterized by a high K_{ow} and binds readily to sediment (*29*). CHPY degrades much faster than DDT, but it can persist in nature >400 days (*29*). Sediment-associated chlorpyrifos is highly toxic to the marine copepod *Amphiascus tenuiremis* (*30*), and densities of *M. littorale* were significantly reduced in whole-community microcosm experiments dosed with CHPY (*14*). Dichlorodiphenyltrichloroethane (DDT) is a persistent organochlorine pesticide commonly present as a historical contaminant in U.S. aquatic sediments, even though its use is now prohibited in the United States and many other developed countries. The voltage-sensitive sodium channel is the primary molecular target site for DDT. DDT induces in axons multiple-spike discharges and eventual death in insects, crayfish, fishes, and amphibians. DDT decreases the longevity and reproductive success of a meiofaunal gastrotrich (*31*) and a harpacticoid copepod (*32*).

In this study, three naturally occurring *M. littorale* genetic lineages were exposed to an aqueous pesticide mixture in the laboratory and assayed for genetically linked differential survival. This study supplements the results of the aforementioned field genetic survey of *M. littorale* (*25*) that found haplotype frequency differences in copepods from pristine versus polluted sediments.

Materials and Methods

Sampling Location. Extensive collection and analysis of *M. littorale* from the South Carolina coast revealed that Buck Hall Recreational Reserve (McClellanville, SC) is the only sampled site in South Carolina where the three reported mitochondrial lineages of *M. littorale* exist in approximately equal frequencies (*22*). Buck Hall Reserve is a largely uncontaminated estuarine site with total PAH < 88 ng/g and undetectable levels of PCBs and pesticides.

Spiking Procedures. Pesticide stock solutions were prepared using technical-grade DDT (mixed isomers) and chlorpyrifos (CHPY) (99.9% purity) obtained from Ultra Scientific, North Kingstown, RI. Prior to use, all experimental glassware was acid-cleaned, rinsed 4 times with deionized water, and then rinsed 3 times with acetone and dichloromethane. Artificial seawater media were filtered (0.45 μm) and aerated (DO > 85% saturation) prior to addition of pesticides and/or carrier. A salinity of 12 ppt was used to coincide with field salinity at time of copepod collection. Individual CHPY and DDT stock solutions in acetone were added to volumetric flasks and diluted with seawater to desired concentrations. Treatment solutions were transferred to glass beakers and allowed to equilibrate for 1 h on a magnetic stir plate with a Teflon-coated stir bar prior to addition of test organisms. CHPY and DDT stock solutions were analyzed on a Hewlett-Packard model 5890 gas chromatograph with an electron capture detector. The instrument was operated in splitless mode using helium as the carrier gas. Injection port and detector temperatures were 250 and 320 °C, respectively. A DB-1 60 m × 0.25 mm i.d., 0.25 μm film thickness column (J&W Scientific) was operated at an initial temperature of 110 °C for 1 min with a 10 °C/min ramp to 300 °C and a 5-min hold.

Aqueous Exposures. Adult *M. littorale* were collected during June 1999 from a mudflat located on the intracoastal waterway in the Buck Hall Recreational Reserve, south of McClellanville, SC (33°1′15″ N, 79°34′56″ W). Additionally, copepods were collected at the same site on three different sampling dates, June 1998−1999, to provide baseline information on the seasonal fluctuations of the frequencies of the mitochondrial lineages. Similar initial frequencies of the copepod haplotype groups from Buck Hall were necessary to ensure that starting experimental genotypic diversity was equivalent across treatments and controls.

Copepods were collected by hand from the top centimeter of sediment during low tide, transported back to the laboratory, and extracted from sediments using fiber-optic lights (*33*). A range-finder test was performed to determine a mixture concentration that would result in approximately 10% survival in the definitive test and also yield sufficient numbers of organisms for DNA and statistical analysis. In the definitive experiment, copepods were exposed to a single pesticide treatment (45 ng/mL CHPY and 6 ng/mL DDT) and an acetone carrier control treatment (<0.5 mL/L). Definitive test treatments were replicated six times each. For both treatment and control replicates, 600 adult copepods (450 females and 150 males) were randomly selected and added to glass crystallizing dishes containing 150 mL of the seawater:pesticide mixture. This sex ratio (3:1, female:male) was representative of approximate field sex ratios at the time of copepod collection. Experimental dishes were loosely covered and held in the dark in an environmental chamber at 20 °C for 24 h. At test completion, surviving copepods were counted and collected under a stereo dissection microscope. Survivors were defined as copepods with normal swimming ability. All surviving *M. littorale* were preserved in 95% ethanol and held at 4 °C for DNA processing.

Over 1000 copepods were genetically screened in this study, including 212 copepods collected from Buck Hall during the three sampling dates. DNA from individual copepods was extracted as described (*34*). Two microliters of the extracted DNA was used as a template to amplify 425 bp of cytochrome *b* via the polymerase chain reaction (PCR) procedure (*35*). For the DNA amplification, the "universal" cytochrome *b* primers developed by Merritt et al. (*36*) were used and followed previous procedures (*24*). After completion of the DNA amplification, a portion of the PCR reaction (13 μL) was digested with the enzyme *Dpn*II. Samples were incubated overnight (37 °C) in order for complete digestion of the PCR product to occur. The digested samples were then electrophoresed on 2% gels (1× TBE buffer) and stained with ethidium bromide. RFLP patterns were visualized and photographed under UV illumination. Enzymatic digestion of the 425 bp of cytochrome *b* with *Dpn*II results into three RFLP patterns, diagnostic of the three mitochondrial groups (*25*).

Statistical Analyses. For the copepods collected during three sampling sites from Buck Hall, a two-way ANOVA model (haplotype group * time) without replication (*37*) was utilized to assess if there was a significant temporal component in the frequency of the three mitochondrial lineage groups. For the definitive experiment, data from replicate dishes within a treatment were pooled because analysis by three-way ANOVA (treatment * haplotype group * replicate) revealed no significant difference ($P > 0.05$) at the "replicate" level of the model. "Treatment" signifies the control and pesticide exposure levels of the model; "haplotype group" encompasses the three mitochondrial groups *li*I, *li*II, and *li*III; and "replicate" includes the six replicates per treatment. Before statistical analysis, frequencies of mitochondrial groups were converted with the arcsine square root transformation to meet ANOVA data assumptions. Tukey's Studentized range test was used to test for significant differences between and within treatments with regard to frequencies of the three haplotype groups. All statistical tests were performed using the General Linear Model procedures of SAS (*38*).

Results

Field Frequencies of the Three Mitochondrial Lineages. No significant differences in frequencies of the three mitochondrial groups were observed between June 1998−1999 at the Buck Hall, SC, site ($P > 0.05$; Figure 1). The least common mitochondrial group, *li*II, comprised approximately 25−30% of the processed *M. littorale*. Neither *li*I nor *li*III

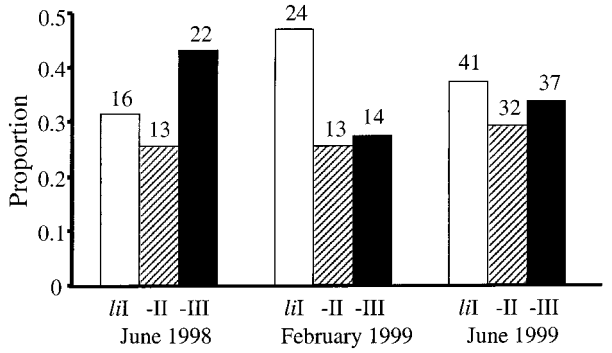

FIGURE 1. Temporal variation of frequencies of the three haplotype groups (*li*I, *li*II, and *li*III) of field collected *Microarthridion littorale* from Buck Hall Reserve, SC. Numbers above bars represent processed specimens.

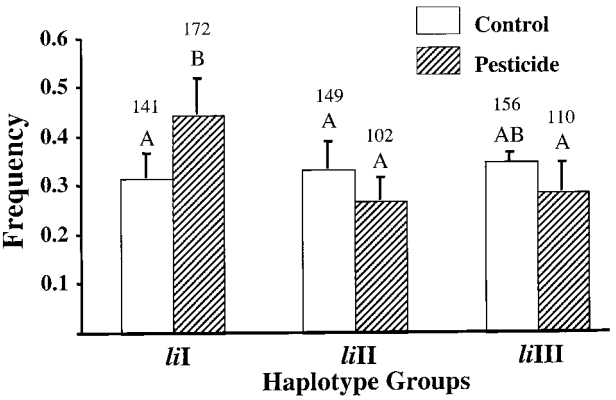

FIGURE 2. Proportion of *Microarthridion littorale* survivors by haplotype group (*li*I, *li*II, and *li*III) pooled from the six replicates of the control and pesticide treatment. Error bars represent one standard deviation from the mean. Letters above bars denote results from the Tukey's test. Bars with no similar letters are significantly different at the $\alpha = 0.05$ level. Numbers above the bars denote the number of processed individuals of a haplotype group for all replicates in the particular treatment.

ever exceeded 50% of the total number of the sampled *M. littorale* among the three sampling periods (Figure 1).

Definitive Pesticide Exposure Experiment. All of the surviving individuals of the pesticide treatment and approximately 10% of the surviving individuals of the control treatment were processed for DNA extraction, amplification, and digestion ($n_{total} = 854$). From the control and treatment replicates, we processed 73−75 and 54−73 specimens, respectively. Twenty specimens (16 females and 4 males) over both treatments did not provide sufficient DNA for analysis. These 20 specimens were not included in genetic analyses; however, they were included in estimations of overall *M. littorale* survival rates per treatment. Overall survival in controls and pesticide treatments was 98 ± 1% and 11.22 ± 1.17%, respectively. Totals of eight males survived in the six replicates of the pesticide treatment and were only included in estimations of overall *M. littorale* survivorship.

We did not observe significant replicate-to-replicate variability in the control and pesticide treatment. The frequency of *li*I, *li*II, and *li*III was approximately the same among the survivors of the control treatment (Figure 2). Significant mitochondrial group differences occurred between treatments (G-test, 2 df, $P < 0.05$, Figure 2). Comparisons between control and pesticide treatment frequencies for each mitochondrial group indicated that the frequency of *li*I was higher in the pesticide treatment than in the control (Tukey's test, $P < 0.05$; Figure 2). Both *li*II and *li*III were reduced under pesticide exposure relative to the control, but the decrease was not significant (Tukey's test, $P > 0.05$, Figure 2).

Discussion

Pesticides and other anthropogenic toxicants are a relatively recent environmental influence in geological time. But toxicants are becoming widespread globally and no doubt will become an increasingly predominant structuring and selective force in ecological communities (*39*). Since persistent contamination is a prevalent and common characteristic of many sedimentary environments, these systems provide a unique opportunity to experimentally study the processes of natural selection and evolution directly.

The differential susceptibility of the *M. littorale li*I, *li*II, and *li*III haplotypes to pesticides in the experiment described here may explain the frequency of these mitochondrial groups observed for sediment-dwelling copepods in 10 field sites (*25*). In three contaminated sites in Charleston Harbor, SC (Diesel Creek, Shipyard Creek, and Newmarket Creek), the haplotype groups *li*II and *li*III comprised <17% of the standing genetic stock of *M. littorale*. Conversely, in two "clean" sites (Beaufort, GA, and Buck Hall, SC), *li*II and *li*III made >50% of the population (*25*). The differences in the frequency distribution of *li*I, *li*II, and *li*III along the South Carolina coast may be explained by a multitude of reasons, including the presence/absence of toxic contaminants in the sampled habitats (*25*). The experimental results presented here represent a potential mechanism for observed field population patterns if these populations undergo similar episodes of toxicant-induced mortality.

Our results also suggest caution should be exercised in considering *M. littorale* as one homogeneous taxonomic group for toxicological studies. The copepod *M. littorale* has been an integral part of many ecological (e.g., refs *17*, *18*, and *40*−*44*) and ecotoxicological studies (e.g., refs *14*−*16*, *20*−*23*, and *45*). The majority of these studies utilized *M. littorale* from North Inlet, SC, where *li*I is the predominant mitochondrial group and comprises 81% of the total sample (*25*). The mitochondrial group *li*III is the only other mitochondrial group encountered in North Inlet. Because North Inlet is relatively homogeneous in terms of mitochondrial group diversity, earlier conclusions reached from studies including North Inlet, SC, *M. littorale* should not be affected by the pitfalls of genetic nonhomogeneity at the population level.

How do the experimental conditions relate to the *M. littorale* natural habitat? Our laboratory experiments were performed under controlled laboratory conditions, far from the complexity observed in the native habitat of *M. littorale*. A lack of sediments and short duration of exposure in pesticides were unrealistic relative to what *M. littorale* encounter in nature. *M. littorale* is an obligatory meiobenthic copepod and is in constant contact with the sediment matrix throughout its life cycle. Additionally, in the field, *M. littorale* are exposed to contaminants that differ in variety and concentration as compared to the two pesticides used in this study. The differential sensitivity exhibited by the three haplotypes may be specific only to the pesticide mixture used here rather than to chemical stressors similar to the field conditions. The initial study (*25*) used a coarse measurement of contamination, comprised of all available data for chemical stressors, rather than using the concentration levels of DDT and CHPY. However, since both pesticides are associated with the sediment, it is possible that the sensitivity exhibited by the haplotype groups shown here is an indication of sediment-bound toxicants in general. Furthermore, the route of exposure may be different in the field relative to the laboratory conditions since sediment ingestion and direct contact with contaminated detritus and sediment may facilitate contaminant uptake. Despite these obvious differences between experimental and field conditions, fre-

quencies of mitochondrial groups observed in the pesticide treatment were significantly different from the control treatment in a 24-h exposure, and these patterns were similar to those observed in the more generic mixed contaminant field setting.

The potential of toxicants to permanently alter the genetic composition of *M. littorale* is likely to be more pronounced over chronic exposures since toxicants represent only a part of the selective forces active in estuarine environments. Estuarine ecosystems are dynamic where water depth, salinity, temperature, pH, and grain size distributions often change hourly. All of these abiotic variables exert additional selective pressure on estuarine organisms because of the magnitude of the fluctuations and time scales in estuaries.

In the *M. littorale* example, for the frequency changes of *li*I, *li*II, and *li*III to be evolutionarily significant, they must also link either to a significant reduction of genetic diversity generally or lead to significant negative changes in population parameters such as fitness. Dominance of one haplotype in a population can result in reduction of genetic variation that may negatively affect the overall (species) fitness. Additional experimental evidence is needed to associate specific gene frequency changes to fitness changes; e.g., test if survivors of the *li*I haplotype are relatively more or less fit in clean environments than the more sensitive copepods.

Evolution is defined, from a population genetics perspective, as the change of gene frequencies over time. In a strong, acute laboratory exposure, significant changes in gene frequencies of *M. littorale* were induced in 24 h by a mixture of two pesticides. In nature, unless a catastrophic mortality event occurs (e.g., an oil spill or pesticide-induced fish kill), such changes likely happen in the span of many generations.

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