

Reaping the benefits of an anthropogenic contaminant: the evolution of copper tolerance in a marine invader

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Reaping the benefits of an anthropogenic contaminant: the evolution of copper tolerance in a marine invader.



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Thesis submitted in fulfilment of the requirements for the degree of Doctor of

Philosophy within the University of New South Wales

September 2010

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The global spread of fouling invasive species continues despite the use of antifouling biocides on vessel hulls. Furthermore, previous evidence suggests that non-indigenous species (NIS) introduced via hull fouling may have been selected for metal tolerance. Consequently, this process of selection may have then enhanced their introduction and establishment success in metal-polluted environments. This thesis examines the basis of copper tolerance in the non-indigenous bryozoan *Watersipora subtorquata*, and whether this successful NIS has evolved to benefit from a common contaminant in the marine environment.

Using a series of laboratory-based experiments I found that tolerance to copper in *W. subtorquata* is a heritable genetic trait that varies on an individual basis. While there was no difference in tolerance between sites within an estuary for larval *W. subtorquata*, there was a significant interaction between parental colony and copper, with large variation in the response of colony offspring within sites. Larval size differed significantly both between sites and between colonies and was positively correlated with tolerance. In adult *W. subtorquata* colonies there was individual variation in tolerance to a gradient of copper concentrations and in the ability to recover from exposure to copper. Growth during and after exposure appear to be separate traits, while a trade-off between growth in control and copper environments during exposure suggests a cost to tolerance. The genetic variation present within this species indicates that further evolutionary change is possible under strong directional selection.

The relationship between W. subtorquata and copper was further examined in field-based experiments. Copper appears to act as a settlement cue for *W. subtorquata* larvae by significantly increasing recruitment, although short term fitness costs were associated. Exposure to copper during initial settlement decreased recruit size and resulted in greater post-settlement mortality. In contrast, long term exposure to copper post-settlement significantly increased growth and reproductive fitness. The negative consequences of initial exposure during recruitment appear to be outweighed by the benefits of long term exposure which significantly increased overall fitness.

In summary I have found that tolerance to copper is a heritable trait in *W. subtorquata*. Moreover, in an entirely novel finding, it appears that *W. subtorquata* has evolved to benefit directly from copper pollution, as well as indirectly through reduced competition from less tolerant species.

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Abstract

The global spread of fouling invasive species continues despite the use of antifouling biocides on vessel hulls. Furthermore, previous evidence suggests that non-indigenous species (NIS) introduced via hull fouling may have been selected for metal tolerance. Consequently, this process of selection may have then enhanced their introduction and establishment success in metal-polluted environments. This thesis examines the basis of copper tolerance in the non-indigenous bryozoan *Watersipora subtorquata*, and whether this successful NIS has evolved to benefit from a common contaminant in the marine environment.

Using a series of laboratory-based experiments I found that tolerance to copper in *W*. *subtorquata* is a heritable genetic trait that varies on an individual basis. While there was no difference in tolerance between sites within an estuary for larval *W*. *subtorquata*, there was a significant interaction between parental colony and copper concentration, with large variation in the response of colony offspring within sites. Larval size differed significantly both between sites and between colonies and was positively correlated with tolerance. In adult *W*. *subtorquata* colonies there was individual variation in tolerance to a gradient of copper concentrations and in the ability to recover from exposure to copper. Growth during and after exposure appear to be separate traits, while a trade-off between growth in control and copper environments during exposure suggests a cost to tolerance. The genetic variation present within this species indicates that further evolutionary change is possible under strong directional selection.

The relationship between *W. subtorquata* and copper was further examined in fieldbased experiments. Copper appears to act as a settlement cue for *W. subtorquata* larvae by significantly increasing recruitment, although short term fitness costs were associated. Exposure to copper during initial settlement decreased recruit size and resulted in greater post-settlement mortality. In contrast, long term exposure to copper post-settlement significantly increased growth and reproductive fitness. The negative consequences of initial exposure during recruitment appear to be outweighed by the benefits of long term exposure which significantly increased overall fitness.

In summary I have found that tolerance to copper is a heritable trait in *W. subtorquata*. Moreover, in an entirely novel finding, it appears that *W. subtorquata* has evolved to benefit directly from copper pollution, as well as indirectly through reduced competition from less tolerant species.

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Chapter 1 General Introduction

Invasive species are considered one of the greatest threats to native communities (Carlton & Geller 1993; Vitousek et al. 1997). From an ecological perspective, the impact of non-indigenous species (NIS) on local communities can range from reduced biodiversity (Wilcove et al. 1998) to dramatic habitat modification by ecosystem engineers (Crooks 2002). Consequently, it is important to understand what makes a species a successful invader. This requires a mixed disciplinary approach that looks at both the biology of the invader and the process of invasion, as the process can have evolutionary implications for the species (Henery et al. 2010). For example the initial mechanism of species entrainment and transfer is of considerable importance (Hulme et al. 2008) as this can select for species traits that influence invader success in the new environment (Suarez & Tsutsui 2008).

Specific physiological traits may mediate a species' ability to successfully invade a new environment (i.e. its 'invasiveness' Sakai et al. (2001)) and although multiple traits have been put forward, a definitive set of attributes is yet to be outlined as consistently present across taxa (Hayes & Barry 2008). One trait that has recently been affiliated with a number of NIS is an increased tolerance to metal pollution, particularly in the marine environment (Dafforn et al. 2009; Piola & Johnston 2009; Liu & Pang 2010). While invasive species have provided the opportunity to study evolution (Sakai et al. 2001; Huey et al. 2005; Lee & Gelembiuk 2008), there has been little research on the evolutionary response of marine or aquatic invasives to the novel stress of heavy metal pollution. Therefore it is important to understand the basis of metal tolerance, i.e. whether it is a genetically based heritable trait that can respond to further selection (Pease et al. 2010). Particularly, as this trait is likely to be pertinent at multiple stages of

the invasion process (entrainment, transport, establishment and spread, Piola et al. (2009)).

Metal pollution

Marine and estuarine environments in particular can be vulnerable to metal pollution (Hall et al. 1998) that enters the system through numerous sources including the use of antifouling biocides (Weis & Weis 1996; Srinivasan & Swain 2007), sewage discharge and industrial effluent (Apte & Day 1998; Hall et al. 1998). Concentrations of metals typically reach their highest in areas of urban development such as harbours, ports and marinas (Hall & Anderson 1999; Matthiessen et al. 1999; Dafforn et al. 2009). Copper is one of the more prevalent metals in these environments and is particularly toxic to marine invertebrates (Hall et al. 1998). Depending on the proximity and source of contamination dissolved copper can reach concentrations of 50 μ g L⁻¹ in marine environments, particularly near surfaces coated in copper-based antifouling paints (Valkirs et al. 2003; Schiff et al. 2004).

The impacts of metal pollution can be dramatic, the most obvious being a significant reduction in species richness (Pollard & Yuan 2006; Johnston & Roberts 2009). Yet tolerance to metal pollution has evolved rapidly in numerous species in marine, freshwater and terrestrial habitats (Macnair 1987; Medina et al. 2007). The occurrence of site-specific heavy metal pollution can expose populations to intense selection for multiple generations resulting in population-specific resistance (Klerks & Weis 1987), to metals such as copper (Piola & Johnston 2006b) and cadmium (Klerks & Levinton 1989; Hendrickx et al. 2008). The marine habitats most affected by metal pollution are also the most vulnerable to species introductions, as they are focal points for recreational and commercial shipping activities (Floerl et al. 2009). And while metal

1. Introduction

pollution is negatively impacting native communities (Pollard & Yuan 2006; Johnston & Roberts 2009), an increased prevalence of NIS has been found in these environments (Piola & Johnston 2008a; Dafforn et al. 2009).

History of selection in marine NIS

The ability of multiple NIS to tolerate copper pollution may be a consequence of selection during the invasion process, particularly the mode of transport. A predominant vector for many invasive species in the marine environment is shipping (Carlton 1996; Ruiz et al. 2000), with organisms transported via hull fouling and ballast water. The use of antifouling paints deters most species from settling and growing on vessel hulls, hence reducing NIS transfer, but some species are tolerant of these paints and are transported more often (Piola et al. 2009). Historically copper has been the most commonly used biocide within antifoulant paints and it is still widely used today (I.M.O. 2001; Piola et al. 2009). For many marine NIS an introduction via hull fouling has created an interesting exposure regime where copper has the potential to act as a selective pressure at multiple stages of the invasion process (Sakai et al. 2001). Firstly a species must either adapt or be pre-adapted to copper polluted conditions prior to transportation (Henery et al. 2010), which may potentially be highly toxic (Valkirs et al. 2003), then endure and survive the journey. This is followed by introduction and establishment into a new environment which may be similarly impacted by metal pollution, such as many harbours, ports and marinas (Birch & Taylor 1999; Matthiessen et al. 1999; Dafforn et al. 2009).

Thesis Aims

The prevalence of NIS in metal polluted areas has been attributed to greater tolerance to copper, supported by evidence from laboratory and field studies (Piola & Johnston

2006b; Piola & Johnston 2006a; Dafforn et al. 2008; Piola & Johnston 2008a; Dafforn et al. 2009; Piola & Johnston 2009). Greater tolerance to pollution may enable opportunistic utilisation of resources made available through the loss of less tolerant native competitors (Rygg 1985; Johnston & Keough 2002). An alternative, more radical hypothesis, posits that organisms directly benefit in some manner from contaminant exposure. This hypothesis is generally considered unlikely for highly toxic contaminants such as metals (DeAngelis 1996), however, some successful NIS may have evolved to benefit from anthropogenic contaminants. The fundamental question of whether this tolerance is a heritable genetic trait or acclimation to polluted conditions has not been answered. By understanding this relationship that has evolved between non-indigenous fouling organisms and copper, we can discern the reasons for their success.

In this thesis I address the questions underlying tolerance to copper in a marine NIS using the invasive bryozoan *Watersipora subtorquata* (d'Orbigny 1852). This was achieved using evolutionary and toxicological methodologies combined with field- and laboratory-based ecological experiments. Novel methods were developed from ecotoxiological techniques to investigate the evolutionary basis of copper tolerance, in an attempt to ascertain the ecological benefits that tolerance conveys. An emphasis of this project was to incorporate evolutionary concepts into current ecological issues, namely through the use of quantitative genetics, to achieve a greater understanding of the changes we are seeing taking place in the marine environment.

Thesis Outline

Chapter 2 compares copper tolerance in *W. subtorquata* larvae between individuals and populations, and relates tolerance to larval size. Using a quantitative approach, tolerance

was found to be a heritable trait. Chapter 3 investigated the genetic basis for tolerance to a gradient of copper concentrations in adult *W. subtorquata* colonies. This involved a novel technique of cloning individual colonies to test for a genotype by environment interaction. Chapter 4 discerned the recruitment patterns of *W. subtorquata* larvae in the field into clean and copper polluted conditions. This chapter gives the first indications of a strong positive relationship between the contaminant and the larvae of a NIS. Chapter 5 determined the short and long term fitness consequences of choosing to recruit into a copper polluted habitat. This used a field based experiment to build on the recruitment patterns observed in the previous chapter.

Chapter 2-5 of this thesis have been prepared in the form of stand-alone manuscripts for publication in peer-review journals. As such, there may be some repetition between chapters. Chapter 2 is currently in review.

Chapter 2 Variable heavy metal tolerance in an introduced sessile invertebrate

Abstract

The global spread of fouling invasive species is continuing despite the use of antifouling biocides. Furthermore, previous evidence suggests that non-indigenous species (NIS) introduced via hull fouling may be capable of adapting to metal-polluted environments. Using a laboratory based toxicity assay, we investigated tolerance to copper in the nonindigenous bryozoan Watersipora subtorquata from four source populations. Individual colonies were collected from four sites within Port Hacking, (Sydney, Australia) and their offspring exposed to a range of copper concentrations. This approach, using a fullsib, split family design, tests for a genotype by environment (G X E) interaction. Settlement and complete metamorphosis (recruitment) were measured as ecologically relevant endpoints. Larval sizes were also measured for each colony. Successful recruitment was significantly reduced by the highest copper concentration of 80 μ g L⁻¹. While there was no difference in pollution tolerance between sites, there was a significant G X E interaction, with large variation in the response of colony offspring within sites. Larval size differed significantly both between sites and between colonies and was positively correlated with tolerance. The high level of variation in copper tolerance between colonies suggests that there is considerable potential within populations to adapt to elevated copper levels, as tolerance is a heritable trait. Also, colonies that produce large larvae are more tolerant to copper, suggesting that tolerance may be a direct consequence of larger size or a pleiotropic consequence or duplication of genes that lead to larger larvae.

Introduction

Pollution and the global spread of invasive species are both major causes of biodiversity loss and environmental change (Carlton & Geller 1993; Cohen & Carlton 1998), with substantial ecological implications. The marine environments most susceptible to introduced species are harbours and estuaries where many invasives are introduced by the shipping trade (Floerl et al. 2004; 2009). Harbours and estuaries are also subject to high levels of anthropogenic disturbance, particularly in the form of heavy metal pollution which enters the system through numerous sources including industrial waste (Apte & Day 1998; Hall et al. 1998) and antifouling biocides (Weis & Weis 1996). Copper, which is particularly toxic to marine invertebrates, is one of the more prevalent heavy metals in these environments (Hall et al. 1998). An essential element for metabolism in many marine organisms, at higher concentrations copper often becomes lethal (Bryan 1971). Whilst metal contamination reduces diversity in all marine habitats (Johnston & Roberts 2009) there is evidence that invasive species diversity is not affected in hard substrate communities (Piola & Johnston 2008a). It has been hypothesised that a number of fouling non-indigenous species (NIS) have some form of metal tolerance (Piola & Johnston 2006a; Dafforn et al. 2009).

While invasive species have provided the opportunity to study evolution (Sakai et al. 2001; Huey et al. 2005; Lee & Gelembiuk 2008), there has been little research on the evolutionary response of marine or aquatic invasives to heavy metal pollution. If invasive species have the capacity to rapidly adapt to a commonly and increasingly used biocide like copper (I.M.O. 2001; Piola et al. 2009) then even greater concerns are raised regarding the impact that invasives have on the ecology of native assemblages. The efficacy of antifoulant paints may also be reduced if particular species or

populations acquire tolerance to the active biocides, resulting in greater economic and ecological costs.

Tolerance to anthropogenic contaminants has evolved rapidly in numerous species, most notably as pesticide and herbicide resistance in agriculture (Scarabel et al. 2007). The occurrence of site-specific heavy metal pollution in terrestrial, aquatic and marine habitats can expose populations to intense selection for multiple generations (Macnair 1987; Medina et al. 2007), resulting in population-specific resistance (Klerks & Weis 1987) such as cadmium resistance in exposed populations of the wolf spider *Pirata piraticus* (Hendrickx et al. 2008) and aquatic oligochaete *Limnodrilus hoffmeisteri* (Klerks & Levinton 1989).

The increasing occurrence of heavy metal pollution in marine environments (Hall et al. 1998) and the use of antifouling biocides on the hulls of ships which are a major vector for introduced marine species (Piola et al. 2009), means heavy metals can act as a selective pressure during the process of species introductions (Floerl et al. 2005; Piola et al. 2009). In fact a range of marine NIS have been identified as having a positive affiliation with heavy metal pollution (Dafforn et al. 2008). These include a variety of bryozoans, ascidians, algae and crustaceans (Dafforn et al. 2008, 2009). This affiliation may be due to several mechanisms, firstly the capacity for adaptation, a previous history of selection or most likely a combination of both. The inherent ability to endure and adapt to a broad or fluctuating range of environmental conditions is a notable trait in some successful NIS (Sakai et al. 2001; Lee & Gelembiuk 2008). The combination of adaptive capacity and a history of exposure may select for greater plasticity in novel environments translating to tolerance of heavy metal pollution as a novel stressor (Agosta & Klemens 2008; Lee & Gelembiuk 2008). This may partially explain the

propensity for tolerance in introduced species while many indigenous species may lack the capacity to respond (Macnair 1987; Byers 2002).

Phenotypic plasticity is increasingly recognised as an important component influencing contemporary evolutionary responses to anthropogenic change (Carroll et al. 2007; Crispo 2008), particularly in the form of parental effects which can impact upon developing phenotypes through manipulation of reproductive traits in response to environmental conditions (Räsänen & Kruuk 2007; Badyaev & Uller 2009). Plasticity in reproductive traits such as fecundity and offspring size is commonly seen in range of taxa (for example birds: (Reed et al. 2009), plants: (Galloway et al. 2009) and insects (Fox et al. 1999)) and is increasingly being used as a proxy for maternal and offspring fitness (Marshall & Uller 2007), particularly for species in which other fitness traits may be difficult to quantify. With variation in offspring size documented both within and between individuals as well as populations (Marshall & Keough 2008) this variation is attributable to an interaction between parental genotype and environmental conditions (Bernardo 1996). For example, offspring size in invertebrates has been shown to increase when the mother is under stress, such as competition (Allen et al. 2008), pollutants (Marshall 2008) and food availability (Newlon et al. 2003), as a result of maternal provisioning.

In species that do not have a discrete reproductive output period, fertility measurements can be difficult. In these systems offspring size and tolerance are measurable traits that can be directly linked to both maternal and offspring fitness (Marshall & Uller 2007). The encrusting bryozoan *Watersipora subtorquata* (d'Orbigny 1852) is a trickle spawner, where larvae are released over time, with each colony containing larvae that are at different stages of maturation (personal observation) therefore making overall or

lifetime fecundity measures for an individual colony difficult. *W. subtorquata* is relatively insensitive to copper (Piola & Johnston 2006a; Piola & Johnston 2009) and has been described as a 'foundation species' due to its ability to recruit to surfaces coated in antifoulant paint where it creates a less toxic secondary surface for other fouling organisms to settle on (Floerl et al. 2004). It can also form large 3D structures which provide habitat for epibiota (Floerl et al. 2004; Stachowicz & Byrnes 2006) and can dominate hard substrate with high densities (Sellheim et al. 2010). This species has a cosmopolitan distribution; its native range is uncertain but thought to be in the Caribbean (Mackie et al. 2006). The similarities between *W. subtorquata* and many fouling marine NIS, such as method of introduction, comparable physiological traits to other colonial organisms (such as brooded offspring and vegetative growth in ascidians) and a global distribution (Mackie et al. 2006) make this species an appropriate test organism for examining heavy metal tolerance within and between introduced populations.

Tolerance to contaminants has generally been assessed at a species or population level (Johnston In press), including for *W. subtorquata* (Piola & Johnston 2006a), and rarely at the individual level. A novel way to assess individual tolerance is to test offspring, as full or half siblings, independently in multiple environments in a genotype by environment (G X E) design (Falconer & Mackay 1996). This enables us to test whether there is a genetic basis to tolerance and estimate the heritability of the trait (Galletly et al. 2007; Pease et al. 2010). Detailed studies, such as this one, are important because in order for selection to cause evolutionary change there must be heritable variation among individuals. This study aimed to determine whether tolerance to copper is consistent within and between populations of the non-indigenous species *W. subtorquata*, and whether tolerance is correlated with offspring size.

Methods and Materials

Sample collection and site descriptions

Colonies of *W. subtorquata* were collected from four sites within Port Hacking estuary (34°7'S, 151°10'E) south of Sydney in New South Wales, Australia (Fig. 2.1); Burraneer Marina, Dolans Marina, Private pontoon and Cronulla swimming enclosure. Port Hacking estuary is a recreational estuary with no commercial shipping activity, but is still impacted by anthropogenic pollution. Copper accumulation in



Figure 2.1 Sampling locations within Port Hacking estuary. Mature *Watersipora subtorquata* colonies were collected at the four sites: 1. Burraneer Marina; 2. Dolans Marina; 3. Private pontoon; 4. Cronulla swimming enclosure.

experimentally deployed oysters have previously found copper levels within this section of the estuary to be elevated approximately three times beyond natural oceanic levels due to urbanisation (Dafforn et al. 2009). Sites were separated by a minimum of 0.6 km. Colonies at all sites were collected from artificial structures such as floating pontoons, fixed pilings and netting. *W. subtorquata* colonies are most fecund during the summer months, but can still produce larvae throughout the year. They brood their larvae for two weeks before spawning lecithotrophic larvae that are competent to settle immediately (Marshall & Keough 2008).

Colonies were collected during February and March 2008 and were maintained in individual containers at 20°C for up to 3 d without light except during spawning. Spawning was induced after 1-3 d of collection by exposing the colonies to light and stopping aeration for approximately 1 h, stimulating the release of larvae. Larvae were collected using a pipette and preserved for larval size measurements or used for the copper toxicity assay. This was repeated for two consecutive days if insufficient numbers of larvae were produced on the first attempt, as *W. subtorquata* is a trickle spawner releasing larvae slowly over successive days. Colonies were maintained and spawned in individual containers to ensure maternal parentage was known.

Copper Tolerance

Copper tolerances of the larvae of individual *W. subtorquata* colonies were tested by measuring larval settlement and metamorphosis after a 3 d exposure to copper concentrations of 0, 40 or 80 μ g L⁻¹. These concentrations represent relevant values in polluted aquatic environments, particularly near surfaces painted with antifoulant biocides (Valkirs et al. 2003; Schiff et al. 2004). Copper solutions were prepared by adding copper II sulphate anhydrous to Milli-Q water to create a stock solution of 1 g L⁻¹ Cu in freshwater. This was then diluted in filtered (0.2 μ m) and autoclaved seawater to the two experimental concentrations of 40 and 80 μ g L⁻¹ Cu. Filtered and autoclaved seawater was used as the control treatment (0 μ g L⁻¹ Cu). All equipment was acid washed in 5% nitric acid for a minimum of 24 h then thrice rinsed in Milli-Q water prior

to use. The plastic 35 mm diameter Petri dishes used in the assay were pre-soaked for 24 h in the appropriate copper solution prior to commencing the experiment.

Whilst 20 colonies were collected from each site and spawned as previously described, only five colonies from each site produced enough larvae for the copper assay. A full-sib, split family design (Becker 1984) was used to detect variation among colonies, with colony as family/genotype and copper concentration as environment (Genotype X Environment interaction). From each of these colonies an individual larva was placed in a plastic Petri dish containing one of the three copper concentrations; 0, 40 and 80 μ g L⁻¹ (control, low and high). There were six replicate larvae per treatment. Exposure to treatments was maintained for 3 d, with the water within the Petri dish changed on day two. Survival to settlement and metamorphosis was assessed on day 3 and was defined as a fully metamorphosed zooid, complete with orifice (Piola & Johnston 2006a), and is hereafter referred to as successful settlement.

Larval size

To compare offspring sizes between colonies an independent subset of larvae from each of these colonies was also preserved in 10% formaldehyde with seawater. Once preserved, larvae were then photographed with a digital camera (PixeLINK) through a dissecting microscope (Olympus). Larvae were photographed with the oral pole (Wisely 1958) oriented upwards to maintain consistency of measurement. The total area was estimated to the nearest 0.01 mm² using the program Scion Image. Replicate numbers differed between colonies, ranging between four and eight for each colony. Two colonies (one each from Burraneer and Cronulla swimming enclosure) only produced enough larvae for the copper assay and are without larval size data.

Statistical Analyses

Copper assay

Using settlement success as a proxy, tolerance to copper was compared between sites and colonies. As settlement success was binomial, a partly nested three-factor, mixed model PERMANOVA (Anderson 2001) was used, with Euclidean distance to calculate the similarity matrix (factors: Site and Colony(Site) random, Copper: fixed) and was run for 9999 permutations. PERMANOVA is a distance based permutational analysis of variance, which calculates a pseudo F-statistic for univariate data when analysed using Euclidean distance (Anderson 2001). Because there was no significant effect of site or interaction between site and copper treatment (P > 0.25) the final model does not contain these terms. Broad sense heritability H^2 , was calculated for successful settlement for each copper concentration using the formula for a balanced full-sibling design (Becker 1984). Variance components were obtained from one-way ANOVA's performed for each copper concentration, using PERMANOVA (Anderson 2001).

Larval sizes

Larval size was compared between site and colony using a two-factor nested analysis of variance (site and colony were both random factors, with colony nested in site). The number of fecund individuals differed between the sites, resulting in an uneven number of colonies included in the analysis, as two colonies only produced enough larvae for the copper assay. Data were analysed using a two-factor nested PERMANOVA (Anderson 2001) with Euclidean distance to calculate the similarity matrix, and then run for 9999 permutations using the same design as above. Post-hoc pair-wise comparisons were then performed using PERMANOVA to compare between sites. Significance was set at $\alpha \leq 0.05$.

Settlement success at 80 μ g L⁻¹ Cu was compared to average larval size within each colony to determine if there was any correlation between size and tolerance using a randomisation test (Monte Carlo) for significance. Only the high copper treatment was compared as it was the most lethal, significantly reducing settlement making it statistically comparable, as well as being a more extreme indicator of a colony's tolerance. Sites were pooled as tolerance was found not to differ between them.

Results

Copper assay

Copper significantly reduced successful settlement and increased mortality ($F_{2,32}$ = 105.18, P < 0.001). The average settlement within a colony ranged from 93 ± 2% and 82 ± 5% in the control and low copper treatments respectively, to 18 ± 4% in high copper (mean across sites ± SE) (Fig. 2.2). There was a significant G X E interaction, with colony interacting significantly with copper treatment ($F_{32,307}$ = 1.60, P = 0.028). Settlement success differed between colonies in both the low and high copper treatments (Fig. 2.3a-d), ranging from 33 - 100% and 0 - 50% respectively. High settlement success in 40 µg L⁻¹ Cu did not predict settlement success in 80 µg L⁻¹ Cu, with no linear response between the copper treatments. There was no difference between sites nor an interaction between site and copper treatment as P > 0.25. Broad sense heritability for successful settlement was highest in the low copper treatment ($H^2 = 0.38$) but reduced dramatically in the high copper ($H^2 = 0.05$) and control treatments ($H^2 \sim 0$).



Figure 2.2 Copper assay: effect of 0, 40 and 80 μ g L⁻¹ Cu in seawater on successful settlement and metamorphosis of *Watersipora subtorquata* larvae at each site. Values are mean settlement \pm SE for colony at each site (n = 5 colonies). Letters represent significant differences between sites in post-hoc pair-wise comparisons ($\alpha = 0.05$).



Figure 2.3 Individual colony tolerance and larval size. Total successful settlement per colony at the four sites for each copper concentration (0, 40 and 80 μ g L⁻¹ Cu) at (a) Burraneer Marina, (b) Dolans Marina, (c) Private pontoon and (d) Cronulla swimming enclosure (n = 6 larvae), 0 represents no settlement, (e) mean larval size per colony (± SE) at each site (n = 4 - 8 larvae) and (f) the correlation plot between mean larval size and settlement at 80 μ g L⁻¹ Cu for each *Watersipora subtorquata* colony. *Italicised letters* represent significant differences between sites in post hoc pair-wise comparisons ($\alpha = 0.05$).

Larval sizes

Larvae varied widely in size between sites within the estuary (from 0.034 to 0.101 mm²) and within individual colonies, with the greatest maximum difference between siblings being 0.066 mm². Average larval size for each colony covered a similar range from 0.054 to 0.084 mm², with colonies differing significantly ($F_{14,113} = 3.211$, P < 0.001) (Fig. 2.3e). Sites were significantly different ($F_{3,14} = 5.159 P = 0.016$), with one site in particular driving the difference. Colonies from Private pontoon had significantly larger larvae than colonies from Burraneer Marina and Dolans Marina (Fig. 2.3e). The comparison of average larval size to successful settlement in 80 µg L⁻¹ Cu found colonies which produced larger larvae were more tolerant of high copper levels (R = 0.448, P = 0.036) (Fig. 2.3f).

Discussion

The increasing prevalence of non-indigenous sessile invertebrates in metal polluted estuaries, whilst many native species are declining (Piola & Johnston 2008a; Piola et al. 2009) suggests some form of heavy metal tolerance. This study aimed to discern whether a NIS, the bryozoan *W. subtorquata*, varies in heavy metal tolerance and offspring size production within and between populations, and whether there is any relationship between tolerance and offspring size. We found that both offspring size and tolerance varied greatly between individuals, with colonies which produced larger offspring correlating with greater tolerance to high levels of copper. The significant genotype by environment interaction between colony and copper implies a genetic basis to tolerance, which may be heritable. Our results found similar effects of copper on *W. subtorquata* offspring to previous studies where high levels of copper (\geq 50 µg L⁻¹) significantly reduced survival (Piola & Johnston 2006a) and successful metamorphosis

(Ng & Keough 2003). These findings also further demonstrate *W. subtorquata's* greater tolerance to copper compared to native species (Dafforn et al. 2009; Piola & Johnston 2009) and the potential for further selection for this trait in polluted environments.

Tolerance differed on an individual basis, with each colony interacting differently with the various levels of copper. Interestingly high settlement in clean and low copper conditions was not indicative of tolerance to high copper treatments, as there were no strong patterns of settlement. Differential tolerance between populations has been found in other species, but these experiments have compared clean and heavily polluted sites (Durou et al. 2005; Piola & Johnston 2006b; Johnston In press) where selection pressures are polarised. Tolerance at multiple contamination levels within a half sibling design has not been examined (although see Marshall (2007)), instead focus has been on an overall population response (Daka & Hawkins 2004).

A number of mechanisms have been identified for handling heavy metal pollution; such as decreased uptake (Gale et al. 2003) and increased detoxification (Rainbow et al. 2009) which may incorporate the non-enzymatic proteins metallothioneins (Amiard et al. 2006). The exact processes utilised by bryozoans have not been explored but many invertebrates employ at least one tolerance mechanism, which can even differ between populations of the same species (Mouneyrac et al. 2003; Daka & Hawkins 2004). The lack of consistency that we observed between colonies in their tolerance across copper levels not only precludes predicting overall tolerance but also suggests that there may be several mechanisms involved (Galletly et al. 2007). These may not be consistent between individuals considering that various pathways have been discerned for multiple populations of the same species (Daka & Hawkins 2004). It is possible that these nonindigenous populations have been founded by individuals from many source areas that
have experienced distinct exposure and selection regimes, resulting in several mechanisms for resistance.

One potential mechanism for tolerance could be the use of matrotrophy. Numerous factors have been found to influence offspring size through maternal provisioning, such as maternal size, resource availability (Lampert 1993), competition (Marshall & Keough 2009) and predation (Tollrian 1995). Offspring size is a strong predictor of settlement and post-settlement success in many marine organisms (Bernardo 1996; Marshall & Keough 2007) including W. subtorquata (Marshall & Keough 2004), with variability in larval sizes anticipated due to the interaction between parental genotype and environmental conditions (Bernardo 1996). Bryozoans in particular have evolved to utilise reproductive plasticity to deal with variable environmental conditions, such as competition (Allen et al. 2008; Marshall & Keough 2008; Ostrovsky et al. 2009), therefore it is likely that maternal provisioning has become a strategy for dealing with novel anthropogenic disturbances. In another bryozoan species, *Bugula neritia*, colonies exposed to toxicants responded by producing larger, more tolerant offspring (Marshall 2008), and continued selection in contaminated environments may be strengthening the relationship between pollutants and maternal response. Another way to deal with unpredictable environmental conditions is to produce an array of offspring sizes within a brood; bet-hedging (Crean & Marshall 2009). Considering the variability that was present within some broods, it is possible that bet-hedging is a strategic way to deal with fluctuating heavy metal pollution levels. As larger lecithotrophic larvae are capable of longer swimming times, faster growth and quicker reproductive maturation rates post settlement due to greater energy reserves (Marshall & Keough 2003; 2007), they would be at an advantage in a stressful, polluted environment.

Although colonies that produce larger larvae appear to be more tolerant to higher levels of contamination, continual production of large offspring is costly, reducing overall fecundity (Smith & Fretwell 1974), and unnecessary if pollution levels are inconsistent. Although matrotrophy no doubt evolved to deal with other environmental and biological stressors, reproductive plasticity has potentially contributed to the success of this species in novel and anthropogenically polluted environments (Räsänen & Kruuk 2007; Whitney & Gabler 2008). Considering the proposed vector for the translocation of *W*. *subtorquata*, via hull fouling (Floerl & Inglis 2005; Mackie et al. 2006), it is possible that this species has been under selection not only for an overall increased heavy metal tolerance (Floerl et al. 2004) compared to most native species, but also 'broad organismal plasticity' (Lee & Gelembiuk 2008). Inconsistent and fluctuating levels of heavy metal pollution expose populations within a generation to a heterogeneous selection regime (Morgan et al. 2007; Lee & Gelembiuk 2008), resulting in selection for individuals that can utilise this phenotypic plasticity as a mechanism for tolerance.

Conclusion

Ultimately, the within-population variation suggests that heavy metal tolerance is a heritable trait under selection which can rapidly evolve in novel environments (Reznick & Ghalambor 2001), such as a boat hull coated in copper based antifouling paint. Whilst tolerance is not consistent in every individual, plasticity to deal with fluctuating environmental conditions is a trait that appears to be perpetually selected for (Morgan et al. 2007) and it is likely that manipulation of maternal provisioning (Crean & Marshall 2009; Ostrovsky et al. 2009) may be ameliorating the effect of heavy metal pollution. With heavy metal pollution an increasing disturbance on a global scale (Birch et al.

2008) combined with the ever increasing spread of NIS (Vitousek et al. 1997) we can expect to see a greater prevalence of tolerant populations of invasive species.

Chapter 3 Copper and clones: investigating metal tolerance in an invasive marine species using a novel approach

Abstract

The global spread of fouling invasive species continues despite the use of antifouling biocides. Furthermore, previous evidence suggests that non-indigenous species (NIS) introduced via hull fouling may have been selected for metal tolerance. Consequently, this process of selection may have then enhanced their introduction and establishment success in metal-polluted environments. This study aimed to investigate individual variation in tolerance to and ability to recover from exposure to copper in an invasive marine bryozoan, Watersipora subtorquata. Using a novel assay technique we cloned colonies of this organism into multiple fragments to independently test multiple environments in a genotype by environment design. Genotypes were exposed to a gradient of copper concentrations; control, 25, 50, 75, 100 and 125 μ g L⁻¹, and growth measured during exposure and after a recovery period. There was a significant genotype X environment interaction in growth during exposure and recovery. Overall W. *subtorquata* was found to be capable of tolerating periodic exposure to copper concentrations of up to 50 ug L^{-1} . For higher concentrations fewer genotypes were able to maintain or increase growth either during or after exposure. We found individual variation in tolerance to a gradient of copper concentrations and in the ability to recover from exposure to copper. Growth during and after exposure appear to be separate traits, whilst a trade-off between growth in control and copper environments during exposure suggests a cost to tolerance. Overall the genetic variation present within this population indicates that further evolutionary change is possible under strong directional selection.

Introduction

What makes a species a successful invader is a fundamental and reoccurring question that requires investigation from both an ecological and evolutionary angle (Facon et al. 2006). It is also a question of great practical relevance given that many of the impacts of non-indigenous species (NIS) on native communities are detrimental. These impacts range from reducing biodiversity to dramatic habitat modification by ecosystem engineers (Wilcove et al. 1998; Crooks 2002). Hence there is a great need to understand which physiological traits mediate a species' ability to successfully invade a new environment (i.e. its 'invasiveness' Sakai et al. (2001)). Multiple traits have been proposed, although a definitive set of attributes is yet to be outlined as consistently present across taxa (Hayes & Barry 2008). One trait that has recently been associated with a number of invasive species is an increased tolerance to metal pollution (Zhang et al. 2008; Dafforn et al. 2009; Liu & Pang 2010). This trait is likely to be pertinent at multiple stages of the invasion process (entrainment, transport, establishment and spread, Piola et al 2009). Therefore it is important to understand the basis of metal tolerance, i.e. whether it is a genetically based heritable trait that can respond to further selection (Chapter 2; Pease et al. 2010).

Metal pollution is a result of many anthropogenic activities ranging from industrial and mining waste (Apte & Day 1998; Gale et al. 2003) to agriculture (Keskin 2010). The marine environment is particularly vulnerable, receiving additional input from sewage discharge, urban run-off and antifouling biocides (Scanes 1996; Fabris et al. 1999; Valkirs et al. 2003). The impacts of metal pollution can be dramatic, the most obvious being a significant reduction in species richness (Pollard & Yuan 2006; Johnston & Roberts 2009). In contrast, tolerance to metal pollution may develop rapidly in some

species (Macnair 1987; Medina et al. 2007), usually as a result of site specific pollution exposing a population to intense selection for multiple generations (e.g. mountain birch (Eranen 2008), wolf spider (Hendrickx et al. 2008) and aquatic oligochaete (Klerks & Levinton 1989)). Recently, tolerance to copper pollution has been associated with a number of invasive species , most predominantly in the marine environment (Dafforn et al. 2009). A greater prevalence of NIS has been observed in habitats impacted by metal pollution (Piola & Johnston 2008a; Dafforn et al. 2009; Crooks et al. 2010), furthermore comparisons between analogous indigenous and NIS have identified greater tolerance in the latter (Piola & Johnston 2009). This ability to tolerate copper is thought to be a consequence of species introductions via hull fouling, a major vector for invasive marine species (Wasson et al. 2001; Floerl & Inglis 2005), due to the long term use of copper based antifoulant biocides on ship hulls (Piola et al. 2009).

A consequence of an introduction via hull fouling is the potential for copper to act as a selective pressure at numerous stages of the invasion process (Sakai et al. 2001; Piola et al. 2009). Firstly a species must either adapt or be pre-adapted to novel conditions prior to transportation (Henery et al. 2010), which may potentially be highly toxic (Valkirs et al. 2003), then endure and survive the journey. This is followed by introduction and establishment into a new environment which may be impacted by metal pollution, as many harbours, ports and marinas are (Birch & Taylor 1999; Matthiessen et al. 1999; Dafforn et al. 2009). As a consequence, exposure to copper may fluctuate dramatically during the life of an organism. Inconsistent exposure regimes may select for genotypes that are capable of responding to these fluctuating conditions (Lee & Gelembiuk 2008), in comparison to tolerance evolving in response to site-specific pollution where conditions are potentially more consistent (Klerks & Levinton 1989).

Tolerance to contaminants has generally been assessed at a population level (Johnston In press), although research has begun to assess tolerance at an individual level (Chapter 2). These detailed studies are important because in order for selection to cause evolutionary change there must be heritable variation among individuals. Many studies of the heritability of tolerance involve testing parents and offspring, as full or half siblings, to estimate the heritability of the trait (Galletly et al. 2007; Pease et al. 2010). Another more novel way of measuring individual tolerance is to use a modular organism which can be manipulated to independently test multiple environments in a genotype by environment (G X E) design (Newlon et al. 2003; Monro & Poore 2009). Using a G X E design to test for an interaction, we can infer whether the genetic basis of traits differs across environments by looking at the specific combinations of genetic and environmental factors that influence a trait (Falconer & Mackay 1996; Galletly et al. 2007).

Many invasive marine species are clonal organisms that reproduce sexually but grow vegetatively, for example bryozoans and ascidians, allowing an individual, or genotype, to be cloned into fragments (Newlon et al. 2003). These clones can then be individually exposed to different environments, thus testing a genotype's response to multiple contaminant concentrations while maintaining independence and precluding any influence of a previous exposure. Because of this, modular organisms are a useful system to estimate genetic variation when a breeding design is unachievable due to physiological constraints. By doing this we can test whether there is a G X E interaction and therefore a genetic basis to tolerance. Variation among genotypes, indicated by a significant G X E interaction, may also reveal the extent of variation available for selection to act on (Falconer & Mackay 1996).

The invasive bryozoan Watersipora subtorquata (d'Orbigny 1852) is highly tolerant to copper (Piola & Johnston 2009) and has been found to actively recruit into high copper environments (Chapter 4 & 5). This bryozoan has a cosmopolitan distribution; its native range is uncertain but thought to be in the Caribbean (Mackie et al. 2006). Capable of growing on copper-based antifoulant paints, it provides a less toxic secondary surface upon which other organisms can settle, facilitating the transfer of other hull-fouling sessile invertebrates beyond their natural distribution (Floerl et al. 2004). It reproduces sexually, releasing lecithotrophic larvae which settle within approximately 24 hours of release (Wisely 1958; Marshall & Keough 2004). Upon settlement the larvae metamorphose into a single zooid, or ancestrula, from which a colony forms by vegetative growth. Each zooid within the colony is a genetic clone of the founding larvae, capable of feeding and reproducing, with a connective system between them (Shepherd & Thomas 1982). Being a modular organism, individual colonies/genotypes can be fragmented into clones that are capable of recovery and subsequent growth, making it an ideal test species with which to examine environmental tolerances. Many fouling NIS also share similarities with W. subtorquata, i.e. are clonal organisms, inhabit the same marine environments and are likely to have undergone similar modes of introduction, making the results of this study comparable to other species.

Invasive species have long been recognised as useful subjects in which to study evolution (Grinnell 1919; Lee & Gelembiuk 2008), but research into how they adapt to novel stressors, such as metal pollution, remains rare (Galletly et al. 2007). In order to understand the potential for such adaptation, it is valuable to understand the genetic architecture of copper tolerance. This is especially true in invasive species, such as *W*. *subtorquata*, in which selection fluctuates between periods of high copper exposure and low copper. We asked whether there is an interaction between genotype and

environment (i.e. copper contamination) on colony growth, as growth is an important predictor of fitness. By cloning *W. subtorquata* genotypes and exposing them to multiple copper environments we were able to examine this interaction, which indicates whether there is genetic variation in tolerance to copper (Lynch & Walsh 1998). The presence of additive genetic variation (Blows & Hoffmann 2005), will then indicate whether there is the capacity for further evolution of tolerance, particularly in highly impacted environments.

Materials and Methods

Genotype culturing and cloning

To investigate copper tolerance in an invasive species, *W. subtorquata* colonies were reared from the offspring of field collected adult colonies. Initially, gravid colonies were collected from Burraneer Marina Port Hacking estuary ($34^{\circ}7'S$, $151^{\circ}10'E$) south of Sydney in New South Wales, Australia. Colonies were collected in May 2008 and maintained in individual containers at 20^{0} C for up to 3 d without light. Spawning was induced by exposing the colonies to light and stopping aeration for approximately 1 h, stimulating the release of larvae. Each container, containing a single field collected colony, was lined with an acetate sheet (that had been pre-soaked in seawater for a minimum of 24 h) upon which multiple larvae from that parent colony settled on. Once larvae had successfully settled and metamorphosed, the acetate sheets were sectioned and individual recruits were cultured in separate containers. Multiple siblings were maintained from each parent colony, although only one sibling from each colony was used for the copper tolerance experiment. This sibling was later chosen from each family based on its size and ability to be cloned. Each individual was maintained in a separate aerated container and fed the microalga *Isochrysis galbana* (clone T.Iso) five

days a week, with seawater changed on a weekly basis. Culturing was conducted in a constant temperature room set at 20^{9} C, using filtered sterilised seawater (details below). Individuals were maintained as such until they had grown large enough on the acetate sheet for the colony to be cut into twelve pieces, or clones. This number of clone fragments per genotype was decided by the size of the initial colonies. Once large enough (approximately 1.5 cm in diameter), each colony/genotype was sliced into pieces approximately 8 mm² using MicroPoint FeatherLite^(TM) scissors (to reduce tissue damage), ensuring that each piece had a distinct and equally sized growing edge. Hereafter each cloned colony is referred to as genotype. Each clone fragment was then attached to the bottom of a Petri dish (35 mm diameter) using a minuscule smear of superglue on the bottom of the acetate. Once cemented, the Petri dish was rinsed once before being filled with seawater containing *I. galbana* at a concentration of 10^5 cells mL⁻¹, which was replaced every 24 h from this point on. Colonies were allowed to recover for 7 d before commencing the copper tolerance assay.

Copper treatments

Copper solutions were prepared using analytical grade copper II sulphate anhydrous. The experimental concentrations were prepared on a daily basis from a stock solution of 1 g L⁻¹ Cu in Milli-Q water. From this an initial solution of 1000 μ g L⁻¹ Cu in Milli-Q water was prepared daily from which the experimental treatments 25, 50, 75, 100 and 125 μ g L⁻¹ Cu in seawater were then diluted. For a food source *I. galbana* was incorporated as a component of the copper and control solutions at 10⁵ cells mL⁻¹. All equipment was acid washed in 5% nitric acid for a minimum of 24 h then thrice rinsed in Milli-Q water prior to use. All seawater used throughout this experiment was filtered to 5 μ m (Pentair Mechanical Filter Module) and sterilised using a UV light steriliser

(Emperor Aquatics Ultraviolet Sterilizer, 50 watt). Salinity, pH and dissolved oxygen were monitored for all solutions using a YSI 556 MPS® (Yellow Springs, OH, USA) before being used experimentally to ensure no differences between treatments. Water samples were collected from each treatment every 3 d throughout the exposure period to determine the total Cu. From these, random samples from the three treatments: control, 50 and 100 μ g L⁻¹ (n=3) were chosen for analysis. These samples were analysed at the Australian Government National Measurement Institute in Sydney, Australia, using ICP-AES (detection limit of 5 μ g L⁻¹). These analyses found copper concentrations to be close to the nominal values (control < 5 μ g L⁻¹, 50 = 54.3 ± 0.9 μ g L⁻¹ and 100 = 106.7 ± 3.3 μ g L⁻¹).

Copper tolerance assay

After the initial recovery period two clone fragments from each genotype were randomly assigned to each of the six experimental treatments (environments), control seawater, 25, 50, 75, 100 and 125 μ g Cu L⁻¹ (n = 2 replicates per concentration within a genotype). Clone fragments were exposed to the appropriate solution for a 9 d period, with the treatment solution replaced daily with fresh solution. After the exposure period the clone fragments recovered for a period of 9 d, with all treatments given the control solution containing microalgae (10⁵ cells ml⁻¹) on a daily basis. A total of eleven genotypes were cloned and tested for copper tolerance which, due to differing growth rates between genotypes, were tested in three time blocks (January, February and May 2009). Based on the methods and techniques developed by Piola and Johnston (2009), this exposure regime aimed to mimic the fluctuating conditions that a colony may be exposed to such as pulse and press pollution events.

Throughout the experiment clone fragments were photographed to assess zooid survival and growth as an indicator of copper tolerance, using a dissecting microscope (Leica Model: M205C) and digital camera (Leica Model: DFC290). After the initial cloning recovery period, the clone fragments were photographed on day 1 when the copper exposures began, day 10 when the recovery period began and day 18 at the end of the recovery period. Images were digitally analysed, measuring the area of live zooids to the micrometer using Leica Application Suite (LAS Version 3.6). From these photos two periods of growth were calculated, growth during exposure and growth during recovery, as a proportion of area at the end compared to the beginning of each period (the change in area relative to the initial area at the beginning of each period). A growth rate of 1 denotes no change in area, > 1 is an increase and < 1 a decrease. Due to the method of cloning, live zooids were easily delineated with fragments growing flat against the base of the Petri dish. Only live zooids were measured, which were tinged orange and had obvious internal organs. Zooids damaged on the edge of the fragment whilst cutting were not included in the measurements even if still orange after the initial recovery period, as they were incapable of growth and had reduced survival.

Statistical analysis

To determine whether copper tolerance differs between genotypes and to test for a G X E interaction, growth rate during exposure and recovery was analysed using a nested three factor analysis of variance (random: block and genotype(block), fixed: environment). Data were checked for normality and homogeneity of variances using frequency histograms and residual plots. Tests for homogeneity of variances, such as Levene's test, were inappropriate because there were only two replicates within an environment for each genotype. Data were analysed untransformed.

3. Copper and clones

The genetic variance co-variance matrix (**G**) across copper treatments was estimated using the method described in Galletly et al (2007). Block and environment were included in the model as fixed variance effects, whilst genotype(block) was a random factor. Eigenvectors were calculated by diagonalization of the G matrix, with each dimension tested for significance in the model using factor analytical modelling (Galletly et al. 2007). To explore the common genetic basis of growth during exposure and recovery, genetic correlations were calculated for each environment using the ANOVA method used by Astles et al (2006). Variance components were extracted from two-way ANOVAs performed for each environment, with genotype and growth period as the two factors. The standard deviation was unable to be estimated to test the significance of genetic correlations (r_g) because there were only two clone fragment replicates within an environment. This number of clone fragments per genotype per treatment was limited by the size of initial colony, as only 12 fragments could be cloned from each.

Measurement efficacy

To determine that any effects found were not caused by measurement error, measurement efficacy was quantified by repeatedly measuring random fragments. This was done for 30 fragments, with three measurements taken per fragment, to compare variation within and between fragments using a one-way ANOVA. We checked data for normality and homogeneity of variances using frequency histograms, residual plots and Levene's test. Data were found to have a bimodal frequency distribution and unevenly spread residuals, which were corrected by a natural log transformation, although variances were still found to be heterogeneous after transformation. Therefore we analysed the transformed data and accepted significant results when P < 0.01.

Fragments were found to differ significantly in size, with minimal within fragment variance, and measurement repeatability was $99.97 \pm 0.007\%$ indicating that measurement efficacy was high (Table 3.1).

Table 3.1 Measurement efficacy. Data were natural log transformed. *P*-values in bold represent significant differences at $\alpha = 0.01$, P = 0.000 denotes values < 0.001

Source	df	MS	F	Р
Fragment	29	0.56	15353.81	0.000
Error	60	$3.67E^{-05}$		

Results

All clone fragments survived the cloning process and exhibited signs of growth and repair during the initial recovery period. There was no mortality during and after exposure to copper, with all clone fragments surviving even in the highest concentration environments. Most genotypes exhibited new zooid formation, with negative growth (i.e. <1) usually being attributed to death of older fully formed zooids that were behind the growing edge. When growth was impacted and zooid formation either reduced or halted budding zooids began turning black, although there were no obvious deformities or mutations observed.

Growth during exposure was > 1 for the control and 25 μ g L⁻¹ Cu treatments, although there were two exceptions, with a genotype in each environment that had a growth rate of < 1 (Fig 3.1a). In the lowest copper environment (25 μ g L⁻¹), the growth rates between genotypes ranged the least from 0.97 to 1.18, a much narrower range than any of the other environments. In the 50 μ g L⁻¹ treatment and those with higher copper

3. Copper and clones

concentrations, most genotypes had reduced growth during exposure to copper, although there were always two to four genotypes that exhibited a growth rate of > 1. The genotypes that were capable of maintaining or increasing in size in a higher concentration environment generally did so in more than one of them.

Growth during exposure varied between genotypes, with a significant G X E interaction between genotype and copper environment (Table 3.2a). This is apparent when graphing the reaction norms, or growth of each genotype in each environment (Fig. 3.1a), where some genotypes showed enhanced growth in lowest copper treatment of 25 μ g L⁻¹ compared to in the seawater control. There was also a significant interaction between block and copper treatment (Table 3.2a).

During the recovery period, growth was > 1 for 80% of the genotypes in the control environment but only 64% in 25 μ g L⁻¹, with four genotypes having a rate of < 1 (Fig 3.1b). This environment, 25 μ g L⁻¹, also displayed the greatest variation in growth, with growth rates ranging between 0.86 and 1.57. Compared to growth during exposure, only five genotypes had a growth rate of < 1 in the 50 μ g L⁻¹ environment. Similar numbers of genotypes exhibited a > 1 growth rate in the 75 – 125 μ g L⁻¹ environments, ranging from four to two in the highest two environments.

A significant G X E interaction between genotype and copper treatment was also found in growth during recovery (Table 3.2b), with growth in general still lower in the high copper environments despite the exposure having ceased. Different genotypes varied in their ability to recover from exposure, with one genotype in particular dramatically increasing growth following exposure to the low copper treatment (Fig 3.1b). There was no effect of block during the recovery period (Table 3.2b).



Figure 3.1 Reaction norms for each genotype across the six environments, (a) during the exposure period and (b) during the recovery period. Each line represents a genotype. A growth rate of 1 denotes no change in area, > 1 is an increase and < 1 a decrease. There were 11 genotypes used.

Table 3.2 ANOVA results for growth during (a) exposure and (b) recovery, showing the significant G X E interaction. *P*-values in bold represent significant differences at $\alpha = 0.05$, P = 0.000 denotes values < 0.001

Source	df	MS	F	Р
a. Growth during exposure				
Copper	5	0.31	6.28	0.007
Block	2	0.43	8.18	0.011
Genotype(Block)	8	0.02	1.11	0.381
Block*Copper	10	0.05	2.42	0.024
Genotype (Block)*Copper	40	0.02	1.71	0.027
Error	66	0.01		
b. Growth during recovery				
Copper	5	0.34	8.21	0.003
Block	2	0.01	0.03	0.967
Genotype (Block)	8	0.28	9.65	0.000
Block*Copper	10	0.04	1.44	0.200
Genotype (Block)*Copper	40	0.03	2.93	0.000
Error	66	0.01		

3. Copper and clones

Genetic variance was greatest in the 50 μ g L⁻¹ copper environment, followed by the control, then decreased in all copper environments above (Table 3.3a). This followed a similar pattern for growth during recovery, with the genetic variance peaking in the 50 μ g L⁻¹ copper environment then decreasing sequentially in the environments on either side (Table 3.3b). Growth in all environments with copper ($\geq 25 \mu$ g L⁻¹) negatively covaried with growth in the control environment during exposure (Table 3.3a), but these covariances were positive for growth during recovery (Table 3.3b).

The diagonalization of the G matrix for growth during exposure showed one significant dimension of genetic variance, the eigenvector g_{max} which explained 61.7% of the genetic variance (Table 3.4a). The low dimensionality of the genetic variance was supported by the fact that the G matrix was ill conditioned: only two of the remaining five eigenvectors, g_2 and g_3 , were positive, explaining 34.9% and 3.1% of the overall variance respectively. The first eigenvector showed a clear difference between the control environment, which had a negative loading, compared to the copper environments (Table 3.4a), consistent with the pattern of positive and negative covariances.

For growth during recovery, g_{max} explained 80.2% of the genetic variation followed by 14.1%, 4.2% and 1.5% for the subsequent positive eigenvectors (g_2 to g_4) (Table 3.4b). The final two eigenvectors were negative; again indicating an ill conditioned G matrix with fewer dimensions than originally measured traits. All environments were positively loaded on g_{max} with the highest loading in the 50 µg L⁻¹ copper environment, corresponding with the genetic variance at this treatment. In both of the diagonalised G matrices only the first dimension, g_{max} , was statistically significant (growth during exposure: $\chi^2 = 16.7$ df = 6 P = 0.011, growth during recovery $\chi^2 = 77.9$ df = 6

(a) During	g Exposure					
	Control	25	50	75	100	125
Control	0.0169	-0.0001	-0.0067	-0.0045	-0.0063	-0.0111
25		0.0015	0.0091	0.0033	-0.0004	0.0015
50			0.0223	0.0051	-0.0019	0.0038
75				0.0001	0.0039	0.0032
100					0.0009	0.0046
125						0.0042
(b) During	g Recovery	r				
	Control	25	50	75	100	125
Control	0.0311	0.0281	0.0384	0.0231	0.0171	0.0151
25		0.0471	0.0513	0.0322	0.0197	0.0137
50			0.0634	0.0490	0.0410	0.0366
75				0.0423	0.0383	0.0317
100					0.0364	0.0298
125						0.0305

Table 3.3 Genetic variance covariance (G) matrix of growth (a) during exposure and (b) during recovery. Genetic variances are on the diagonal and in bold, while covariances are above the diagonal. Environments are in μ g L⁻¹ Cu.

Table 3.4 Diagonalization of the six environments (a) during exposure and (b) during recovery, showing the eigenvectors, loadings of each environment and the percentage of total variance by each eigenvector. Eigenvectors in bold indicate a statistically significant dimension. Environments are in μ g L⁻¹ Cu.

		% of total						
Eigenvector	Eigenvalue	variance	Control	25	50	75	100	125
(a) During Exposure								
g_{\max}	0.0348	61.7	-0.5728	0.2177	0.6523	0.2400	0.1435	0.3476
g_2	0.0196	34.9	0.5469	0.2912	0.6348	-0.0284	-0.3380	-0.3132
g_3	0.0019	3.4	0.3992	0.4319	-0.1860	0.5892	0.5079	0.1199
g_4	-0.0021	0	0.1690	0.4316	-0.1793	-0.3485	-0.3316	0.7222
g_5	-0.0032	0	0.4291	-0.6317	0.3113	-0.0995	0.3221	0.4542
g_6	-0.0050	0	-0.0285	0.3099	0.0888	-0.6805	0.6273	-0.1968
(b) During Re	ecovery							
g_{\max}	0.2060	80.2	0.3091	0.3983	0.5641	0.4379	0.3682	0.3168
g_2	0.0362	14.1	-0.3414	-0.6069	-0.1377	0.2384	0.4607	0.4764
g_3	0.0109	4.2	0.8120	-0.4670	0.0231	-0.2485	-0.1066	0.2210
g_4	0.0037	1.5	-0.2725	0.1329	0.2239	-0.2627	-0.5474	0.6995
g_5	-0.0002	0	-0.1282	0.0673	0.3044	-0.7785	0.5280	-0.0393
g_6	-0.0058	0	-0.1943	-0.4824	0.7208	0.1213	-0.2496	-0.3647



Figure 3.2 Reaction norms for each genotype across the exposure and recovery period, within the six environments, (a) control (b) 25 μ g L⁻¹ Cu (c) 50 μ g L⁻¹ Cu (d) 75 μ g L⁻¹ Cu (e) 100 μ g L⁻¹ Cu and (f) 125 μ g L⁻¹ Cu. Each line represents a genotype. A growth rate of 1 denotes no change in area, > 1 is an increase and < 1 a decrease. There were 11 genotypes used. Please note different y-axes scales.

Table 3.5 Genetic correlations between the two growth periods: during exposure and during recovery, within each environment. MS is the Mean square for Genotype (G), Environment (E), Genotype X Environment and the Error (Err), whilst V is Variance. n = 2 E = 2 Environments are in μ g L⁻¹ Cu.

	MS_G	$MS_{G^{\ast}E}$	MS _{Err}	V_{G}	$V_{G^{*E}}$	r _g
Control	0.118	0.014	0.01	0.026	0.002	1.08
25	0.053	0.041	0.011	0.003	0.015	-0.25
50	0.045	0.088	0.011	-0.011	0.039	0.22
75	0.029	0.042	0.008	-0.003	0.017	0.16
100	0.034	0.060	0.015	-0.007	0.023	0.22
125	0.029	0.066	0.01	-0.009	0.028	0.25

P < 0.001), and therefore considered to display significant genetic variation for the two growth periods.

Genotypic responses to each environment were variable across the two growing periods, with no obvious patterns (Fig 3.2a-f). Responses varied from an increase in growth during exposure followed by a decrease during recovery, to the opposite where growth was greater during recovery, whilst some genotypes maintained constant growth throughout both periods. These patterns of growth also varied between environments with the same genotypes. The only environment where growth rates were relatively consistent was in the control environment (Fig 3.2a). Genetic correlations between growth during and after exposure found a high positive genetic correlation for growth within the control environment (Table 3.5). All other environments with copper had much lower r_g values (Table 3.5), indicating little correlation between growth during exposure and during recovery within each of the environments.

Discussion

Tolerance to metal pollution has been suggested as an important trait contributing to invasion success for a number of marine species (Piola et al. 2009), yet little is known about how invaders have adapted to copper as a novel stressor (Galletly et al. 2007). This study aimed to investigate metal tolerance in a marine NIS, *W. subtorquata*, and whether there is genetic variation in this trait. Using a novel assay technique we found individual variation in tolerance to a gradient of copper concentrations and the ability to recover from exposure to copper. Most genotypes were capable of tolerating concentrations up to 50 μ g L⁻¹, where the genetic variance was also greatest. Growth during and after exposure appear to be separate traits, while a trade-off between growth in control and copper environments during exposure suggests a cost to tolerance. The variable partitioning of growth in response to exposure suggests that *W. subtorquata* has undergone a fluctuating selection regime that has selected for differing mechanisms to tolerate press and pulse copper pollution events. Furthermore the presence of genetic variation in tolerance to ecologically relevant copper concentrations parallels tolerance and mortality patterns in juvenile *W. subtorquata* (Chapter 2, 4 & 5). Overall the genetic variation present within this species indicates that further evolutionary change is possible under strong directional selection.

Pollution events can vary dramatically in frequency, intensity and duration, creating fluctuating selection regimes (Beck 1996). Even within an impacted site, such as a marina where there are multiple sources of copper pollution (Weis et al. 1998; Srinivasan & Swain 2007), disturbance events can cause a further spike in exposure (Knott et al. 2009). Hence selection for copper tolerance will vary depending on the temporal and spatial exposure regimes. It is possible that fluctuating selection might contribute to maintaining the high levels of genetic diversity that I report in this chapter. These fluctuating conditions, compared to a constant exposure regime often experienced in chronically polluted environments (Klerks & Levinton 1989) may also select for the ability to survive and then recover from a pulse pollution event.

A potential mechanism for withstanding pulse pollution events is to partition growth in response to the duration of the stress, as a form of adaptive plasticity (Ghalambor et al. 2007). Within each copper environment genotypes differed in how they partitioned maximum growth; during exposure, during recovery, or maintenance of a relatively constant growth rate across the two periods. These alternative patterns of growth in response to copper exposure suggest that multiple mechanisms or traits may have evolved to tolerate copper pollution, depending on the strength of the disturbance

(Galletly et al 2007). For example genotypes that allocated resources to post exposure growth would be more capable of recovery from a pulse pollution event. In comparison genotypes that grew strongly during exposure are likely to flourish under continuous or long-term copper exposure (Klerks & Levinton 1989). The lack of genetic correlation between each copper environment during exposure and recovery, implies that growth during contamination and recovery afterwards are largely separate traits (Falconer & Mackay 1996). These traits may have then become optimised under different selection regimes, dependant on the duration and intensity of exposure events. The evolution of multiple mechanisms to withstand different exposure concentrations and durations is difficult to test, but has been suggested for another invasive marine invertebrate *Styela plicata* (Galletly et al. 2007).

Larvae and juveniles are more susceptible to contaminants (Xie et al. 2005), and the reduced tolerance and genetic variation in adult colonies to higher concentrations reflects early life history mortality. Genetic variation was greatest at 50 μ g L⁻¹, a concentration that is ecologically relevant to early life history stages. In an ecological context, a concentration of copper such as 50 μ g L⁻¹ in the marine environment is most likely to correspond with the exposure an individual would receive when recruiting to a contaminated surface such as an antifoulant coated boat hull (Valkirs et al. 2003). *W. subtorquata* larvae actively recruit to copper polluted surfaces such as these, but then suffer high mortality and consequently experience strong selection (Chapter 4 & 5). Laboratory studies have also shown that tolerance to similar concentrations is a heritable trait in larval *W. subtorquata* (Chapter 2) compared to higher concentrations that significantly reduce survival beyond metamorphosis (Wisely 1958; Piola & Johnston 2006a; Chapter 2).

Tolerance to metal pollution is often associated with a fitness cost (Medina et al. 2007). This is expressed as a reduction in fitness for tolerant genotypes when not exposed to the pollutant, for example reduced fecundity and growth (Shirley & Sibly 1999; Hendrickx et al. 2008). A cost of copper tolerance was found in this study, although not overly obvious, from the negative covariance and gmatrix loading between growth in control and copper environments during exposure. The exact mechanisms used to tolerate copper are unknown in *W. subtorquata*, although the synthesis of metal binding proteins (metallothioneins) has been suggested or identified for many invertebrate species (Amiard et al. 2006). Increased production of metallothioneins is likely to be metabolically costly (Roesijadi 1992). Associated costs can become more apparent when comparing between highly tolerant and intolerant populations, which have been chronically exposed for multiple generations (Shirley & Sibly 1999; Piola & Johnston 2006b). A population of *W. subtorquata* that has experienced strong selection due to copper pollution is likely to exhibit costs associated with tolerance, which may also manifest in traits other than growth. For example field experiments manipulating copper exposure regimes found that colonies which recruited to, but where then removed from, a copper polluted environment had lower growth and fecundity then individuals that remained exposed (Chapter 5). Clearly, W. subtorquata has evolved to tolerate exposure to copper, but there is a trade-off between growth and tolerance, implying that tolerance is a costly trait to maintain.

Conclusion

The NIS *W. subtorquata* appears to have evolved multiple mechanisms to tolerate a range of pollution events. Alternative patterns of growth in response to copper exposure, where growth is partitioned either during exposure or recovery, indicate they

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have adapted to withstand pulse and press exposure events. This ability to tolerate periodic exposure to high concentrations of copper would explain its persistence in copper polluted environments, such as harbours, ports and marinas. Additionally the variation in tolerance to copper indicates evolutionary potential within *W. subtorquata* to respond to further directional selection. Regardless of whether this species was preadapted to metal polluted environments prior to being globally transported, it is apparent that tolerance to copper is an advantageous trait that is under selection in anthropogenically impacted habitats. With metal pollution an increasing disturbance on a global scale (Birch & Taylor 1999) we can expect to see a greater prevalence of copper tolerant populations of invasive species.

Chapter 4 A common contaminant increases establishment

success of a marine invader

Abstract

The spread of non-indigenous species by human vectors has exposed species to novel conditions such as metal pollution, which may reduce or enhance invasion success. As successful establishment is influenced by propagule pressure and disturbance we investigated how recruitment of the invasive bryozoan *Watersipora subtorquata* is affected by copper pollution. Using Perspex plates left untreated or painted with a copper-based antifoulant, we quantified settlement patterns onto control and copper environments after one day, then assessed recruit survival and fitness after one week. Copper significantly increased recruitment, despite greater post-settlement mortality and reduced fitness. These results show a strong positive affiliation between larval *W. subtorquata* and high levels of copper although there are associated fitness costs. Overall, it appears that copper can potentially enhance success at multiple stages of the invasion process, including facilitating transport and establishment, by increasing the supply and retention of individuals into anthropogenically disturbed environments.

Introduction

Anthropogenic transport vectors have rapidly increased the rate at which species are introduced into new environments (Ruiz et al. 2000; Hulme et al. 2008). The impact of non-indigenous species (NIS) on local communities can be difficult to quantify but often the effects are detrimental, ranging from reduced biodiversity (Wilcove et al. 1998), to dramatic habitat modification by ecosystem engineers (Crooks 2002). Consequently, it is important to understand the factors that enhance success at various stages of the invasion process (Kolar & Lodge 2001). One factor that is being increasingly recognised as pertinent for successful establishment is propagule pressure (Lonsdale 1999; Puth & Post 2005). Propagule pressure describes a measure of the number of individuals released into an area to which they are not indigenous (Carlton 1996). Therefore a greater supply of invasive propagules should increase the likelihood of establishment and, subsequently, invasion success (Lockwood et al. 2005). Many NIS are introduced into anthropogenically disturbed environments which can, in most cases, reduce the propagule pressure necessary for successful invasion by freeing resources (Davis et al. 2000; Lockwood et al. 2005). A more novel prediction is that anthropogenic disturbance will act directly to change the parameters of propagule pressure and directly facilitate species invasion.

A particularly detrimental form of disturbance is contamination from anthropogenic sources (Foster & Charlesworth 1996). Pollutants such as metals, pesticides and oils can have profound impacts on communities, mostly by reducing species richness (Johnston & Roberts 2009; Brittain et al. 2010). Interestingly, tolerance to toxicants has also been observed, often in pest species for whom the toxicant was initially intended (Scarabel et al. 2007). The prevalence of NIS in polluted environments (Dafforn et al. 2009) suggests two things; firstly that contaminants themselves may be facilitating species introductions, through direct disturbance and the removal of native biomass. Secondly that due to a prior history of exposure and selection, certain NIS may be more tolerant to contaminants than native species within the receiving communities (Piola & Johnston 2008a; Piola & Johnston 2009). Tolerance to anthropogenic contaminants has evolved rapidly in numerous species, particularly after site-specific pollution results in a population being exposed to intense selection for multiple generations (Macnair 1987; Medina et al. 2007).

Marine and estuarine environments in particular are very vulnerable to an increasingly common and toxic form of contamination: metal pollution (Hall et al. 1998). Entering the system through numerous sources including the use of antifouling biocides (Weis & Weis 1996), sewage discharge and industrial effluent (Hall et al. 1998), concentrations of metals typically reach their highest in areas of urban development such as harbours, ports and marinas. These habitats are also the most vulnerable to species introductions, as focal points for recreational and commercial shipping activities (Floerl et al. 2009). Boat hulls are a major vector for marine NIS and are often coated with copper-based antifoulant paints (Floerl & Inglis 2005), therefore recruitment to and survival on hulls implies selection for copper tolerance in the fouling organisms being transported (Piola et al. 2009). This, coupled with increased propagule pressure from repeated introductions (Carlton & Geller 1993) and a recipient community with a lowered invasion resistance due to anthropogenic disturbance (e.g. by increasing resource availability (Davis et al. 2000)), may explain the increased presence of NIS in environments contaminated by metals. For example a number of marine NIS have been found to have a positive affiliation with metal contamination, an affiliation that may enhance further range expansion (Dafforn et al. 2009).

With propagule pressure increasingly recognised as a pertinent factor in the establishment and spread of NIS (Lockwood et al. 2005), it is important to understand how this component of the invasion process is affected by metal pollution. Recruitment is considered a key step in the introduction of a species, with increased invasion success linked to increased propagule pressure and disturbance (Clark & Johnston 2009). This study aimed to determine whether copper affects the recruitment process of an invasive species, the encrusting bryozoan *Watersipora subtorquata* (d'Orbigny, 1852). *W. subtorquata* is highly tolerant of copper (Piola & Johnston 2006a), capable of

recruiting to surfaces coated in antifoulant paint where it creates a less toxic secondary surface for other fouling organisms to settle on (Floerl et al. 2004). By providing a nontoxic refuge, W. subtorquata has been suggested to be capable of causing an 'invasion meltdown' by facilitating the transfer of other hull-fouling sessile invertebrates beyond their natural distribution (Floerl et al. 2004) (although see Simberloff (2006)). This bryozoan has a cosmopolitan distribution; its native range is uncertain but thought to be in the Caribbean (Mackie et al. 2006). W. subtorquata shares numerous similarities with many fouling NIS, for example method of introduction, comparable physiological traits with other colonial organisms (such as brooded offspring and vegetative growth in ascidians) and a global distribution. Therefore W. subtorquata is an appropriate organism for examining how a common metal pollutant, copper, affects the recruitment patterns of an invasive species. We did this by investigating how high and control copper environments influence larval settlement, post settlement survival and morphological characteristics of W. subtorquata recruits. We found that high copper contamination strongly influenced recruitment patterns and morphological traits, with implications for continued range expansion by human vectors.

Methods and Materials

To investigate the effect of copper on recruitment processes, we designed this experiment to mimic the settlement surface provided by a vessel's hull recently painted with copper based antifouling biocide. Incomplete coverage of antifouling paint is common, due to the docking procedures preventing access to the entire hull, and creates a scenario which has been linked to the spread of invasive species (Piola & Johnston 2008b). It also simulates surfaces provided by permanent structures within urbanised

estuaries that have been coated with biocides. Antifoulant biocides can leach dissolved copper at an initial rate of approx 25-65 μ g/cm²/day (Valkirs et al. 2003), resulting in concentrations that are lethal to many invertebrates and inhibit recruitment (Hall et al. 1998).

We deployed settlement plates at Burraneer Marina in Port Hacking estuary (34°7'S, 151°10'E) south of Sydney in New South Wales, Australia, during January and February 2009. This site has a high abundance of *W. subtorquata* colonies growing on artificial substrate and is an open marina. To encourage recruitment of *W. subtorquata* panels were hung horizontally at 1m below mean low tide level with the settlement surface facing down, randomly located in shaded areas. Panels were treated as a replicate; either control or copper, to prevent any contamination between treatments. For ease of experimental manipulation, each panel had four 11 by 11 cm Perspex settlement plates attached that had been roughened to encourage settlement. For the copper treatment, settlement plates were painted with a 2 cm wide border of the commercially available copper-based antifoulant paint International® Micron Extra, whilst on control panels the settlement plates were untreated. There were five panels per treatment and the entire experiment was repeated a month later at the same location (subsequently called trial 1 and 2).

W. subtorquata release larvae from early in the day usually upon exposure to light and the larvae begin to settle immediately upon release (Marshall & Keough 2003). Hence, we deployed plates late in the afternoon to effectively sample the following day's recruitment. After approximately 40 h plates were pulled out of the water, photographed and all *W. subtorquata* recruits circled with a graphite pencil for later identification and to monitor survival. We surveyed the entire plate including the border and counted any

recruits that settled there. Although recruitment of other species into either environment was minimal, we removed any non-*W. subtorquata* recruits by scraping before redeploying the plates for one week. After one week the plates were removed from the water and all surviving recruits from the initial settlement period were counted and photographed live with a digital camera (Leica Model: DFC290) using a dissecting microscope. Settler size, or the first zooid formed as a result of successful metamorphosis (Piola & Johnston 2006a), has previously been shown to be a good predictor of initial larval size (Marshall & Keough 2003). Settler size has also been shown to significantly influence survival and growth of recruits during the initial three weeks after settlement (Marshall & Keough 2004). This initial zooid, or ancestrula, is easily delineated in small colonies due to the distinctive pattern of colony formation. We measured ancestrula length, from the top of the operculum to the distal edge of the zooid (length mm), and overall colony size (area mm²). These morphological traits, ancestrula length and overall colony size, were measured to the nearest μm using the program Leica Application Suit Version 3.6, before converting to mm.

Statistical Analysis

We compared initial settlement, final recruitment and post-settlement mortality between environments (copper and control) using a two factor analysis of variance (trial: random, environment: fixed). Data were analysed the data using a two factor mixed model PERMANOVA (Anderson 2001).Because there was no significant effect of the interaction between trial and environmental treatment (P > 0.2) for each variable the final model does not contain this term. PERMANOVA is a distance based permutational analysis of variance, which calculates a pseudo F-statistic for univariate

data when analysed using Euclidean distance (Anderson 2001). We analysed the data as density per cm^2 and ran the model for 9999 permutations.

To test the effect of environment on morphology, we analysed colony size and ancestrula length using a two-factor mixed model multivariate analysis of variance (MANOVA) (trial: random environment: fixed). Ancestrula length and colony size were averaged for each panel, giving five replicates per treatment. We checked the data for homogeneity of variances and co-variances using Levene's and Box's test. Because there was no significant effect of the interaction between trial and environmental treatment (P > 0.2) the final model does not contain this term.

Results

High numbers of *W. subtorquata* larvae recruited to the settlement plates, with densities ranging from 0.03 - 0.10 and 0.07 - 0.30 recruits per cm², on control and copper panels respectively. Initial settlement was significantly greater on copper painted panels ($F_{1,17}$ = 16.78, P < 0.001)(Fig. 4.1a). Post-settlement mortality was also significantly greater in the copper environment ($F_{1,17}$ = 35.96, P < 0.001)(Fig. 4.1c), with mortality reaching 49% on one panel compared to 0-17% on control panels. Despite the increased mortality, recruitment after one week was still significantly greater on copper painted plates ($F_{1,17}$ = 9.58, P = 0.007) with more than double the density of recruits (Fig. 4.1b). There was no difference between trials for initial settlement ($F_{1,17}$ = 1.77, P = 0.217), final recruitment ($F_{1,17}$ = 1.64, P = 0.225) or post-settlement mortality ($F_{1,1}$ = 0.10, P = 0.759).



Figure 4.1 Density per cm² of *Watersipora subtorquata* recruits (a) after one day of recruitment and (b) one week after initial recruitment, and (c) the mean mortality (\pm SE) on control and copper environments for each trial. Values are mean \pm SE



Figure 4.2 The morphological traits: (a) ancestrula length (mm) and (b) colony size (mm²), of recruits that survived to one week in control and copper environments at each trial. Values are mean \pm SE.

Of the *W. subtorquata* recruits that survived to 1 week, the majority exhibited growth with more than 96% having multiple fully formed feeding zooids. Overall, colonies appeared healthy with active lophophores, although there were a few mutated individuals in the copper environment. Recruit morphology was significantly impacted by the copper environment (Wilks' $\lambda = 0.06$, $F_{2,16} = 129.73$, P < 0.001) for both traits. Colonies in the copper environment were significantly smaller in size ($F_{1,17} = 6.75$, P = 0.019) and had shorter ancestrula length ($F_{1,17} = 246.49$, P < 0.001) (Fig. 4.2a-b) compared to colonies in the control environment. Morphology also differed significantly between trials (Wilks' $\lambda = 0.58$, $F_{2,16} = 5.78$, P = 0.013), but this was attributable to ancestrula length ($F_{1,17} = 5.24$, P = 0.035) and not colony size ($F_{1,17} = 1.61$, P = 0.221) (Fig. 4.2a-b). There was no significant interaction between trial and environmental treatment (P > 0.2).

Discussion

With the continued spread of NIS occurring at a global scale it is important to understand whether anthropogenic disturbances are facilitating the invasion process, particularly as disturbance is predicted to enhance the establishment and persistence of NIS when combined with an increased supply of propagules (Lockwood et al. 2005). In the marine environment many habitats are impacted by metal pollution (Hall et al. 1998), habitats in which NIS are becoming increasingly prevalent (Dafforn et al. 2009). This study aimed to investigate how an invasive species, *W. subtorquata*, is responding to the novel disturbance of copper pollution, particularly as *W. subtorquata* has the potential to facilitate the invasion of other sessile marine species (Floerl et al. 2004). We found that copper significantly increased recruitment of *W. subtorquata* larvae; although this increased recruitment was accompanied by greater post settlement mortality.

Despite increased recruitment in the copper environment, surviving recruits demonstrated different morphology with reduced ancestrula length and colony size. Whilst previous research has found *W. subtorquata* to be capable of recruiting near or onto copper-based antifoulant coated surfaces (Floerl et al. 2004), this study has shown a dramatic increase in recruitment to panels with copper, but with fitness costs associated. The positive affiliation between *W. subtorquata* and copper has consequences for two components of the invasion process. Firstly, it is likely to promote the continued spread of the species as there is an increased likelihood of recruitment onto a vector surface, such as a ship hull. Secondly, it is likely to further facilitate recruitment into environments that are impacted by copper contamination, notably most urbanised harbours and ports. Therefore copper contamination has the capacity to directly enhance propagule pressure by increasing the supply and retention of individuals into anthropogenically disturbed environments.

Recruitment is a fundamental demographic process that can strongly affect population dynamics (Minchinton & Scheibling 1991; Myers & Harms 2009). For many marine invertebrates recruitment includes the process whereby larval offspring exert preferential settlement choices, choosing habitat in response to environmental cues (Pawlik 1992). Despite the toxicity of copper (Hall et al. 1998), settlement was significantly greater in the copper environment, with densities reaching up to three times higher than in the control environment. These densities remained substantially greater, by more than double, even after greater post-settlement mortality in the copper environment. This is rather unusual as high copper contamination (20-30 μ g l⁻¹) has been shown to reduce recruitment for an array of marine invertebrate species (Johnston et al. 2003), suggesting that *W. subtorquata* may be responding to copper as a settlement cue, or that copper is inducing settlement. Furthermore, very few
contaminants have been linked with an increase in population density through enhanced recruitment. Such a positive response is usually restricted to nutrient additions, for example enhanced recruitment of oyster and barnacle larvae (Minchinton & McKenzie 2008), to which the effects have been attributed to the contaminant either mimicking settlement cues or enhancing habitat quality.

Given the increased recruitment in the copper environment despite the availability of numerous clean alternate substrates, it seems plausible that *W. subtorquata* larvae may be using copper pollution as a settlement cue. Especially as this species has been shown to exhibit settlement preferences regarding substrate types (Marshall & Keough 2003). Hull fouling has been identified as an important vector for this cosmopolitan invasive species (Floerl et al. 2004), which suggests this positive affiliation is a result of historical exposure and selection. This affiliation may be a direct influence of copper or an indirect effect. For example changes to bacterial biofilm composition caused by copper contamination can reduce recruitment or biofilm inductiveness (Bao et al. 2010), a potentially important effect considering biofilms are recognised as an important settlement cue for many invertebrate larvae (Pawlik 1992).

Early life history stages can be particularly susceptible to contaminants because they are periods of rapid growth and metamorphosis. In conjunction with greater recruitment, mortality was also significantly greater in the copper environment, with an average of almost 30% of the recruits dying after settlement. This is unsurprising considering the toxic effect of copper on many organisms (Hall et al. 1998) and its specific use as a biocide in the marine environment (Piola et al. 2009). Instead, the combination of high recruitment and high mortality suggests that copper is exerting strong selection on each cohort of recruits after settlement. When a contaminant has a negative impact on the

recruitment process, it can cause rapid shifts in the genotypic composition of a population, selecting for more tolerant individuals (Eranen 2008). This has been seen in both the aquatic and terrestrial environment, where populations of acutely exposed organisms have rapidly evolved tolerance (e.g. aquatic oligochaete (Klerks & Levinton 1989), wolf spider (Hendrickx et al. 2008) and mountain birch (Eranen 2008)). Additionally, the high mortality observed here suggests that copper tolerance is not consistent throughout this introduced population, indicating a likely opportunity for further selection of this trait. This selection for tolerant genotypes might enhance the capacity for some genotypes to survive in highly contaminated environments and as this would most likely include the hull of a vessel, it may augment this species invasion potential.

Morphologically, recruits differed dramatically between the control and copper environments, having shorter ancestrula and smaller overall colony size in the contaminated conditions. These differences indicate that the recruits that are capable of surviving in a copper environment may be suffering short and long term fitness effects (Ng & Keough 2003). Overall, morphological differences between the two environments are not surprising, assuming the high mortality levels have selected for tolerant genotypes (Eranen 2008). Comparisons between populations with and without a history of exposure have found physiological differences such as reduced size and changes in reproductive strategy (Shirley & Sibly 1999; Hendrickx et al. 2008), which are attributable to reallocation of resources to deal with the metabolic costs of tolerance (Shirley & Sibly 1999). Therefore reduction in size may indicate a trade-off between growth and tolerance, which would then lead to longer term impacts on reproduction and fitness.

Offspring size is strongly linked with individual fitness, with general trends predicting greater survival and fitness in larger offspring for a variety of organisms (Smith & Fretwell 1974). For organisms that have a larval stage, recruit size after metamorphosis is a pertinent measure, as it represents larval history and size as well as future fitness potential (Pechenik et al. 1998). With ancestrula length as a representative measure of initial recruit size, the disparity in lengths between the two environments indicates that copper is affecting the recruitment process beyond simple mortality. The most likely point of greatest impact would be during metamorphosis, with copper disrupting the process and changing ancestrula morphology, which is further indicated by the presence of malformed recruits. When not immediately lethal, contaminants can delay or facilitate metamorphosis in many organisms (e.g. amphibians (Gross et al. 2007)). The differences in ancestrula length between trials would most likely reflect variation in environmental conditions of the parent colony during brooding period, offspring size being a result of parental environment and genotype (Bernardo 1996), resulting in variation between larval/recruit sizes over time.

Conclusion

Despite high mortality and fitness costs, *W. subtorquata* larvae recruited considerably more into the copper environment, where numerous individuals were capable of surviving beyond the initial recruitment process. Hence we observed an anthropogenic disturbance directly increasing propagule pressure of a NIS. The reduction in size for two morphological traits suggests a physiological cost to tolerating such contaminated conditions, affecting metamorphosis and growth, although it is unknown how this will impact long term fitness. These recruitment patterns provide insight into how this species has become such a successful and cosmopolitan invader (Mackie et al. 2006),

especially if responding to metal pollution as a settlement cue. And whilst these recruitment patterns initially appear to be a maladaptive habitat choice, a consequence of choosing to recruit to a copper contaminated environment is an intense increase in propagule pressure if individuals survive to reproduce. It also indicates that the spread of this species will not be curbed through the use of copper-based antifoulant paints, instead increasing use may actually be facilitating invasion of copper-tolerant genotypes. Considering that other sessile marine invertebrates already show a positive affiliation with metal pollution (Dafforn et al. 2009; Piola et al. 2009) there is the possibility that this relationship has already evolved and/or has the evolutionary potential to evolve further in other invasive species. In this study we have identified the novel effect of how an anthropogenic contaminant can directly facilitate species invasion by changing the parameters of propagule pressure. Therefore, with the increasing prevalence of metal pollution on a global scale (Foster & Charlesworth 1996), we can only anticipate an increasing spread of copper tolerant invasive species.

Chapter 5 The long and the short of it: the costs and benefits of copper pollution on fitness in a tolerant marine invader.

Abstract

The global spread of fouling invasive species continues despite the use of antifouling biocides. Furthermore, previous evidence suggests that non-indigenous species introduced via hull fouling may have been selected for metal tolerance, which has enhanced their introduction and establishment success in metal-polluted environments. We investigated the effect of copper pollution on multiple life history traits in the invasive bryozoan *Watersipora subtorquata*. We manipulated exposure to copper at two stages, during settlement and post settlement, to create four selection regimes in a fieldbased experiment. Copper significantly increased recruitment, although exposure to copper during initial settlement decreased recruit size and resulted in greater postsettlement mortality. Long term exposure to copper post-settlement significantly increased growth and reproductive fitness. The negative consequences of initial exposure during recruitment appear to be outweighed by the benefits of long term exposure which significantly increased overall fitness. The positive affiliation between W. subtorquata and copper has consequences for numerous components of the invasion process and would explain the current global distribution of the species. This affiliation is likely to promote the continued spread of the species via hull fouling, as well as enhance introduction and establishment success.

Introduction

Invasive species are considered one of the greatest threats to native communities (Carlton & Geller 1993; Vitousek et al. 1997). From an ecological perspective, the 60

impact of non-indigenous species (NIS) on local communities can range from reduced biodiversity (Wilcove et al. 1998) to dramatic habitat modification by ecosystem engineers (Crooks 2002). Consequently, it is important to understand the numerous stages within the invasion process and what factors may enhance success at each step (Kolar & Lodge 2001). The initial mechanism of species entrainment and transfer is of considerable importance (Hulme et al. 2008) as this can select for species characteristics that influence invader success in the new environment (Suarez & Tsutsui 2008).

Transport vectors are a major transfer mechanism for many unintentionally introduced species (Hulme et al. 2008). Within the marine environment a predominant vector for many invasive species is shipping (Carlton 1996; Ruiz et al. 2000), with organisms transported via hull fouling and ballast water. The growing globalisation of trade has lead to an increase in shipping frequency, resulting in a greater transfer of NIS into new environments (Ruiz et al. 2000). Up to 70% of species introductions have been attributed to hull fouling (Wasson et al. 2001; Eldredge & Carlton 2002; Hewitt 2002). The use of antifouling paints has prevented many species from settling and growing on vessel hulls and hence reduced NIS transfer, yet there are a number of organisms that have survived transportation and successfully invaded new locations despite antifouling practices (Piola et al. 2009).

Historically copper has been the most commonly used biocide within antifoulant paints and it is still widely used today (I.M.O. 2001; Piola et al. 2009). A consequence of the long term use of copper appears to be an increased tolerance in a number of marine NIS to concentrations of copper that are toxic to many other organisms (Dafforn et al. 2008; Piola & Johnston 2008a; Piola & Johnston 2009; Crooks et al. 2010), particularly invertebrates (Hall et al. 1998). Tolerance to metal pollution has evolved rapidly in

numerous species (Macnair 1987; Medina et al. 2007), in marine, aquatic and terrestrial habitats. The occurrence of site-specific heavy metal pollution can expose populations to intense selection for multiple generations resulting in population-specific resistance (Klerks & Weis 1987), to metals such as copper (Piola & Johnston 2006b) and cadmium (Klerks & Levinton 1989; Hendrickx et al. 2008). For many invasive marine species introduction via hull fouling has created an interesting exposure regime where copper has had the potential to act as a selective pressure at numerous stages of the invasion process (Sakai et al. 2001). Enhanced establishment and persistence in metal polluted environments (Chapter 3; Dafforn et al. 2009) is a beneficial consequence of selection for copper tolerance in these non-indigenous hull-fouling species.

Marine and estuarine environments in particular are very vulnerable to metal pollution (Hall et al. 1998) that enters the system through numerous sources including the use of antifouling biocides (Weis & Weis 1996; Srinivasan & Swain 2007), sewage discharge and industrial effluent (Apte & Day 1998; Hall et al. 1998). Concentrations of metals typically reach their highest in areas of urban development such as harbours, ports and marinas (Hall & Anderson 1999; Matthiessen et al. 1999; Dafforn et al. 2009). These habitats are also the most vulnerable to species introductions, as focal points for recreational and commercial shipping activities (Floerl et al. 2009). And whilst the impacts of metal pollution can be dramatic, the most obvious being a significant reduction in species richness (Pollard & Yuan 2006; Johnston & Roberts 2009), an increased prevalence of NIS has been found in these environments (Piola & Johnston 2008a; Dafforn et al. 2009).

Greater tolerance to pollution may enable opportunistic utilisation of resources made available through the loss of less tolerant native competitors (Rygg 1985; Johnston &

Keough 2002). An alternative, more radical hypothesis, posits that organisms directly benefit in some manner from contaminant exposure. This hypothesis is generally considered unlikely for highly toxic contaminants such as metals (DeAngelis 1996), however, it remains possible that some successful NIS have evolved to actually benefit from anthropogenic contaminants. By understanding the relationship that has evolved between non-indigenous fouling organisms and copper, we can discern the reasons for their success.

The invasive bryozoan Watersipora subtorquata (d'Orbigny 1852) is highly tolerant to copper in its adult form (Chapter 3; Piola & Johnston 2009). With a cosmopolitan distribution attributed to shipping, its native range is uncertain but thought to be in the Caribbean (Mackie et al. 2006). Capable of growing on copper-based antifoulant paint, it provides a less toxic secondary surface which other organisms can settle upon, facilitating the transfer of other hull-fouling sessile invertebrates beyond their natural distribution (Floerl et al. 2004). It reproduces sexually, releasing lecithotrophic larvae that settle within 24 hours of release (Wisely 1958; Marshall & Keough 2004). Interestingly, *W. subtorquata* has been found to actively recruit into copper environments in significantly higher numbers, despite short term fitness costs including higher post-settlement mortality and a physiological reduction in size (Chapter 4). It is unclear whether recruiting to an environment polluted with copper would actually benefit individuals in the long term, as there are multiple initial fitness costs (Chapter 4), yet this species is a highly successful invasive (Floerl et al. 2004; Mackie et al. 2006). Therefore, to discern whether this success is attributable to evolutionary adaptation or opportunistic resource utilisation, it is important to identify the effect of copper exposure on long term fitness, especially reproductive success. To investigate the effect of copper on the recruitment process and long term fitness, a transplant

experiment was designed that created four selection regimes with increasing exposure to copper. This experiment aimed to investigate the impact of copper pollution on short and long term fitness, from initial settlement to adult reproduction. We found that high copper contamination strongly influenced recruitment patterns and morphological traits in the short term, with negative fitness costs, but long term exposure positively influenced growth and reproduction.

Materials and Methods

Exposure regime

To identify the short and long term fitness benefits of choosing a copper environment, exposure to copper was manipulated at two phases: firstly the initial recruitment period and secondly; during growth and reproduction, in a field based experiment. These changes in exposure mimic pulse and press pollution events, where an individual may be subjected to fluctuating copper concentrations at various or multiple life history stages. This was done using the method developed by Piola and Johnston (2009), using Perspex settlement plates with detachable collars to manipulate copper exposure. To expose settlement plates to copper, the Perspex collars were treated with two coatings of copper-based antifoulant paint (International® Micron Extra), while untreated collars were used as a procedural control. Settlement plates (11 by 11cm) were roughened to encourage settlement, to which the collars were attached using cable ties to form a 2 cm border.

Exposure to copper was manipulated at two stages in the field (Fig. 5.1). In the first stage, control and copper treatment settlement plates were exposed for 40 h, during initial settlement. In the second stage, half of the settlement plates from the two initial treatments, copper and control, were changed to the opposite treatment by removing and

replacing collars with the corresponding painted/unpainted ones. This experimental transplant occurred after the initial 40 h and was maintained for 8 – 10 weeks. This created four exposure regimes: 1. Control to Control, 2. Control to Cu, 3. Cu to Control, 4. Cu to Cu.

Settlement plates were deployed at Burraneer Marina in Port Hacking estuary ($34^{\circ}7'S$, $151^{\circ}10'E$) south of Sydney in New South Wales, Australia, during February 2010. This site has a high abundance of *W. subtorquata* colonies growing on artificial substrate and is an open marina. For ease of manipulation, settlement plates with collars (hereafter referred to as plates) were attached to panels. Each panel had four plates of the same treatment attached, from a single regime (1 – 4), to prevent any copper contamination between treatments. There were 20 plates, attached to five panels, per regime, although replicate numbers later became uneven due to settlement patterns and mortality.

To encourage recruitment of *W. subtorquata*, panels were hung horizontally at 1 m below mean low water level with the settlement surfaces facing down. Panels were randomly located in shaded areas of the marina. *W. subtorquata* release larvae from early in the day usually upon exposure to light and the larvae begin to settle immediately upon release (Marshall & Keough 2003). Hence, we deployed plates late in the afternoon to effectively sample the following day's recruitment. After approximately 40 h plates were pulled out of the water, the collars removed and all *W. subtorquata* recruits circled with a graphite pencil for identification and to monitor survival. Each recruit was photographed with a digital camera (Leica Model: DFC290) using a dissecting microscope and a photo taken of the overall plate to individually track and identify each recruit, with each individual given an identification number.



Figure 5.1 The four exposure regimes: 1. Control to Control, 2. Control to Cu, 3. Cu to Control, 4. Cu to Cu. Exposure to copper was manipulated at two stages. In the first stage, control and copper treatment settlement plates were exposed for 40 hours, during initial settlement. In the second stage, half of the settlement plates from the two initial treatments, copper and control, were changed to the opposite treatment by removing and replacing collars with the corresponding painted/unpainted ones. This experimental transplant occurred after the initial 40 hours and was maintained for 8-10 weeks.

Although recruitment of other species into either environment was minimal, any non-*W*. *subtorquata* recruits were removed by scraping before redeploying the plates.

Plates from each initial treatment were randomly assigned to control or copper exposure for the second stage of the experiment, in the transplant design to create the exposure regimes, and the collars re-attached accordingly. By randomising plates between the initial and second stage, plate was treated as a replicate without being nested in panel for all analyses involving both stages, although copper and control treatments were kept separate to prevent contamination. Due to recruitment being lower on the initial control plates, plate allocation was stratified to ensure even replication in regime 1 and 2, resulting in some panels having only three settlement plates. Before redeployment all panels were covered in plankton mesh (212 μ m) to prevent recruitment of other fouling organisms. The mesh was propped up off the settlement plates to allow recruits to grow unhindered and water to flow through.

A second census assessed survival after 10 d of redeployment (14 d after initial settlement) with plates brought back into the laboratory temporarily for this process. Because collars were left on to minimise handling disturbance, plates were kept in 50 L tubs with aeration and frequent water changes to prevent high copper concentrations, with copper and control treatments contained separately. Each recruit was classified as either alive, dead or crushed due to handling. All crushed recruits were noted and discarded from analysis. After the census, recruits were culled to leave only ≤ 8 individuals per plate, to minimise the effects of intraspecific competition on growth. Panels were then redeployed within 24 h of collection, with plates in the same configuration as previously and covered in clean plankton mesh. Panels were randomly

redistributed within the site after every census and mesh change. Panels were monitored on a weekly basis and the mesh, while never observed to be clogged or blocking water flow, was changed weekly to minimise any fouling and sedimentation. Plates and panels were also gardened to remove any fouling organisms, although the mesh appeared to be too fine for most larvae to penetrate.

After 8 weeks of deployment plates were collected from the field with their collars still attached and brought back to the lab to assess survival, growth and fecundity of the remaining recruits. Plates were collected in batches of five panels, with one panel per regime, over a two week period. Collection was staggered to allow for fecundity counts before attempting to obtain larvae through spawning from each plate (using methods described in Chapter 2). Unfortunately, very few larvae were collected and fecundity measurements were restricted to identifying colonies with gravid zooids to give a percentage of fecund colonies per plate. Each plate was photographed using a digital camera to measure final colony size as an indicator of growth.

Image analysis

Initial recruit size, or the first zooid formed as a result of successful metamorphosis (Piola & Johnston 2006a), was measured using the image processing program Leica Application Suit (LAS Version 3.6). We measured two dimensions: ancestrula length (mm), from the top of the operculum to the distal edge of the zooid, and width (mm), the widest point of the zooid. These measurements were multiplied together to give an initial recruit area/size (mm²). Final colony size (mm²) was measured using digital photos analysed in the image processing program Image J. Colonies that had merged with or were hindered by another colony were excluded from analysis, although very

few colonies were in contact with another colony. Any colonies that had been mechanically damaged by the collar or screw were also excluded from the analysis.

Statistical Analysis

Initial settlement

Initial settlement was compared between copper and control environments using a oneway fixed-factor ANOVA. Total settlement was pooled for each panel, with panel treated as the replicate, removing plate from the analysis. This included all individuals that recruited within the initial deployment period and was treated as density per cm². Data were tested for normality and homogeneity of variances using frequency histograms and Levene's test. Initial settlement had bimodal distribution and uneven variances which were unable to be fixed by transformation. As ANOVA is considered robust to uneven variances and non-normality (Underwood 1997) data were analysed untransformed and the significance lowered to $\alpha = 0.01$. These and all following analyses were run in the statistical program PASW Statistics version 18.

Short term fitness

Survival at two weeks was compared between each selection regime using a one-way fixed-factor ANOVA. As plates had been randomised between the initial and second stage, data were analysed as percent survival per plate, with plate as the replicate. Data were tested for normality and homogeneity of variances using frequency histograms and Levene's test. Data were skewed and residuals were unevenly distributed due to the high frequency of zeros in the regimes 1 and 2, which were unable to be fixed by transformation. As above, data were analysed untransformed and the significance lowered to $\alpha = 0.01$. A SNK test then identified the significant differences between regimes post-hoc.

The ancestrula size of recruits that survived compared to the size of recruits that died in each selection regime was analysed using a fixed two-factor ANOVA (Regime: fixed and Survival: fixed). Mortality was very low in regimes 1 and 2 (initial control environment), resulting in insufficient replicates and an unbalanced design when analysing the full data set (n = 6 and n = 3 respectively for sizes of recruits that died). As this was a planned comparison specifically to determine whether recruits suffering mortality in the regimes with an initial exposure to copper differed in size, a reduced data set containing only regimes 3 and 4 was analysed. Data were analysed as the average ancestrula size for recruits that either survived or died on each plate. Data were tested for normality and homogeneity of variances using frequency histograms and Levene's test. As this was a paired comparison, the full data set was first run to estimate the Error term for which the reduced data model MS values were tested over to calculate Fishers *F* statistic. Fitness relating to recruit size was estimated using the program Glmswin10 with a cubic spline calculation (Schluter 1988).

Long term fitness

Colony mortality, colony size and fecundity after seven weeks were compared between regimes using a one-way fixed factor ANOVA. Colony mortality data were analysed as percent mortality and colony size as average colony size (mm²) per plate. Fecundity was measured as the proportion of reproductive colonies per plate. All data sets were tested for normality and homogeneity of variances using frequency histograms and Levene's test, with plate as the replicate. Mortality and fecundity were analysed untransformed and the significance lowered to $\alpha = 0.01$ due to uneven variances and non-normal distributions respectively. Colony size data were log(x+1) transformed. A SNK test was then used to identify the significant differences between regimes post-hoc.

Results

Initial settlement

High numbers of *W. subtorquata* larvae recruited to the settlement plates, although densities ranged from as low as three to as high as 189 individuals on control and copper panels, respectively. Initial settlement was significantly greater in copper environments, with an average density of 0.36 recruits per cm² compared to a density of 0.03 recruits per cm² in the control environment (Table 5.1a, Fig. 5.2).

Short term fitness

After the initial settlement period and the experimental transplant of plates, survival at two weeks varied significantly between the four exposure regimes (Table 5.1b). Mortality was dependent on the initial environment with three times as many recruits dying in the regimes that began with copper exposure (~30% mortality in regimes 3 and 4), whilst almost all recruits survived in the regimes with an initial control environment (1 and 2) (Fig. 5.3a). Thus the final environment appeared to have no effect on survival during this two week period, despite the longer duration of copper exposure.

Overall, recruit size was also dependent on the initial environment, with individuals that settled in the initial control environment (regime 1 and 2) being much larger than recruits initially exposed to copper (regime 3 and 4), regardless of survival status (Fig. 5.3b). For recruits initially exposed to copper in regime 3 and 4 where mortality was greatest, a planned comparison compared sizes between individuals that survived or died. The individuals that died were significantly smaller than the recruits that survived, with fitness increasing with recruit size (Table 5.1c, Fig. 5.3b-c).

Table 5.1 ANOVA results for (a) Initial settlement in control and copper environments, (b) Short term mortality in all exposure regimes, (c) Recruit size comparing the size of recruits that survived or died in exposure regimes with an initial copper environment: 2 and 4, (d) Long term mortality in all exposure regimes (e) Final colony size in all exposure regimes and (f) Reproductive fitness in all exposure regimes, represented by the proportion of fecund colonies. *P*-values in bold represent significant differences at $\alpha = 0.01$, *P* = 0.000 denotes values < 0.001

Source	df	MS	F	Р
a. Initial Settlement				
Initial environment	1	0.554	82.20	0.002
Error	18	0.007		
b. Short term mortality				
Regime	3	0.273	5.25	0.003
Error	69	0.052		
c. Recruit size	_	a. a. i — 05		
Regime	1	9.94E ⁻⁰³	0.13	0.716
Survival	1	0.005	7.25	0.008
Regime*Survival	1	$2.98E^{-05}$	0.04	0.842
Error	104	$7.50E^{-04}$		
d. Long term mortality				
Regime	3	0.017	1.07	0.367
Error	64	0.016		
e. Final Colony Size	2	1.40	44.04	0.000
Regime	3	1.48	41.84	0.000
Error	60	0.035		
f. Reproductive Fitness				
Regime	3	1.699	23.67	0.000
Error	59	0.072		



Figure 5.2 Initial density per cm² of *Watersipora subtorquata* recruits after one day of recruitment to control and high copper environments. Values are mean \pm SE

While average recruit sizes were significantly different, size distributions overlapped between the initial exposure regimes (Fig. 5.3d). Recruits that settled into the control environment had the broadest size range, $0.16 - 0.34 \text{ mm}^2$, and this distribution fully encompassed the sizes of live and dead recruits in the copper environment. The individuals that settled into the initial copper environment and survived ranged from $0.20 - 0.27 \text{ mm}^2$, which overlapped partially with the size distribution of recruits that died $0.17 - 0.27 \text{ mm}^2$, although size frequencies differed.

Long term fitness

Very few colonies died after the second census with overall mortality only 5%. There was no significant difference between regimes, as mortality ranged between 0 and 8% across the four regimes and was highly variable within regimes (Table 5.1d).

All surviving colonies exhibited growth but were significantly affected by the different exposure regimes (Table 5.1e, Fig. 5.4a-b). The largest colonies were growing in the regimes which were exposed to copper during the second stage; 2 and 4, compared to colonies in regimes 1 and 3 (second stage control environment). In regimes 2 and 4 colonies averaged $226 - 324 \text{ mm}^2$ and were exhibiting bright red live tissue with active feeding zooids. Multiple colonies had actually grown onto the antifoulant coated collars, with no obvious detrimental effects of the copper. Colonies in these regimes differed significantly in size indicating an effect of the initial environment as colonies in regime 4, which were exposed to copper during settlement then copper in the final stage, were significantly smaller than regime 2 (control-copper treatment) (Fig. 5.4a). In comparison, colonies in regime 1 and 3 were significantly smaller (74 – 126 mm²), appearing less healthy with fewer live zooids.



Figure 5.3 Short term fitness of *Watersipora subtorquata* recruits under the four exposure regimes. (a) Mortality at two weeks (mean \pm SE), (b) Size of recruits mm² that survived and died within each regime (died: n = 6 for regime 1, n= 3 for regime 2)(mean \pm SE) (c) Survival as a function of recruit size (mm²) with a 95% confidence interval (dotted lines), (d) Size frequency distribution for: all recruits that settled in initial control environment (black diamonds), recruits that settled and survived in initial copper environment (grey triangles) and recruits that settled and died in the initial copper environment (black squares). *Italicised letters* represent significant differences between regimes and survival within regimes in post hoc comparisons ($\alpha = 0.05$).

Colonies began showing signs of reproduction from approximately six weeks after initial settlement, with obvious gravid zooids and new recruits settling within the mesh covered panels. Reproductive fitness differed significantly between regimes and was largely dependent on the final environment (Table 5.1f, Fig. 5.4c-d), with long term exposure to copper having a strong positive effect on the number of colonies that were fecund. The proportion of colonies that were reproductively active was greatest in regimes exposed to copper in the second stage, 2 and 4, averaging 63 – 87% (Fig. 5.4c). Regimes 2 and 4 differed significant but both were significantly greater than the proportion of reproductive colonies in regimes 1 and 3, which were unexposed to copper in the long term (Fig. 5.4c). The proportion of fecund colonies in regimes 1 and 3, at only 25% and 16% respectively, did not differ significantly (Fig. 5.4c). The difference between regime 2 and 4 indicate an effect of the initial environment, as early exposure to copper reduced reproduction, while colonies only exposed to copper during the final environment had significantly greater reproductive fitness.

Discussion

The transport of numerous marine NIS via hull fouling has selected for species characteristics that can influence invader success, in particular tolerance to copper through the use of copper-based antifouling paints. Many NIS are becoming increasingly associated with metal pollution in marine environments and this study aimed to investigate whether an invasive species, *W. subtorquata*, has evolved to benefit from copper pollution. We found copper significantly increased settlement of *W. subtorquata* larvae, although this was accompanied by higher mortality and



Figure 5.4 Long term fitness of *Watersipora subtorquata* recruits under the four exposure regimes. (a) Colony size (mean \pm SE), (b) Reaction norm for growth in selected (initial copper) and unselected (initial control) treatments in the final control and copper environments (c) Reproductive fitness, the proportion of reproductive colonies within each regime (mean \pm SE), (d) Reaction norm for reproductive fitness in selected (initial copper) and unselected (initial control and copper environments. *Italicised letters* represent significant differences between regimes in post hoc comparisons ($\alpha = 0.05$).

physiological effects on recruit size. These short term costs of recruiting to a copper polluted environment were off-set by considerable long term benefits, as long term exposure to copper significantly increased growth and the proportion of colonies that became reproductive. The positive affiliation between *W. subtorquata* and copper has consequences for numerous components of the invasion process. Firstly, it is expected to promote the continued spread of the species as there is an increased likelihood of recruitment onto a vector surface, such as a ship hull. Secondly, it is likely to further facilitate recruitment into environments that are impacted by copper contamination, notably most urbanised harbours and ports. The increased long term growth and reproduction in copper polluted environments will further enhance success at the introduction and establishment stage, as well as persistence within the new environment.

Short term fitness

Recruitment strongly affects population dynamics (Minchinton & Scheibling 1991; Myers & Harms 2009) and is important at multiple stages of the invasion process, including uptake onto transport vectors, introduction and establishment followed by range expansion (Lockwood et al. 2005). For many marine invertebrates recruitment involves larval offspring exerting preferential settlement choices, choosing habitat in response to environmental cues (Pawlik 1992). Despite the toxicity of copper (Hall et al. 1998), settlement was significantly greater in the initial copper environment, with average densities reaching over ten times higher than in the control environment. Considering that recruitment is severely inhibited in similar conditions for many other fouling organisms (Johnston et al. 2003), this unusual response suggests that *W*. *subtorquata* may be responding to copper as a settlement cue (Chapter 4). There are

ecological consequences to these settlement patterns at multiple stages of the invasion process. Larvae are more likely to settle, firstly, onto an antifoulant coated boat hull and secondly into polluted habitats such as harbours, ports and marinas, which correspondingly increases the likelihood of transfer to a new location and successful establishment if the location is anthropogenically impacted.

Early life history stages can be particularly susceptible to contaminants because they are periods of rapid growth and metamorphosis. Whereas an initial exposure to copper greatly increased settlement, post settlement mortality was also significantly greater, regardless of the final environmental conditions. This clarifies that the initial environment in which the larva settles and metamorphoses is of greater influence on survival than later conditions. This high mortality is unsurprising considering the toxic effect of copper on many organisms and its specific use as a biocide in the marine environment (Weis & Weis 1996; Srinivasan & Swain 2007). A consequence of high recruitment followed by high mortality is that copper is exerting strong selection on a cohort of recruits post-settlement. When a contaminant has a negative impact on the recruitment process, it can cause rapid shifts in the genotypic composition of a population, selecting for more tolerant individuals (Medina et al. 2007). This has been seen in both the aquatic and terrestrial environment, where populations of acutely exposed organisms have rapidly evolved tolerance (Klerks & Weis 1987; Eranen 2008). As tolerance is known to be variable within this species (Chapter 2 & 3), strong selection for tolerant genotypes is likely to occur in environments where copper contamination reaches high concentrations on either a periodic or continual basis, such as pulse pollution events or antifoulant painted surfaces (Valkirs et al. 2003; Warnken et al. 2004).

A physiological reduction in size was a further cost of recruiting into a copper contaminated environment. Offspring size is strongly positively linked with individual fitness for a variety of organisms (Smith & Fretwell 1974), yet recruits that settled into an initial copper environment were significantly smaller. Recruit size after metamorphosis is a measure representing larval history and size, as well as future fitness potential for organisms that have a larval stage (Pechenik et al. 1998). When not immediately lethal, contaminants can delay or facilitate metamorphosis in many organisms (e.g. amphibians (Gross et al. 2007)). The differences in recruit size yet the overlapping size frequency distributions between copper and control initial environments suggest that copper is affecting the metamorphosis process beyond simple mortality. And as a result of the stress from copper exposure during metamorphosis the ancestral zooid ends up reduced in size. It is possible that only larvae which are initially large enough to endure the stress would be capable of surviving settlement in a copper environment, with smaller larvae not having the energetic reserves to survive beyond metamorphosis (Marshall et al. 2003). Therefore in copper polluted environments fitness is still positively linked with larval size, as observed in the fitness curve and also supported by previous findings which found greater tolerance in colonies with a larger average offspring size (Chapter 2).

Exposure to stress during early life history stages, such as delayed settlement, limited food and contaminants, can have negative impacts on long term fitness measures including survival and growth (Pechenik et al. 1998; Ng & Keough 2003). This carryover effect was evident for growth and reproduction as while long term exposure to copper had a positive effect and increased fitness, this effect was greatest for colonies which had not been exposed during recruitment. Although there were negative

consequences of short term initial exposure, they were not consistent across regimes and these effects were far outweighed by the differences caused by long term exposure.

Long term fitness

Metal contamination is commonly associated with negative fitness impacts, such as higher mortality, reduced growth and lower fecundity (Shirley & Sibly 1999; Johnston et al. 2003). In comparison the fitness benefits of long term exposure to copper post settlement on *W. subtorquata* were strongly positive. Colonies were, on average, more than double the size and appeared much healthier (more active red zooids). Reproductive fitness followed a similar pattern, with the proportion of reproductive colonies almost three times greater in copper environments. These effects were observed in the absence of competition for space or food from other organisms.

Direct positive effects of a contaminant on fitness are rarely observed (although see Harper et al. (1997)). Occasionally they are seen in comparisons between organisms that have and haven't experienced strong selection through exposure. For example tolerant populations can exhibit faster growth, shorter maturation times and higher fecundity under contaminated conditions compared to non-tolerant populations (Shirley & Sibly 1999; Eranen 2008). In contrast, fitness was substantially increased by copper exposure post recruitment for both the selected (initial copper) and unselected (initial control) treatments. In theory these colonies would be expected to be outperformed by individuals that had not been selected for tolerance and maintained in a control environment (regime 1) (e.g. Shirley and Sibly (1999)), making these results even more novel.

There are many implications of an increase in growth and fecundity in a NIS when exposed to copper. Firstly, for individuals that have recruited onto an antifouled ship hull this will directly increase the number of propagules that are likely to be released in a new location. This may effectively increase the propagule pressure of any inoculation (Lockwood et al. 2005). Secondly, whilst *W. subtorquata* is generally considered a poor competitor (Floerl et al. 2004), greater fitness in a polluted environment compared to many native species (Piola & Johnston 2009) will ensure establishment success. In habitats that experience continuous or frequent pollution events this species is likely to persist, leading to further spread along pollution gradients (Piola et al. 2009).

Conclusion

The overall reduced fitness of this invertebrate in a clean (control) environment shows that *W. subtorquata* experiences fitness costs when not exposed to copper. This was consistent for both the selected and unselected populations, these being the individuals that recruited to the initial copper and control environments respectively. So although the short term costs of copper on recruitment appear contradictory to the long term benefits, by recruiting into a copper polluted environment overall reproductive output and therefore fitness is significantly increased. This suggests that *W. subtorquata* has evolved to benefit directly from copper pollution, rather than simply utilising resources made available through reduced competition from less tolerant species.

Tolerance in *W. subtorquata* may be the result of the widespread use of copper-based antifouling paints, or it may result from selection in more generally contaminated ports and harbours. Regardless, the global distribution of this invader in anthropogenically impacted habitats is undoubtedly a consequence of the current and historical practice of copper-based antifouling paint use. The continued use of copper-based antifoulant paints will only enhance the spread of this species and enable its persistence in

contaminated environments. This may apply to any fouling organisms that have experienced a similar evolutionary response.

Chapter 6 Summary

I found that tolerance to copper is a heritable trait in the non-indigenous bryozoan *Watersipora subtorquata*. It appears that *W. subtorquata* has evolved to benefit directly from copper pollution, rather than simply utilising resources made available through reduced competition from less tolerant species. Adult tolerance reflected larval tolerance in laboratory studies, which were supported by recruitment patterns found in manipulative field experiments. A positive affiliation between *W. subtorquata* larvae and copper polluted environments resulted in greater long term fitness despite short term costs. This species has also evolved multiple mechanisms to withstand pulse and press copper pollution events. The genetic variation I observed in these traits suggests that further exposure to copper pollution will result in rapid evolutionary change.

Evolution in the marine environment

This research sheds light on the evolutionary impact of metal pollution on a marine nonindigenous species (NIS), which in turn has ecological implications for native communities. Copper-based antifouling paint has been recognised as a selective pressure during the invasion process for hull-fouling organisms (Piola et al. 2009), but the exact mechanisms of tolerance, be it acclimation or a heritable trait, have not been explored (although see Galletly et al. (2007)).

For many organisms that have evolved tolerance to metal pollution, selection has resulted from site-specific exposure over multiple generations (Eranen 2008; Hendrickx et al. 2008). In contrast, selection for copper tolerance in marine NIS is likely to be less consistent and may fluctuate dramatically during the invasion process as well as throughout the life of an organism (Sakai et al. 2001; Piola et al. 2009). In addition,

many sessile marine organisms have distinctly different juvenile (mobile) and adult (sessile) stages (Pechenik 1999), which may vary in tolerance to copper (Xie et al. 2005). My research shows that larval tolerance to copper in *W. subtorquata* corresponds between laboratory-based toxicity assays and field-based experiments using copperbased antifoulant paint. Larvae and juveniles are more susceptible to contaminants (Xie et al. 2005), and the early life history mortality at higher copper concentrations reflected the reduced tolerance and genetic variation found in adult colonies at similar concentrations. This shows that exposure to commercially available copper-based antifouling paints directly selects for tolerant genotypes at the larval stage, which then delineates tolerance ranges in adults.

Pollution events in the marine environment can vary dramatically in frequency, intensity and duration. Even within an impacted site, such as a marina where there are multiple sources of copper pollution (Weis et al. 1998; Srinivasan & Swain 2007), disturbance events can cause a further spike in exposure (Knott et al 2010). Storms can create pulse pollution events due to sediment resuspension and urban run-off being contaminated with multiple pollutants, including metals such as copper and zinc (Fabris et al. 1999; Knott et al. 2009). On a finer spatial scale, proximity to pollution sources (e.g. antifoulant coated surfaces (Valkirs et al. 2003)) will result in heterogeneous exposure between individuals that may only be a few meters apart (Addison et al. 2008). These fluctuating conditions, compared to a constant exposure regime often experienced in chronically polluted environments (Klerks & Levinton 1989) may also select for the ability to survive then recover from a pulse pollution event. I found that *W. subtorquata* appears to have evolved multiple mechanisms to tolerate a range of pollution events. By partitioning growth between periods of exposure or recovery they

have adapted to withstand pulse and press pollution events. This explains their ability to colonise habitats ranging from antifouled surfaces to uncontaminated rocky reefs.

Recruitment strongly affects population dynamics (Minchinton & Scheibling 1991; Myers & Harms 2009) and is important at multiple stages of the invasion process, including uptake onto transport vectors, introduction and establishment followed by range expansion (Lockwood et al. 2005). For many marine invertebrates the larval offspring exert preferential settlement choice and choose habitat in response to environmental cues (Pawlik 1992). Contaminants, particularly copper, have been shown to negatively impact settlement (Johnston & Keough 2002). My research found that, despite the toxicity of copper, recruitment was significantly greater in copper environments. This suggests that W. subtorquata larvae are responding to copper as a settlement cue (either directly or through the effect that copper may be having on developing biofilms). This overall increase in recruitment was also accompanied by short term fitness costs including post-settlement mortality. These recruitment patterns appear contradictory, since a positive larval response to a settlement cue implies that there are fitness benefits to choosing that habitat. In comparison, I found that the fitness benefits of long term exposure to copper post settlement on *W. subtorquata* were strongly positive. Although there are short term costs of copper on recruitment, recruiting into a copper polluted environment increased overall reproductive output and therefore fitness. This suggests that W. subtorquata has evolved to benefit directly from copper pollution, to the extent that it appears to use this highly toxic agent as a settlement cue.

Research Novelty

Ecological and evolutionary processes can be studied as distinct fields of research, despite their interdependence. Ecological interactions act as selection pressures causing evolutionary change, which in turn can redefine ecological relationships. Examples of this are invasive species that undergo rapid evolution in response to anthropogenic disturbance. An aim of this thesis was to create a mixed disciplinary approach in an attempt to fully elucidate the evolutionary processes behind the ecological patterns. I did this by incorporating ecological and toxicological techniques into quantitative genetics experimental designs.

Traditionally, tolerance to contaminants has been assessed at a population level (Johnston In press). To estimate the heritability of tolerance I adapted larval settlement toxicity assays by using a split family design, treating colony as the maternal parent. For the adult assays I used a novel approach by cloning *W. subtorquata* colonies and exposing them independently to a gradient of copper concentrations. This demonstrates that modular organisms are a useful system to estimate genetic variation when a breeding design is unachievable due to physiological constraints. These methods allowed me to test for a genotype X environment interaction, which indicate whether genotypes differ in their response to environmental conditions and the potential for evolutionary change. It can also be inferred whether the genetic basis of traits differs across environments by looking at the specific combinations of genetic and environmental factors that influence a trait (Falconer & Mackay 1996; Galletly et al. 2007).

Toxicity testing in laboratory conditions can lack ecological relevance, hence the need for comparative field-based manipulative experiments. I manipulated exposure to

copper during multiple life history stages while controlling for ecological effects, such as competition, to create realistic selection regimes. These selection regimes represented pulse and press pollution events and mimicked selection events that occur throughout the invasion processes, such as during species entrapment and transfer. Thus, I was able to test both the selective pressure of copper and its ecological implications.

Future Directions

The combination of methodologies employed within this thesis are an example of how cross-disciplinary research can yield greater insights into ecological problems. I have shown that a NIS has evolved to benefit from a common contaminant, which is useful in explaining its global distribution. Given the similarities between *W. subtorquata* and many other fouling species in their method of introduction and therefore history of selection, it is likely that other NIS have undergone similar evolutionary changes. The next step is to use this information on the evolutionary response to an anthropogenic disturbance to reduce the spread and impacts of invasive species. For example, the ability to evolve to one contaminant may result in co-tolerance to other stressors, such as to other contaminants or biological tolerances. Identifying these attributes, including the capacity to rapidly adapt, will help us understand the species characteristics that enhance invasion success.

Final Remarks

This thesis aimed to examine the basis of copper tolerance in the non-indigenous species *W. subtorquata*, and whether this successful NIS has evolved to benefit from a common contaminant in the marine environment. By comparing laboratory toxicity assays on multiple life history stages and incorporating quantitative genetics into the experimental designs, I was able to determine the basis of tolerance. These novel

methods resulted in estimates of heritability and genetic variation for tolerance in larval and adult *W. subtorquata*, respectively. These levels of tolerance corresponded with juvenile mortality in field-based experiments. In an unprecedented result, I found copper pollution significantly increased recruitment, which culminated in considerable long term benefits. There were short term costs of recruiting to a copper polluted environment, including greater post-settlement mortality and reduced recruit size. These, however, were off-set by the substantial long term benefits as long term exposure to copper significantly increased growth and the proportion of colonies that became reproductive. Consequently, it appears that this species is significantly advantaged in copper polluted environments.

Tolerance in *W. subtorquata* may be the result of the widespread use of copper-based antifouling paints, or it may result from selection in more generally contaminated ports and harbours. Regardless, the global distribution of this invader in anthropogenically impacted habitats is at the very least, partially attributable to the current and historical practice of copper-based antifouling paint use. The continued use of copper-based antifoulant paints will only enhance the spread of this species and enable its persistence in contaminated environments.

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