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Genetic and life-history trait variation of the amphipod *Melita plumulosa* from polluted and unpolluted waterways in eastern Australia

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ABSTRACT

To monitor genetic diversity and environmental contamination in eastern Australia, toxicity studies have employed the sensitive benthic amphipod *Melita plumulosa*. The goal of this study was to examine the genetic and life-history variability of natural populations of *M. plumulosa* from the Parramatta (polluted) and Hawkesbury (unpolluted) Rivers. The underlying genetics of the populations in these distinct waterways was examined at one mitochondrial (cytochrome *c* oxidase subunit I (COI)) and one nuclear (ribosomal internal transcribed spacer region 1 (ITS1)) locus. Seven unique haplotypes for COI were found amongst animals from the Parramatta River, while animals from the Hawkesbury River showed a complete absence of genetic variation at this locus. At ITS1 a total of two sequence variants were found amongst Parramatta River amphipods and three sequence variants among Hawkesbury River animals, with no common variants across the two river systems. To establish whether genetic differences were associated with organismal responses to toxicant exposure, two life-history trait variables (female head length as an estimator of amphipod size and female fecundity) were analyzed. Life-history trait analyses showed that females from the Hawkesbury River were significantly larger and more fecund. These data have critical implications for toxicity tests, the use of laboratory cultures for testing purposes, and environmental contamination in Sydney Harbor.

1. Introduction

The overall goal of this study is to determine whether genetic variation in the amphipod *Melita plumulosa* is reflective of organism exposure to toxicants in two eastern Australian waterways. Aquatic invertebrates such as amphipods are suitable test species for biomonitoring programs in marine, freshwater and estuarine ecosystems, as they have been demonstrated to be highly sensitive to the presence and effects of toxicants (Amiard et al., 2006; Ford et al., 2003; Manyin and Rowe, 2006; McCready et al., 2005; Neuparth et al., 2005; Scarlett et al., 2007). Furthermore, many amphipod species fulfill the criteria for a sediment-toxicity test organism (Chapman and Wang, 2001; Simpson et al., 2005). In eastern

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Australia, *M. plumulosa* has been proposed as a bioindicator species to monitor marine sediment (King et al., 2006a). This amphipod is a deposit-feeder that lives at the sediment-water interface under rocks or shells, and is common along the intertidal mudflats in both marine and freshwater environments (Lowry et al., 2000). It is sensitive to a variety of sediment-bound metal toxicants including copper, cadmium and zinc (Gale et al., 2006; King et al., 2006a,b, 2005). Currently, however, little is known about the genetic or life-history trait variation of this amphipod.

This study examines molecular and life-history trait variation of amphipods from two major river systems: the Parramatta River and the Hawkesbury River (see Fig. 1). In comparison to other waterways in Australia and around the world, there are elevated levels of contaminants such as petrochemical and metal contaminants in the Parramatta River (Birch, 2000; Birch et al., 2007; McCready et al., 2006). By comparison the Hawkesbury River is an unpolluted waterway, with concentrations of metal and organic toxicants at near background levels (Birch et al., 1998; Birch and Taylor, 1999).

It is hypothesized that exposure to toxicants will result in genetic deviations from a neutral model of mutation, either by increasing the rate of mutation or decreasing the amount of variability within a population (De Wolf et al., 2004; Mulvey et al., 2002; Peles et al., 2003). We compare the genetic variability of *M. plumulosa* at one mitochondrial locus, cytochrome *c* oxidase subunit I (COI), and one nuclear locus, the ribosomal internal transcribed spacer region 1 (ITS1). The N-terminus of the COI gene has been identified as a highly variable locus and is widely considered as a useful genetic marker for the study of maternal lineages and the identification of closely related populations (Hebert et al., 2003; Lefébure et al., 2006; Meyran et al., 1998; Saccone et al., 2006; Witt et al., 2006). ITS1 is a short stretch of non-coding DNA of variable length separating the 18S and 5.8S ribosomal genes. Studies have shown that this sequence is often highly divergent in crustaceans, making it a potentially useful marker for population studies (Chu et al., 2001; Schulenburg et al., 1999).

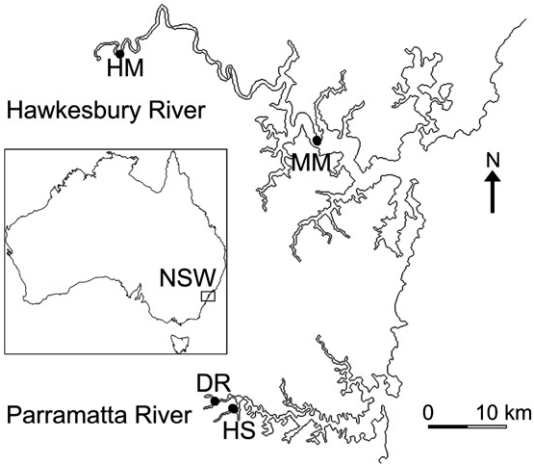

Fig. 1 – Study sites chosen along the Parramatta River and Hawkesbury River, Australia. Parramatta River localities were Homebush Bay South (HS; 33°50′06.9″S, 151°04′39.0″E) and Duck River (DR; 33°49′27.7″S, 151°03′05.0″E), and Hawkesbury River localities were Mooney Mooney (MM; 33°31′56.1″S, 151°11′47.9″E) and Half Moon Bend (HM; 33°25′51.9″S, 150°55′23.9″E).

Toxicant exposure is also expected to affect life-history traits in *M. plumulosa*. Exposure to chemical stressors can lead to decreased body size and growth rates in crustaceans and fish (Ferrando et al., 1996; Fisher et al., 2007; King et al., 2006b) or reductions in fecundity in fish (Cook et al., 2003; Heiden et al., 2006; Jobling et al., 2002a,b) and in amphipods (Ford et al., 2003; Gale et al., 2006). Therefore it is hypothesized that toxicant exposure will result in smaller, less fecund animals. We measure head length as an estimator of amphipod size (Mann and Hyne, 2008), and female fecundity was estimated by counting the number of embryos per female (Mann and Hyne, 2008). If animals collected from a contaminated site or their F1s or F2s are used in life-history trait analyses and toxicity tests, grand-maternal affects may bias the results of toxicology tests (Hercus and Hoffman, 2000; Marcial and Hagiwara, 2007). Alternatively, or in addition, the underlying genetic substructure of the populations from polluted and unpolluted sites may differ and be manifest as life-history trait differences. A study by Schizas et al. (2001) found that different mitochondrial lineages of the marine copepod *Microarthridion littorale* showed different susceptibility to pesticide exposure.

It is further hypothesized that genetic structure at COI is linked with differences in organism size and reproductive output. Organisms exposed to stressors have been shown to divert energy away from processes such as growth and reproduction into stress response pathways (Fisher et al., 2007; Wayne et al., 2007). COI is a component of the electron transport chain; changes in genetic structure at COI may result in altered energy production and thus also altered growth and reproductive output. In this study, it was determined that genetic subdivision exists between amphipod populations across the two river systems. In addition, significant differences in life-history traits were also identified amongst amphipod populations between the two river systems. These findings correlate genetic structure and organismal differences with the presence of elevated concentrations of contaminants.

2. Materials and methods

M. plumulosa were sampled in two localities from each of the Parramatta and Hawkesbury Rivers (Fig. 1). The contaminated sites were Duck River and Homebush Bay South along the Parramatta River, and the uncontaminated sites were Mooney Mooney and Half Moon Bend along the Hawkesbury River (Birch, 2000; Birch et al., 2007; McCready et al., 2006). Amphipods from the Parramatta River were sampled at low tide, in November 2006 and in May, August and November 2007. Two samples were taken from each of the localities on the Hawkesbury River. The Mooney Mooney site was sampled on an unknown date in 2003 (and maintained as a mixed population in culture) and in November 2007. The Half Moon Bend site was sampled in November 2006 and November 2007.

Amphipods were identified and sorted within 24 h of collection (Hyne et al., 2005). For genetic analyses individuals were preserved in ice-cold 100% ethanol (Dean and Ballard,

2001). For life-history-trait analyses 16 cultures from single females (isofemale cultures) were established in December 2006 with animals collected from Homebush Bay South, Duck River and Half Moon Bend, and animals from the mixed population culture originally collected from Mooney Mooney (Hyne et al., 2005). Construction of isofemale cultures reduces genetic variability and allows comparisons to be made both within and between localities and river systems.

2.1. Genetic studies

Immediately prior to extraction, animals were blotted onto filter paper until completely dry. Total DNA was extracted using the Puregene® Genomic DNA Purification Kit following the Isolation from Solid Tissue protocol (Gentra Systems Inc., Minneapolis, USA). Extracted DNA was resuspended in DNase- and RNase-free water and stored at −20 °C.

2.1.1. Mitochondrial DNA studies

A 700 bp sequence from the N-terminus of the mitochondrial (mt) DNA encoded COI gene was amplified by PCR using 10 pmol of each universal primer LCO1490 and HCO2198 (Folmer et al., 1994). PCR products were sequenced in both directions and sequences were edited and aligned using Sequencher 4.5 (Gene Codes, Ann Arbor USA). Synonymous and non-synonymous polymorphisms were calculated using DnaSP 4.0 (Rozas and Rozas, 1999).

Parsimony network analysis of the COI haplotypes was performed using TCS 1.21 (Clement et al., 2000). Parsimonious networks were generated by estimating the age of each haplotype based on their calculated frequencies within a sample of sequences. As sample sizes were small it is likely that some haplotypes were not collected by chance. As a consequence, intermediate sequence variants identified within the network analyses were assumed to be present within the sampled populations (none inferred a premature stop codon).

Nucleotide diversity (π) was calculated as the average number of nucleotide substitutions per site between all possible pairs of sequences, and was compared between river systems and between localities over time. The neutral parameter (θ) was calculated based on the total number of polymorphisms and the number of segregating sites. Two tests of neutrality determined whether sequenced populations deviated from a neutral equilibrium model: Tajima's D (Tajima, 1989) and Fu and Li's F^* (Fu and Li, 1993). Tajima's D is a comparison of the estimates of nucleotide diversity and the number of sites segregating for different nucleotides, where D is expected to be zero for a population at neutral equilibrium. Fu and Li's F^* compares the number of singleton mutations (mutations which occur in only one sequence of a sample of sequences).

2.1.2. Comparison of Mitochondrial and Nuclear DNA

Deviations from neutrality may reflect organismal processes or selection acting on a particular locus. In an attempt to distinguish between these alternatives, two populations were randomly selected for comparative analysis between the mtDNA encoded COI and nuclear encoded ITS1. Four individuals collected in December 2006 were sampled from Homebush Bay South (Parramatta River). Four individuals originally collected in 2003 from Mooney Mooney (Hawkesbury River) were randomly sampled.

COI sequences were amplified and sequenced as described above. ITS1 sequences were PCR amplified using iProof® High Fidelity Polymerase (Bio-Rad Laboratories, Hercules, USA) and the primers: ITS1F68 (5′-GGCACTTAGAGGAAGTAAAAG-3′), ITS1R29 (5′-GGTCTTCATAGCATCCACAG-3′). PCR amplicons were cloned into the vector pCR®-Blunt II (Invitrogen, Carlsbad, USA) and randomly selected colonies were sequenced using the M13 Forward (−20) primer (Invitrogen, Carlsbad, USA). The most common sequence variant identified among the clones sequenced from each individual was taken as the representative sequence of that individual. To determine the minimum number of clones to be sequenced to identify the most common sequence variant, a pilot study was conducted using an individual collected from Mooney Mooney. Based on the ratios of sequence variants within this pilot study, the probabilities at which the most common variant can be identified with respect to the number of clones sequenced were estimated using a standard binomial equation. This approach indicated that 13 clones were needed to identify the most common genetic variant with 95% accuracy and 7 clones were needed to identify the most common variant with 85% accuracy. In this study, 7 clones were sequenced from each PCR amplicon, but if the most common variant could not be resolved additional clones were sequenced until the most common variant was determined. This approach allows for direct comparisons of genetic structure between ITS1 and COI, but does not consider the amount of genetic variation at the level of the individual.

Parsimony network analysis of the COI haplotypes and the most common ITS1 sequence variant identified in each individual was performed using TCS 1.21 (Clement et al., 2000). Nucleotide diversity (π) and the neutral parameter (θ) were calculated and Tajima's D (Tajima, 1989) and Fu and Li's F^* (Fu and Li, 1993) determined.

2.2. Life-history trait analyses

The purpose of the life-history trait analyses was to explore possible links between toxicant exposure and responses at the organismal level. Life-history trait analyses were performed on animals from the 16 isofemale cultures examining female size based on head lengths, and fecundity based on number of embryos per female. Cultures were assayed in August 2007. As the average lifespan of cultured female amphipods are 8± 1 month (Hyne et al., 2005), Homebush Bay South, Duck River and Half Moon Bend cultures were likely to have contained F1, F2 and F3 animals from the field collected founding female. The founding female may or may not have been present.

Twenty randomly selected gravid females from each isofemale culture were digitally photographed under the microscope using the Leica DC 100 v2.41 digital imaging system (Leica Microsystems Imaging Solutions, Cambridge, UK). If fewer than 20 females were present, then all gravid females were imaged. To estimate the size of animals, the head-length of gravid females from each isofemale line was measured as the distance between the center of the eye and the start of the first thoracic segment (Mann and Hyne, 2008; Sheader and Chia, 1970) using

Table 1 – Estimated nucleotide polymorphisms within cytochrome c oxidase subunit I in *Melita plumulosa* collected from the Parramatta and Hawkesbury Rivers

River system	Locality	Collection date	Samples (haplotypes)	Nucleotide diversity		Neutrality tests	
				π (E-3)	θ (E-3)	Tajima's *D*	Fu and Li's *F**
Parramatta	HS	Dec 2006	14 (2)	3.51	2.51	1.38	1.45
		May 2007	10 (4)	4.32	4.51	−0.18	0.00
		Aug 2007	11 (3)	3.13	3.27	−0.16	1.07
		Nov 2007	10 (3)	4.00	3.95	0.06	0.34
	DR	Dec 2006	14 (1)	1.14	2.51	−1.89 *	−2.59 *
		May 2007	10 (2)	1.28	2.26	−1.67	−2.08
		Aug 2007	15 (3)	2.07	3.92	−1.74	−1.99
		Nov 2007	12 (3)	3.72	4.23	−0.48	−0.18
Hawkesbury	MM	2003	4 (1)	0	0	N/A	N/A
		Nov 2007	11 (1)	0	0	N/A	N/A
	HM	Nov 2006	4 (1)	0	0	N/A	N/A
		Nov 2007	10 (1)	0	0	N/A	N/A

Nucleotide diversity (π) and the neutral parameter (θ) were based on the number of segregating sites. Tajima's *D* and Fu and Li's *F** were employed to test whether observed mutation patterns are consistent with a neutral equilibrium model.
HS: Homebush Bay South; DR: Duck River; MM: Mooney Mooney; HM: Half Moon Bend.
N/A: could not be calculated.
* $P<0.05$.

the Leica QWin Standard v2.3 digital image analysis program (Leica Microsystems Imaging Solutions, Cambridge, UK). To estimate fecundity the number of embryos borne by each gravid female was counted.

To assess the differences in life-history traits of animals, nested analyses of variances (ANOVA) were performed using JMP 5.0 statistical software (SAS Institute, Cary, USA) with data from each locality nested within river system. The fecundity count data were logarithmically transformed to normalize the data (Sokal and Rohlf, 1995).

3. Results

3.1. Genetic studies

3.1.1. Mitochondrial DNA studies

Overall, there is no evidence for selection to be operating on COI from amphipods collected from the Parramatta River (Table 1). In contrast, there is no variation at all in amphipods from the Hawkesbury River. This result may reflect organismal processes or selection acting on the mtDNA itself. Sequence analysis of *M. plumulosa* revealed seven unique haplotypes and ten synonymous changes from the Parramatta River, while animals from the Hawkesbury River showed a complete absence of mitochondrial variation at COI (Table 1, Fig. 2). The values of π and θ from Homebush Bay South populations were constant while they increased for the Duck River populations (Table 1).

In the majority of samples both Tajima's *D* and Fu and Li's *F** could not reject a neutral model for each individual sample. However, amphipods collected from Duck River during December 2006 were found to deviate from a neutral model of mutation in both Tajima's *D* and Fu and Li's *F** tests (Table 1). This is more likely to reflect a population level affect rather than selection acting on the mtDNA. When the data are pooled, and the sample size increased, neither Tajima's *D* nor Fu and Li's *F** showed a significant departure from neutrality for Homebush Bay South ($D=0.19$, $F^*=0.13$, $P>0.05$ for both) or Duck River ($D=-1.03$, $F^*=-0.37$, $P>0.05$ for both).

3.1.2. Comparison of Mitochondrial and Nuclear DNA

In an attempt to determine whether the absence of mtDNA variation in animals from the Hawkesbury was reflective of organismal processes or selection acting on the mtDNA, the nuclear encoded ITS1 locus was sequenced from four individuals each from Homebush Bay South and from Mooney Mooney. The individuals from Homebush Bay South had two COI mtDNA haplotypes and two ITS1 sequence variants

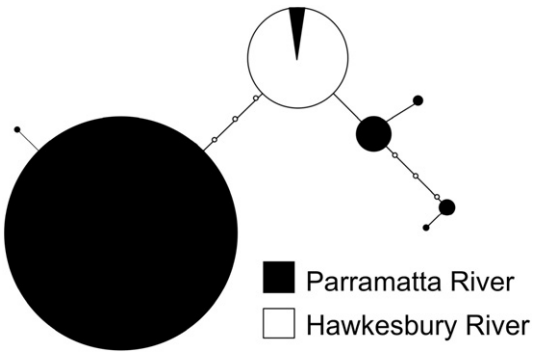

Fig. 2 – Haplotype network of cytochrome c oxidase subunit I for *Melita plumulosa* collected from the Parramatta River in November 2006 and May, August and November 2007 and the Hawkesbury River in November 2006 and November 2007. Each circle represents a unique haplotype connected by a line to those differing by one base pair, and size is proportional to frequency. Nodes on each line represent haplotypes not sampled in this study.

Table 2 – Estimated nucleotide polymorphisms within cytochrome c oxidase subunit I (COI) and the internal transcribed spacer region 1 (ITS1) in *Melita plumulosa* populations collected from the Parramatta River in December 2006 and the Hawkesbury River in 2003

River system	Locality	Sequence [a]	Samples (sequence variants)	Nucleotide diversity		Neutrality tests	
				π (E-3)	θ (E-3)	Tajima's *D*	Fu and Li's *F**
Parramatta	HS	COI	4 (2)	5.32	4.35	2.12	2.01
		ITS1	4 (2)	2.65	2.89	−0.71	−0.60
Hawkesbury	MM	COI	4 (1)	0	0	N/A	N/A
		ITS1	4 (3)	1.79	1.47	1.63	1.28

Nucleotide diversity (π) and the neutral parameter (θ) were based on the number of segregating sites. Tajima's *D* and Fu and Li's *F** were employed to test whether observed mutation patterns are consistent with a neutral equilibrium model.
HS: Homebush Bay South; MM: Mooney Mooney.
N/A: could not be calculated.
[a] COI: cytochrome c oxidase subunit I; ITS1: internal transcribed spacer region 1.

(Table 2). Network analyses implied six COI haplotypes and three ITS1 sequence variants existed (Fig. 3). The four individuals from Hawkesbury River had a single COI haplotype and six ITS1 sequence variants (Table 2). Network analyses implied that one COI and six ITS1 sequence variants existed. A Fisher's exact test comparing the network analyses results showed that the variation at COI from Homebush Bay South was significantly greater than that observed at Mooney Mooney, or the ITS1 variation was less at $P=0.1$ but not at $P=0.05$ (Fisher's Exact = 0.06). As there was a six-fold difference in the implied COI variation but only a two-fold difference in ITS1 we interpreted this trend to suggest that the variation is more likely to reflect selection acting on the mtDNA. An alternative explanation is that ITS1 variation was low in the individuals from Mooney Mooney because the culture had been maintained for four years and the samples were not independent.

Tajima's *D* and Fu and Li's *F** were positive for Mooney Mooney samples, while values of *D* and *F** were negative for Homebush Bay South samples. Neither test could reject a neutral model of mutation for Homebush Bay South or Mooney Mooney samples.

3.2. *Life-history trait analyses*

Analysis of the head lengths of gravid females showed that animals from the Hawkesbury River cultures were larger in size than females from the Parramatta River (Fig. 4). Notably, gravid females from Duck River were smaller than females from all other localities. ANOVA showed that there was a significant

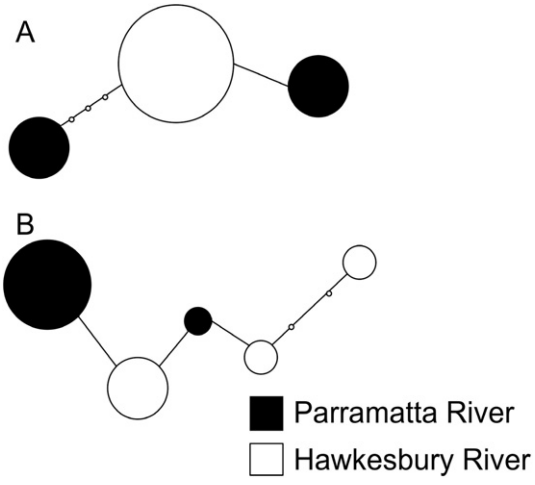

Fig. 3 – Parsimony network analysis of (A) cytochrome c oxidase subunit I and (B) the internal transcribed spacer region 1 for *Melita plumulosa* populations from Homebush Bay South (Parramatta River) and Mooney Mooney (Hawkesbury River). Each circle represents a unique sequence variant connected by a line to those differing by one base pair and size is proportional to frequency. Nodes on each line represent sequence variants not sampled in this study.

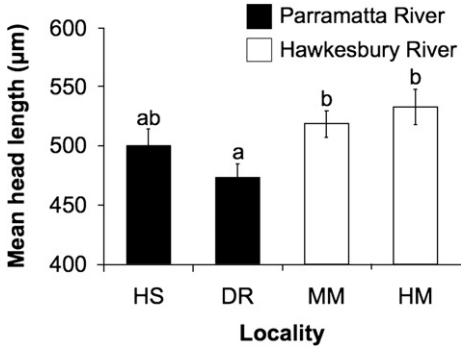

Fig. 4 – Mean head lengths (±standard error) of gravid female *Melita plumulosa* in isofemale cultures started from animals collected along the Parramatta and Hawkesbury Rivers. HS: Homebush Bay South; DR: Duck River; MM: Mooney Mooney; HM: Half Moon Bend. ANOVA showed that there was a significant difference between river systems ($F_{1,182}=8.81$, $P<0.01$) but not locality nested within river system ($F_{2,182}=1.41$, $P<0.25$). Letters above each bar indicate significant differences between localities nested within river system as determined by Tukey's HSD test; for each comparison $Q=2.59$, $\alpha=0.05$. Bars with the same letter do not differ significantly.

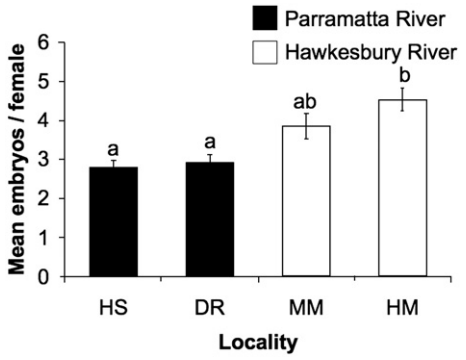

Fig. 5 – Mean fecundity (±standard error) of gravid female *Melita plumulosa* in isofemale cultures started from animals collected along the Parramatta and Hawkesbury Rivers. Fecundity was measured as the number of embryos counted per female. HS: Homebush Bay South; DR: Duck River; MM: Mooney Mooney; HM: Half Moon Bend. ANOVA showed that there was a significant difference between river systems ($F_{1,182}=23.30$, $P<0.01$) but not locality nested within river system ($F_{2,182}=2.77$, $P=0.07$). Letters above each bar indicate significant differences between localities nested within river system as determined by Tukey's HSD test; for each comparison Q=2.59, $\alpha=0.05$. Bars with the same letter do not differ significantly.

difference between river systems ($F_{1,182}=8.81$, $P<0.01$) but not locality nested within river system ($F_{2,182}=1.41$, $P=0.25$).

Analysis of the female fecundity showed that animals from the Hawkesbury River cultures were more fecund than those from the Parramatta River (Fig. 5). Specifically, females from both Duck River and Homebush Bay South showed low fecundity compared to females from Half Moon Bend. ANOVA showed that there was a significant difference between river systems ($F_{1,182}=23.30$, $P<0.01$) but not locality nested within river system ($F_{2,182}=2.77$, $P=0.07$).

4. Discussion

In the modern world, pollution is an increasingly significant problem. Over the past four decades, the long-term consequences of industrial activities on human health and environmental sustainability have gained increasing attention within both the scientific community and environmental regulatory agencies (Bickham et al., 2000). The impact of human activities on biodiversity and the genetic structure of natural populations is of particular concern. As the rapidly expanding human population places increasing pressure on the viability of ecosystems (Vitousek et al., 1997), the need to monitor genetic variability and the rate of loss of biodiversity and species has become a prominent issue.

It was hypothesized that the genetic structure of *M. plumulosa* exposed to industrial contaminants such as metals and/or organic pollutants (e.g. dioxin) would differ significantly from the genetic structure of populations in clean environments. Data presented here suggests that either there is a deficiency of variation at COI in animals collected from the Hawkesbury River or an elevated level of mtDNA variation in the amphipods from the Parramatta River. Levels of variation at ITS1 were similar between the two localities (Table 2). A deficiency of variation may occur if a particular genotype has a selective advantage in a specific environment causing it to increase to high frequency and out-compete all others. One common technique to test for selection is to employ laboratory perturbation cages (Ballard and James, 2004). Genetic hitchhiking may also result from maternally inherited symbionts such as *Wolbachia* (Turelli and Hoffmann, 1991). To investigate the potential for *Wolbachia* to influence the evolutionary dynamics of mtDNA in *M. plumulosa* we tested whether the amphipods were infected with the alpha-proteobacteria (James and Ballard, 2000). There was no evidence for *Wolbachia* infection in these amphipods. A plausible explanation for the greater variation in animals from Parramatta River is that toxicants caused DNA damage and may have, directly or indirectly, caused an increase in variation. Peles et al. (2003) found that the genetic structure of *Lumbricus rubellus* earthworms following chronic exposure to sublethal concentrations of metal toxicants was characterized by a significant increase in new alleles. De Wolf et al. (2004) found high levels of heterozygosity amongst populations of the periwinkle *Littorina littorea* exposed to a range of metal toxicants. Furthermore, Mulvey et al. (2002) reported strong genetic divergence among mummichogs found in polycyclic aromatic hydrocarbon-contaminated sites as compared to those from populations in uncontaminated areas.

It was also hypothesized that toxicant exposure would result in smaller and less fecund animals. This hypothesis was corroborated. To minimize the potential carry-over environmental effects isofemale cultures were created and life history traits of individuals from these cultures tested. However, the original founding female as well as F1, F2 and F3 animals were likely included in the studies so it is not possible to unequivocally determine whether the results are caused by previous exposure to toxicants or whether they reflect genetic differences (Hercus and Hoffman, 2000). Future studies should discard the founding females, F1, and F2 animals to determine whether underlying genetic differences exist. Schizas et al. (2001) reported that different mitochondrial lineages in marine copepods displayed different susceptibility to pesticide exposure. It has also been demonstrated that different strains of the daphnid *Daphnia magna* showed different levels of male neonate production following exposure to hormone mimicking pesticides (Oda et al., 2006).

5. Conclusion

This study has advanced our understanding of the amphipod in eastern Australia and has made an important contribution to our understanding of genetic and life-history trait variation of this amphipod. Individuals collected from polluted sites had higher levels of mtDNA variation and were significantly smaller and less fecund. These data have critical implications for toxicity tests and the use of laboratory cultures for testing purposes. Specifically, the use of genetically homogeneous cultures and animals in toxicity tests is more likely to produce consistent results as significant biases may result from

sampling individuals from sites where selection is operating (Morahan et al., 2007; Schizas et al., 2001).

In Australia, all toxicity tests that employ *M. plumulosa* have sourced animals derived from the Hawkesbury River. At this time, however, it is not clear whether this is an appropriate population because individuals collected from this waterway have low mtDNA variation. The distribution of *M. plumulosa* extends for at least several hundred kilometers south and north of this location and additional sampling is required to determine if this low variation is diagnostic of uncontaminated sites.

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