

# **URCHIN MEDIATED POSITIVE INTERACTIONS**

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## **DECLARATION**

I certify that the work in this thesis entitled “Urchin mediated positive interactions” has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University. I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance that I have received in my research work and the preparation of the thesis itself have been appropriately acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

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## ABSTRACT

There is increasing interest in how positive direct and indirect interactions involving two or more species, maintain community structure. This project aims to investigate the important aspects of three urchin-mediated positive interactions. First we investigated whether the positive relationship between the kelp *Ecklonia radiata* and the herbivorous gastropod *Phasianotrochus eximius* is mediated by a third species, the sea urchin *Holopnuestes purpurascens*, via a facilitation cascade. Both the sea urchin and gastropod are found enmeshed within the laminae of the common kelp *Ecklonia radiata*, which both species consume. Sampling and experiments carried out both in the laboratory and at four sites within Sydney, Australia revealed that both *H. purpurascens* and *P. eximius* were more abundant on kelp than other algal species, and that the gastropod was more abundant on kelp with than without the urchin. When the presence/absence of *H. purpurascens* on kelp plants was manipulated experimentally in the field, *P. eximius* recolonised kelp plants at higher rates when sea urchins were present. Individual *P. eximius* on plants inhabited by *H. purpurascens* were larger than those on plants without the urchin. Adult *P. eximius* used visual cues to locate its two associated species. Gastropods were tethered to plants for 24 hours to test mortality of individuals with as opposed to without sea urchins. Survival of individuals on plants with *H. purpurascens* was greater than on plants without *H. purpurascens* suggesting that the *H. purpurascens* and the refuge it shares with the *P. eximius*, may be protecting the gastropod from predation and/or adverse microclimates. In addition, *P. eximius* was found to not only consume the kelp and epiphytes in which it inhabits but also the excrement of the urchin. Carbon and nitrogen measurements for these three food sources showed a higher nitrogen value for urchin excrement compared to the other food sources, suggesting that there may be a nutritional benefit for the gastropod in associating with the sea urchin. This study provides one of the first demonstrations of a facilitation cascade in a subtidal marine environment. The second part of this project examined the relationship between the isopod



*Exosphaeroma echinensis* and the sea urchin *Evechinus chloroticus* in northern New Zealand. A series of field and laboratory experiments showed that the isopod preferentially inhabits the spines of the sea urchin over other potential habitats. This relationship appears to be commensal in that the sea urchin spines are likely to provide shelter to the isopod. The isopod uses visual cues as opposed to olfactory cues to locate its host and is efficient in host recognition in the field. The final part of this thesis examined the methods of host recognition by the shrimp *Gnathophylloides mineri*, which is symbiotically associated with the sea urchin *Tripneustes gratilla*. *G. mineri* locates its host via olfactory cues emitted into the water column by its host. Within each of the urchin-mediated interactions investigated within this thesis, the sea urchin appears to play a positive role by providing refuge to its associated species. Thus, this study has highlighted the important role that sea urchins can play in mediating positive associations and adds to the growing evidence that positive interactions (both direct and indirect) are a common mechanism by which community structure is maintained. A major focus in ecological literature on sea urchins to date has been on their negative effects, such as overgrazing resulting from reduced predator abundances. This thesis highlights some of the positive effects that some sea urchins can have by providing food and shelter to associated species.

## CHAPTER DECLARATIONS

### **Chapter 1: Introduction**

Jacqueline Bell wrote this chapter. Jane Williamson, Richard Taylor and Melanie Bishop provided constructive comments and editorial advice.

### ***Chapter 2: A facilitation cascade maintains a kelp community***

Jacqueline Bell, Jane Williamson, Richard Taylor and Melanie Bishop conceived and designed the experiments. Jacqueline Bell performed the experiments. Jacqueline Bell analyzed the data. Jacqueline Bell wrote the manuscript, other authors provided editorial advice.

Bell J. B. Bishop, M. J., Taylor, R. B. and Williamson, J. E. (2014) Facilitation cascade maintains kelp community. *Mar. Ecol. Prog. Ser.* DOI 103354/MEPS10727

### **Chapter 3: The role of gastropod behaviour in mediating the establishment and maintenance of a habitat cascade in a temperate Australian kelp forest**

Jacqueline Bell, Jane Williamson, Richard Taylor and Melanie Bishop conceived and designed the experiments. Jacqueline Bell performed the experiments. Jacqueline Bell analyzed the data. Jacqueline Bell wrote the manuscript, other authors provided editorial advice. Peter Schlegel provided assistance in the field. David Balk provided assistance in the laboratory.

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## **Chapter 4: Nutritional Benefits of a Gastropod Cohabiting with a Kelp-Dwelling Sea Urchin: A Case of Coprophagy?**

Jacqueline Bell, Jane Williamson, Richard Taylor and Melanie Bishop conceived and designed the experiments. Jacqueline Bell performed the experiments. Jacqueline Bell analyzed the data. Jacqueline Bell wrote the manuscript, other authors provided editorial advice. Peter Schlegel provided assistance in the field. Ryan Nevatte, Marion Binda and Peter Sampson provided assistance in the laboratory. Sue Lindsay provided assistance in scanning electron microscopy at the Australian Museum. Nicole Vella provided assistance in histology at Macquarie University. Mahamaad Masood provided assistance in the use of the LECO CHN analyzer at the department of Chemical and Biomolecular Sciences at Macquarie University.

## **Chapter 5: The sea urchin *Evechinus chloroticus* as habitat for the isopod *Exosphaeroma echinensis***

Jacqueline Bell, Richard Taylor and Jane Williamson conceived and designed the experiments. Jacqueline Bell performed the experiments. Jacqueline Bell analyzed the data. Jacqueline Bell wrote the manuscript, other authors provided editorial advice. Richard Taylor and Tamson Peebles provided assistance in the field and in the laboratory.

## **Chapter 6: The role of visual and chemical cues in host detection by the symbiotic shrimp *Gnathophylloides mineri***

Jane Williamson wrote the manuscript. Fieldwork and experiments were conducted by Devarajen Vaithilingon and Christine Gleeson. Jacqueline Bell conducted the statistical analyses and assisted in manuscript preparation. Jacqueline Bell and Devarajen Vaithilingon provided constructive comments and editorial advice.

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## **Chapter 7: Discussion**

Jacqueline Bell wrote this chapter. Jane Williamson, Richard Taylor and Melanie Bishop provided constructive comments and editorial advice.

## **Appendix A: Robust age and growth parameters for three heavily exploited shark species off temperate eastern Australia**

Vertebrae samples were collected on-board commercial shark fishing vessels by myself and other NSW DPI scientific observers. Laboratory procedures (i.e. vertebrae cleaning and sectioning) were carried out by myself, with assistance from Jacqueline Bell.

Vertebral ageing data collection was conducted by Pascal Geraghty (Reader 1) and by Michelle Yerman (Reader 2). I was exclusively responsible for all data and statistical analyses, interpretation and written content, and used a range of *R* scripts provided by Alastair Harry including for non-linear growth model fitting and multi-model inference analysis.

Constructive comments on drafts were provided by William Macbeth, Jane Williamson, Jacqui Bell and Alastair Harry.

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## PRESENTATION OF RESEARCH FROM THIS THESIS

### Poster presentations

Australian Marine Science Association 2009 (Wollongong, NSW, Australia)

“The canopy dwelling urchin *Holopneustes purpurascens* makes seaweeds more attractive habitats for the gastropod *Phasianotrochus eximius*”

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### Oral Presentations

Benthic Ecology Meeting 2013 (Savannah, Georgia, USA)

“Facilitation cascade determines a positive interaction in a kelp community”

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NSW DII Fisheries Seminar Series 2010 (Sydney)

“Host Plant Selection by the Gastropod *Phasianotrochus eximius*: direct versus indirect effects”

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## **Media**

Unlikely friendship sets marine researcher on conservation path”

26<sup>th</sup> February 2010

Macquarie University Newsroom

“Science at Snail’s Pace “

6<sup>th</sup> March 2010

The Manly Daily

“Snail finds safe harbour beneath the sea”

24<sup>th</sup> March 2010

The Australian

“Exotic wonderland in our backyard”

6<sup>th</sup> May 2010

The Mosman Daily

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## CHAPTER 1: GENERAL INTRODUCTION

### Positive Associations

Positive interactions between species have been increasingly acknowledged for their importance in contemporary ecological theory (Stachowicz 2001, Bruno et al. 2003, Silliman et al. 2011). Such interactions occur between two or more species when at least one participant benefits and neither are harmed and take place, simply, as either commensal (+, 0) or mutual effects (+, +) (Bertness and Callaway 1994, Stachowicz 2001, Bruno et al. 2003, Hay et al. 2004). A species that has a positive effect on another is referred to as a facilitator (Hacker and Gaines 1997, Bruno et al. 2003). Facilitative or positive interactions tend to be most common in environments with high physical stress and/or where strong consumer pressure exists (Bertness and Calloway 1994, Silliman et al. 2011). Here, facilitators play a positive role by ameliorating environmental stress and by creating complex habitat that can ameliorate effects of competition and/or predation (Stachowicz 2001). Relationships between facilitators and associated organisms may be obligate or facultative, depending on the level of risk to survival for the associated species outside of the relationship (Bertness and Callaway 1994).

One of the most well-studied obligate interactions involving a facilitator species is that between corals and their photosynthetic dinoflagellate symbionts, zooxanthellae (Stat et al. 2008). Photosynthesis by zooxanthellae provides their coral host with carbohydrates, while the resident zooxanthellae receive the majority of nutrients via nitrogenous waste originating from the prey of their carnivorous coral host (Hay et al., 2004). Carbohydrates produced by the association are used by the coral for calcification and growth, allowing them to grow at a rapid rate, necessary for survival (Muscatine and Porter 1977, Muscatine et al. 1981, Hay et al., 2004). By contrast, facultative associations occur when individuals are capable of surviving outside of the association. An interesting example of a facultative association that

occurs in benthic marine invertebrates is the relationship that occurs between a deep sea gastropod and sea anemone (Mercier et al. 2011). The mutual protection from predators that this relationship provides allows both species to exploit food-rich areas more frequently, leading to a nutritional advantage for these individuals (Mercier et al. 2011).

### **Direct and Indirect Effects**

Positive interactions can occur as a result of direct or indirect effects between species. A direct effect occurs as a result of a physical interaction between two species (Wootton 1992), such as provision of shelter or food (Menge 1995). An indirect effect occurs in multispecies assemblages when one species causes a change in a second species that in turn affects a third species (Schmitt 1987, Dill et al. 2003). An example of a positive indirect effect is a ‘facilitation cascade’, which occurs when successive facilitative interactions take place as a result of the formation of new habitat, or the modification of existing habitat (Menge 1995, Altieri et al. 2010). Here, a basal habitat former facilitates an intermediate habitat former, which in turn facilitates a focal species (Altieri et al. 2010). Thomsen et al. (2010) conceptualize a specific type of facilitation cascade, described as a ‘habitat cascade’ and investigate one particular example whereby small invertebrates facilitate habitat for seaweeds, which in turn facilitate habitat for focal species of invertebrates and epiphytes.

The number of studies dedicated to positive interactions, especially those that occur in marine environments (Wootton 2002, Thomsen et al. 2010), are far less than those dedicated to processes such as predation and competition (Bertness et al. 1999, Martinsen et al. 2000). An understanding of positive interactions, and both the direct and indirect pathways along which they occur, is essential in order to accurately predict the impact of potential perturbations on communities and for successful management of marine ecosystems.



## **Marine Communities**

Marine benthic communities inhabit highly dynamic environments (Sousa 2001). Storm surges, wave action, tides and currents as well as biotic factors related to food web dynamics, all contribute to the dynamics of this environment (Schiel 2006). Facilitator species within these systems include foundation species such as kelps (Davenport and Anderson 2007), seagrasses (Pages et al. 2013) and mangroves (Sheridon and Hays 2003). These mitigate environmental stressors for associated species through substrate formation (Bruno and Bertness 2001, Altieri et al. 2007), enhancement of larval settlement (Rule and Smith 2005), provision of food (Bostrom and Matilla 1999), shelter from physical forces such as wave action, tides and currents (Kemppainen et al. 2005), and refuge from predation (Grabowski et al. 2008). These species often form large aggregations whereby facilitation of generally smaller species occurs through the creation of habitat heterogeneity (Bruno and Bertness 2001).

Despite recognition of the importance of positive interactions in marine communities, there are still many positive interactions, especially within marine environments, that are yet to be described, and the complexities that define these relationships, including the motivation to associate with other species, remain poorly understood (Bertness et al. 1999). By identifying both the direct and indirect effects that define positive associations, as well as the methods by which they take place, a more thorough understanding of the establishment and maintenance of the ecological community, as a whole, can be achieved (Martinsen et al. 2000, Wootton 2002, Thomsen et al. 2010). This thesis investigates the direct and indirect effects that define aspects of three sea urchin-mediated positive associations.

## **Sea Urchins as Facilitators**

Sea urchins are considered ‘ecosystem engineers’ in rocky reef ecosystems, having the ability to drastically modify the biogenic structure of habitats by maintaining areas devoid of

macroalgae, namely ‘urchin barrens’ (Bruno and Bertness 2001, Lawrence, 2006). Thus far, most of the ecological literature on sea urchins has focused on the negative impacts that these species can have on biodiversity (Estes 1978, Harrold and Reed 1985, Pinnegar et al. 2000, Carter et al. 2007, Terborgh and Estes, 2010, Byrnes et al 2006). However, sea urchins can also mediate positive interactions, as facilitators (McClosky 1970, Patton et al. 1985, Stebbins 1988, Doti et al. 2008, Williamson et al, 2012). Perhaps the most common and simplest way that sea urchins mediate positive interactions is by providing shelter to other small invertebrates (Dix 1969, Patton et al. 1985, Roberts and Hawkins 1999, Kolm and Berglund 2003). However, for small marine invertebrates, associations with sea urchins can be driven by a range of environmental obstacles that need to be efficiently overcome in order to survive (Poore and Steinberg 1999, Kumagai 2008). These not only include the need for shelter but also finding a reliable and nutritious food source, as well as access to mates, the former two being generally considered the most important driving factors in habitat and/or host choice (Duffy and Hay 1991, Bostrom and Mattila 1999, Orav-Kotta and Kotta 2004, Alfaro 2007). Ideally, an individual will choose a habitat or host that provides all of these attributes (Wootton 2002). Positive interactions that occur between herbivorous species are considered to take place via two broad mechanisms: (1) resource-mediated, where feeding by one herbivore benefits an associated herbivore by changing the attributes of the host plant or (2) refuge-mediated, where the provision of shelter by one herbivore is utilised by another herbivore. Plant-mediated indirect effects or ‘habitat cascades’, involving herbivores typically occur in temporally-separated, spatially-separated or taxonomically distinct species (Damman 1993, Martinsen et al. 2000, Ohgushi 2005).

## **Background and Aims**

The main aim of this thesis is to document and compare different urchin mediated positive associations in Australasia. The sea urchin *Holopneustes purpurascens* occurs in the coastal

waters of New South Wales, Australia (Steinberg 1995). It is commonly found enmeshed within the fronds of the kelp *Ecklonia radiata* where it uses its tube feet to hold onto the laminae of the kelp, creating a refuge around itself (Steinberg 1995). Within this refuge are often aggregations of the small herbivorous prosobranch *Phasianotrochus eximius* (Rogers et al. 2003). The relationship between *H. purpurascens*, *E. radiata* and *P. eximius* is hypothesised to occur as a result of a positive indirect effect in the form of a hierarchical facilitation cascade, whereby *E. radiata* facilitates *H. purpurascens* by providing it with habitat and food, and *H. purpurascens* in turn facilitates *P. eximius* by providing it with shelter and/or a reliable food source. The main aims of this part of my thesis are to: (1) Establish whether the relationship between *P. eximius* and *H. purpurascens* is hierarchical by quantifying the abundance and distribution of both species at representative sites in Sydney, Australia (Chapter 2); (2) Identify the method of host/habitat recognition for *P. eximius* (Chapter 3); and (3) Identify the potential nutritional and/or habitat benefits of this association for *P. eximius* (Chapter 4).

By comparison, in the coastal waters of New Zealand the isopod *Exosphaeroma echinensis* is directly associated with the sea urchin *Evechinus chloroticus*. *E. echinensis* has been observed to inhabit the spines of the sea urchin (Dix 1970). It displays cryptic colouration by way of its brown body and white uropods matching the colour of the urchin spines and thus camouflaging the isopod (Hurley and Jansen 1977). I hypothesise that *E. echinensis* preferentially inhabits the spines of the sea urchin *E. chloroticus* over other more exposed habitats and that this relationship is positive due to the nutritional advantages for *E. echinensis* in associating with *E. chloroticus*. The main aims of this part of my thesis are to: (1) Describe the abundance and distribution of *E. echinensis* at sites close to Leigh, northern New Zealand (Chapter 5); (2) Identify the method of host/habitat recognition for *E. echinensis* (Chapter 5); and (3) Identify the potential nutritional advantages for *E. echinensis* resulting from associating with *E. chloroticus* (Chapter 5).

A similar type of urchin-mediated relationship occurs in the coastal waters of New South Wales, Australia, where the sea urchin *Tripneustes gratilla* is host to the shrimp *Gnathophyllodes mineri*. Unlike the two examples described above, much is known of the ecology of this association (Bruce 1974, Patton et al. 1985, Maciá and Robinson 2009). This species of shrimp inhabits the spines of not only *T. gratilla*, but many other species of urchins, including *T. ventricosus*, *T. ensculentus* and *Pseudoboletia indiana* (Patton et al. 1985). *G. mineri* gains shelter from predation and adverse abiotic conditions by hiding between the sea urchin spines (Patton et al. 1985). The methods of host recognition for *G. mineri* that associate with *T. gratilla* are yet to be identified. Strong selection for the ability to detect and locate an appropriate host is often evident for symbiotic species, since the cost of failing to find a host can amount to predation or a lower reproductive success rate (Williamson et al. 2012). Symbiotic crustaceans often use a combination of cues for host recognition (Huang et al. 2005, Sikkel et al. 2011). I hypothesise that *G. mineri* uses a combination of visual and olfactory cues to identify its host *T. gratilla*. The aims of this final part of my thesis are (1) Quantify the abundance of *G. mineri* on *T. gratilla* versus other morphologically similar sea urchins that overlap in distribution and life history strategies to *T. gratilla*; (2) Identify the part of the urchin test that *G. mineri* usually inhabits; (3) Determine whether *G. mineri* display preferential movement towards *T. gratilla* over other species of potential host sea urchins using visual cues in the absence of chemical cues; and (4) Determine whether *G. mineri* preferentially move towards *T. gratilla* over other potential host sea urchins using chemical cues in the absence of visual cues.

By gaining a better understanding of the roles of sea urchins in mediating positive associations, and the direct and indirect pathways via which they occur, effective management strategies for the conservation of these environments can take place. The role that sea urchins have in facilitating other species should be taken in to consideration when managing marine resources. This thesis will increase the ability to predict the outcome of perturbations such

urchin harvesting, overfishing and climatic events, to the functioning of these urchin environments by not only taking into account the potential effect on sea urchins but also the cascading affects on the species that sea urchins facilitate.

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## CHAPTER 2: A FACILITATION CASCADE MAINTAINS A KELP COMMUNITY

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## **Abstract**

There is increasing interest in how facilitation cascades – linked positive indirect interactions involving at least three species – maintain community structure. Here we investigated whether the positive relationship between the kelp *Ecklonia radiata* and the gastropod *Phasianotrochus eximius* is mediated by a third species, the sea urchin *Holopnustes purpurascens* via a facilitation cascade. Both the urchin and gastropod are found enmeshed within the lamina of the common kelp *Ecklonia radiata*, which the urchin is known to consume. Sampling of urchin and gastropod abundances at four sites in Sydney, Australia, over two years, revealed that both *H. purpurascens* and *P. eximius* were more abundant on kelp than other algal substrates, and that the gastropod was more abundant on kelp with than without the urchin. Large *P. eximius* were found only on plants inhabited by *H. purpurascens*, suggesting that either *P. eximius* is able to survive for longer when it is part of this association or that adults actively move onto this substrate. When the presence/absence of *H. purpurascens* on kelp plants was manipulated experimentally in the field, greater recolonisation by *P. eximius* of kelp with than without urchins was observed. Urchins by contrast did not differentially respond to kelp with and without the gastropod, indicating that the association between the kelp and urchin is maintained by a unidirectional facilitation cascade. Our study adds to growing evidence that facilitation cascades are a common mechanism by which community structure is maintained. Studies are now needed to assess their sensitivity to environmental change.

**Keywords:** indirect effect, plant, herbivore, positive, interaction, marine.

## **Introduction**

Indirect effects, where the effect of one species on another is mediated by one or more additional species, are pervasive in ecological systems (Menge 1995, Abrams and Matsuda 1996, Stachowicz 2001, Wootton 2002, Werner and Peacor 2003, Ohgushi 2008).

Consequently, our ability to predict the responses of ecosystems to perturbations is dependent on understanding indirect effects (Paine 1980, Dambacher et al. 2002, Bolker et al. 2003, Borer et al. 2005, Borrett et al. 2010).

There is growing interest in when and where indirect effects lead to facilitation cascades (Bruno and Bertness 2001, Bruno et al. 2003, Altieri et al. 2010, Angelini et al. 2010). In a facilitation cascade, a basal species facilitates an intermediary species, which in turn facilitates the focal species or community (Altieri et al. 2010, Angelini et al. 2010). In many instances, these cascades arise through the provision of structured habitat by the basal and intermediary facilitators, which are in turn utilized by secondary inhabitants for protection from natural enemies and/or adverse micro-climates (Thomsen et al. 2010). Among these habitat cascades are those underpinned by shelter-building. In this case, a plant facilitates a herbivore by providing food and/or structural habitat, and the herbivore in turn facilitates other organisms by modifying the physical architecture of the plant to provide shelter (Bruno and Bertness 2001, Ohgushi 2005, Thomsen et al. 2010).

Shelter building is widespread in terrestrial systems but there are fewer examples of it in the sea (Ohgushi 2005). This may be because marine herbivory is substantially greater than terrestrial herbivory and often exceeds the amount of primary production of macrophytes (Cyr and Pace 1993). In addition, abiotic factors such as wave action, tides and currents in shallow coastal habitats where marine herbivory is concentrated may make shelters more difficult to build and maintain.

This paper examines how shelter building by the sea urchin *Holopneustes purpurascens* mediates the relationship between the gastropod *Phasianotrochus eximius* and the common kelp *Ecklonia radiata*. *H. purpurascens* is unusual amongst sea urchins in that it is arboreal, inhabiting the canopy of the living algae that it uses as both habitat and food (Steinberg 1995). *H. purpurascens* spawn predominantly in the Australian summer (Williamson and Steinberg 2002), and larvae settle and metamorphose on the foliose red alga *Delisea pulchra* in response to chemical cues, then juveniles later move to the kelp *E. radiata* where they wrap themselves within the algal thallus (Williamson et al. 2000, Williamson et al. 2004). This shift increases the sea urchins' rate of survival, growth and reproductive output (Williamson and Steinberg 2012). The gastropod *P. eximius* occurs on the same host plants as the urchin and the two are usually found together, enmeshed within the fronds of *E. radiata* (Steinberg 1995)

Here we test the hypothesis that the association between *E. radiata* and *P. eximius* is maintained by a facilitation cascade, whereby the kelp *E. radiata* facilitates the intermediary, *H. purpurascens*, and *H. purpurascens* in turn facilitates *P. eximius* by building shelters with kelp blades. First, we tested whether (1) *H. purpurascens* and *P. eximius* were each more abundant on the kelp *E. radiata* than on other algal species and (2) whether *P. eximius* was more abundant on *E. radiata* with than without *H. purpurascens*. Second, we tested the longevity of the shelter built by *H. purpurascens* for *P. eximius* by assessing the frequency of movement of “sheltered” *H. purpurascens* on *E. radiata* over time. Third, the size distribution of *P. eximius* on all host plants was compared to that on *E. radiata* plants containing *H. purpurascens* to assess whether *P. eximius* are likely to recruit directly to, or immigrating post-recruitment to the *E. radiata*/*H. purpurascens* association. As the majority of *P. eximius* were larger in the association than on other species of algae (including *E. radiata* without *H. purpurascens*) it was hypothesised that the gastropods must be actively seeking the sheltered association post-recruitment. Hence, fourthly, a field experiment tested the hypothesis that there would be greater recolonisation of *P. eximius* to *E. radiata* plants with *H. purpurascens*

compared to those without the urchin. This experiment allowed us to distinguish the effects of the sea urchin per se from plant properties that might have made individual *E. radiata* attractive to both *H. purpurascens* and *P. eximius* in the absence of any interaction between the latter two species. Finally, laboratory experiments were conducted to determine if urchins by contrast responded to kelp with and without the gastropod.

## **Methods**

### ***Study site***

Field experiments were done in rocky subtidal habitats (between 1-5 m depth) at Bare Island (33° 59' 38" S, 151° 12' 00" E), Balmoral (33° 83' 02" S, 151° 24' 88" E), Long Bay (33° 96' 50" S, 151° 25' 37" E) and Fairlight (33° 80' 00" S, 151° 27' 54" E) in Sydney, New South Wales, Australia. These sites were sheltered from large waves and comprised irregular patches of barrens and macroalgal habitat in which both *H. purpurascens* and *P. eximius* occurred. Forests of the kelp (*E. radiata*) were common at all sites surveyed, with other macroalgal species including *Sargassum vestitum*, *S. linearifolium*, *Corallina officinalis* and *Delisea pulchra* interspersed throughout, as well as a mosaic of crustose algae encrusting understory rock.

### ***Sources of spatial variation in the abundance of Phasianotrochus eximius and***

### ***Holopneustes purpurascens***

To test the hypotheses that: (1) both *P. eximius* and *H. purpurascens* would be more abundant on *E. radiata* than on the common species of macroalgae, *S. vestitum*, *S. linearifolium* and *C. officinalis*, (2) *P. eximius* would be more abundant on *E. radiata* with than without *H. purpurascens*, and (3) these patterns would persist across seasons and years, sampling was done at the four Sydney study sites over two years. Fairlight and Bare Island were sampled once every season in October, February, April and June, between October 2009 and June



2011 Balmoral was sampled on the first four dates, but was replaced by sampling at Long Bay from October 2010 to June 2011 because, by June 2010, both *P. eximius* and *H. purpurascens* had virtually disappeared from the Balmoral site.

During each sampling period, ten haphazardly-selected replicate plants of the algal species *E. radiata*, *S. vestitum*, *S. linearifolium* and *C. officinalis* were collected from 2-7 m depth at each site using SCUBA. Whole plants were sealed in plastic bags *in situ* then transported back to the laboratory at Macquarie University, where animals were removed for enumeration by twice immersing the plants in fresh water for 30 s. A pilot study in which the abundance of mobile fauna on plants were compared between plants with and without immersion confirmed that this method was 100% efficient in removing all animals greater than 0.5 mm (Bell unpublished data). Within each sample, the numbers of *P. eximius* and *H. purpurascens* were counted.

Separate analyses of variance (ANOVAs) compared the abundances of each of *P. eximius* and *H. purpurascens* among algal substrates and sampling times at each study site. ANOVAs for Bare Island and Fairlight had three orthogonal factors – year (random), season (fixed) and algal species (fixed). ANOVAs for Balmoral and Long Bay each had two factors, season and algal species (each fixed). Two sample t tests were used to test for any significant differences in the abundances of *P. eximius* on plants with *H. purpurascens* compared to plants without *H. purpurascens*. These analyses used data pooled across all algal species at all seasons and sites and were chosen over a correlation because gastropod abundance was not necessarily expected to increase with urchin abundance on plants with urchins. Assumptions of normality and homogeneity of variance were determined using Levene's test of equality of error variances. These assumptions were not met in some instances, even following  $\ln(x + 1)$  transformation of the data. Since ANOVAs are relatively robust to heterogeneous variances (Underwood 1997), the inflated Type I error that resulted from heterogeneous variances was

countered by a more stringent *P*-value of 0.01. Post hoc tests were carried out using a Sidak test of multiple comparisons.

### ***Movement of Holopneustes purpurascens on its host over time***

For *P. eximius* to gain benefit from shelter building by *H. purpurascens*, presumably there would need to be some temporal persistence of shelters and hence urchins on individual *E. radiata*. The temporal stability of urchin numbers on individual *E. radiata* was assessed through a study at Fairlight. In December 2009, fifteen haphazardly-selected *E. radiata* plants were tagged with flagging tape and surveyed at the same time (mid morning) every day for seven consecutive days. The number of *H. purpurascens* on tagged plants was recorded over time. A repeated-measures one-way ANOVA was used to assess the number of urchins on each plant over time following  $\ln(x+1)$  transformation of data. Again, where

Levene's test indicated that the assumptions of ANOVA had not been met the more stringent *P*-value of 0.01 was used.

### ***Size-frequency distribution of Phasianotrochus eximius on kelp with and without***

#### ***Holopneustes purpurascens***

To assess whether *P. eximius* are likely to recruit directly to, or immigrate post-recruitment to the *E. radiata*/*H. purpurascens* association we determined the size distribution of *P. eximius* on host plants with and without *H. purpurascens*. A length-frequency plot was generated from the individuals collected at Fairlight between October 2009 and June 2012 (described above). Individuals were pooled into 1mm size classes. A Kolmogorov Smirnov test was used to test for a difference in the size class frequency distributions of *P. eximius* on *E. radiata* with versus without *H. purpurascens*.

***Post-recruitment movement of Phasianotrochus eximius towards Ecklonia radiata with Holopneustes purpurascens***

To test the hypothesis that the positive relationship between the gastropod and urchin is, in part, maintained by juvenile and adult *P. eximius* immigrating more rapidly to *E. radiata* with than without *H. purpurascens*, we conducted a recolonisation experiment at Fairlight in December 2010. All mobile fauna >1 cm in diameter were removed from 40 haphazardly-selected *E. radiata* by hand while on SCUBA. Two *H. purpurascens* (determined from the average number of urchins per *E. radiata*, see Results) were added to 20 of the defaunated plants that had been randomly assigned to this treatment, with the other 20 plants left free of urchins. To maintain assigned urchin densities, all experimental plants were caged using flexible plastic garden fencing (mesh size 20mm). The mesh was fine enough to prevent urchins from passing through but coarse enough to allow free movement of *P. eximius*. Cages covering the head of the plant were attached to eyebolts in the rocky substratum using bungee cord. This allowed for the natural movement of the cages with the water currents. After 24 h, plants were harvested and sealed in plastic bags (as described above) before being taken back to the laboratory, where plants were immersed in fresh water (as described above) and the number of recolonising *P. eximius* on each plant was counted. The hypothesis that plants with *H. purpurascens* would have a greater abundance of *P. eximius* compared to plants without *H. purpurascens* was assessed using a  $\chi^2$  test.

The process of caging can impact on the number of epifauna (Kenelly 1991, Taylor 1998). To test how complete the recolonisation was on caged plants after 24 hours a separate comparison was made. A greater abundance of *P. eximius* was observed on plants with *H. purpurascens* than without. As a result we chose to test for the effect of caging only on plants with *H. purpurascens*. The abundance of *P. eximius* that had recolonised onto the 10 replicate caged plants with *H. purpurascens* versus 10 replicate uncaged plants with *H. purpurascens*,

representing natural abundances of *P. eximius* in this habitat. This comparison was tested using a simple two sample t-test. Again, assumptions of normality and homogeneity of variance (using Levene's test of equality of error variances) were not met even after data had been  $\ln(x+1)$  transformed so statistical significance was defined as  $P < 0.01$ , in order to reduce the probability of Type I error.

### ***Post-recruitment movement of Holopneustes purpurascens towards Ecklonia radiata with Phasianotrochus eximius***

The field experiment tested whether colonisation of kelp by *P. eximius* is influenced by the presence of *H. purpurascens*. To test the converse – that *H. purpurascens* actively select *E. radiata* with *P. eximius* over kelp without the gastropods - a laboratory experiment was run. Similar sized pieces and weights (30 – 60g) of *Ecklonia radiata* were placed at opposite ends (separated by 14 cm) of a 40L plastic bin filled with filtered seawater (0.22 $\mu$ m). The pieces of kelp were chosen to contain minor grazing scars from *H. purpurascens* as this, presumably, indicates that they are palatable to the urchins. First, no-choice controls in which urchins were offered pieces of gastropod-free *E. radiata* at either end of the tank determined whether, in the absence of choice, gastropods displayed a consistent pattern of movement towards a particular end of the tank. Second, choice treatments where six *P. eximius* (the average number of *P. eximius* per *E. radiata* in the field, see Results) were added to one but not the other piece of kelp assessed selection of one treatment over the other. At the start of each test, one adult *H. purpurascens* (32 to 58mm oral-aboral length) was placed in the centre of the plastic bin (7 cm from either treatment) and given 30 minutes to make a choice between *E. radiata* pieces. An urchin was deemed to have made a choice if it touched one of the pieces of kelp. Positions of the treatments (with or without *P. eximius*) were swapped after 15 replicates to account for any bias between sides and the seawater was changed between replicates (30 replicates in total). New urchins, kelp and fresh seawater were used for each replicate. The

experiment, initially conducted during the day, was repeated at night following evidence to suggest that *H. purpurascens* is more active at night (Williamson et al. *in review*).

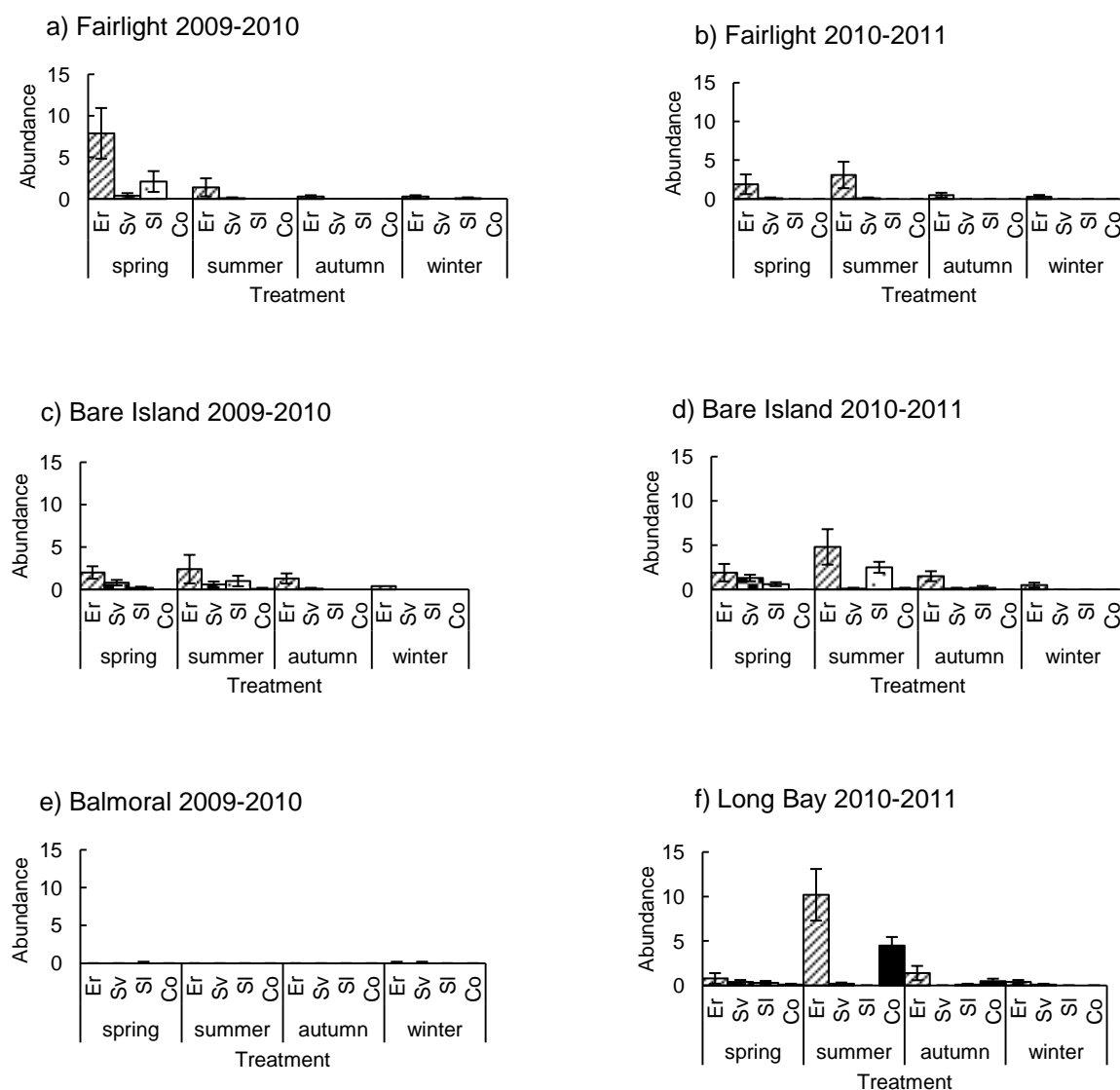
Separate chi-squared tests for differences in the proportion of urchins moving towards one treatment over the other were done for trials run during the day, and the night. These analyses only used urchins that made a choice, and had the null hypothesis that the proportion of urchins moving towards each treatment would not statistically differ from a 50:50 ratio.

## **Results**

### ***Sources of spatial variation in the abundance of Phasianotrochus eximius and***

### ***Holopneustes purpurascens***

Interacting effects of season and algal species on the abundance of *P. eximius* were evident at three of the four sample sites (Figure 1) (Table 1). The fourth site, Balmoral, had low abundances of gastropods throughout the study, which prevented the detection of spatial and temporal patterns (Table 1). In summer, the season of greatest gastropod abundance, gastropods were more abundant on *E. radiata* than on any of the other three algal species at each of the three sites displaying a significant interaction, (post-hoc tests, Table 1). At two of the sites, Bare Island and Fairlight, this pattern of greater abundance on *E. radiata* was also evident at other times of year. Among the other algal species, *C. officinalis* generally supported the fewest gastropods, except at Long Bay. Differences in abundance between *S. vesitutum* and *S. linearifolium* were site and season-specific. In most instances there was no difference in substrate utilization or seasonal variation in abundance across years. The exception was Fairlight, where seasonal patterns varied between the two years (Table 1).



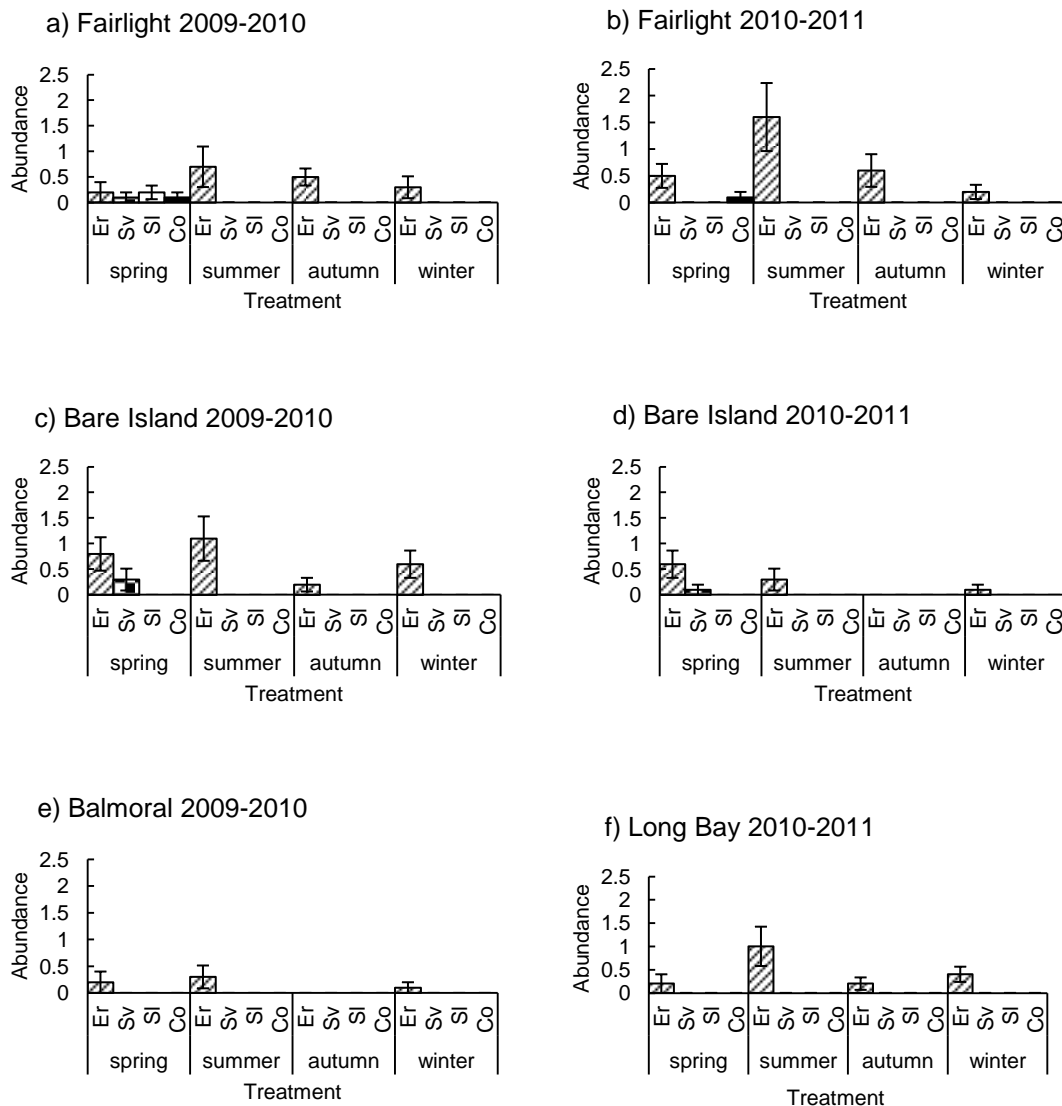
**Figure 1.** Abundance of *Phasianotrochus eximius* (mean +SE number of individuals per plant, N = 10 plants) between 2009 and 2011 at a), b) Fairlight, c, d) Bare Island, e) Balmoral and f) Long Bay on four common alga (Er = *Ecklonia radiata*, Sv = *Sargassum vestitum*, Sl = *Sargassum linearifolium*, Co = *Corrallina officinalis*). *P. eximius* was significantly more abundant on *E. radiata* than the other three algal species at Fairlight, Bare Island and Long Bay (see Table 1).

**Table 1.** Analyses of variance testing for sources of spatial variation in the abundance of *Phasianotrochus eximius* among potential host plant species (Sp, *Ecklonia radiata* [Er], *Sargassum vestitum* [Sv], *S. linearifolium* [Sl] and *Corallina officinalis* [Co]), seasons (S, Spring [Sp], Summer [Su], Autumn [Au], Winter [Wi]) and years (Y, 2009/2010, 2010/2011). Balmoral and Long Bay were sampled only in one year each. Numbers in bold indicate a significant difference at  $p < 0.01$ . Lev = Levene's test of equality of error variances. Sid = Sidak test of multiple comparisons. N = 10.

	d.f.	Bare Island			d.f.	Fairlight		
		MS	F	P		MS	F	P
Year (Y)	1	6.9	2.1	0.146	1	13.6	2.585	0.109
Season (S)	3	27.1	8.4	<b>&lt;0.001</b>	3	37.7	7.2	<b>&lt;0.001</b>
Species (Sp)	3	50.7	7.0	<b>&lt;0.001</b>	3	68.9	13.1	<b>&lt;0.001</b>
Y x S	3	2.9	0.9	0.453	3	26.1	5.0	<b>0.002</b>
Y x Sp	3	2.4	0.7	0.538	3	4.5	0.9	0.465
S x Sp	9	8.5	2.6	<b>0.007</b>	9	20.3	3.9	<b>&lt;0.001</b>
Y x S x Sp	9	2.4	2.4	0.681	9	12.4	2.4	0.014
Residuals	931	3.3			288	5.3		
Lev			5.4	<b>&lt;0.001</b>			15.0	<b>&lt;0.001</b>
Sid								
	S x Sp				S x Sp			
	Sp: Er > (Sv > (Sl = Co))				Su, Au, Wi: Er > (Sv > (Sl = Co))			
	Su, Au, Wi: Er > (Sl > (Sv = Co))				Sp: Er > (Sl > (Sv = Co))			
					Y x S			
					09/10: Er > (Sl > (Sv = Co))			
					10/11: Er > (Sv > (Sl = Co))			
	d.f.	Balmoral			d.f.	Long Bay		
		MS	F	P		MS	F	P
Season (S)	3	0.0	1.2	0.304	3	115.5	17.4	<b>&lt;0.001</b>
Species (Sp)	3	0.0	0.4	0.801	3	83.6	12.6	<b>&lt;0.001</b>
S x Sp	9	0.0	0.9	0.505	9	50.4	7.6	<b>&lt;0.001</b>
Residuals	144	0.0			144	6.7		
Lev			4.4	<b>&lt;0.001</b>			13.2	<b>&lt;0.001</b>
Sid					S x Sp			
					Sp, Au, Wi: Er = Sv = Sl = Co,			
					Su: Er > (Co > (Sv = Sl))			

Like *P. eximius*, *H. purpurascens* displayed variation in abundance among algal substrates in at least some seasons and years, at three of the four sites sampled (Figure 2) (Table 2). The exception was Balmoral - the site of lowest urchin abundance. During all seasons and at all sites where differences among algal substrates were detected, the urchin was more abundant on *E. radiata* than on the other three algal species (Table 2). Again, in all cases where differences in urchin abundance across algal species were observed, urchin abundance was greater on *E. radiata* than on the other algal taxa. Like the gastropod, the urchin displayed stronger patterns of difference among algal substrates in summer, when its abundance was greatest, than in other seasons.





**Figure 2.** Abundance of *Holopneustes purpurascens* (mean +SE number of individuals per plant, N = 10 plants) between 2009-2011 at a), b) Fairlight, c), d) Bare Island, e) Balmoral and f) Long Bay on four common alga (Er = *Ecklonia radiata*, Sv = *Sargassum vestitum*, Sl = *Sargassum linearifolium*, Co = *Corrallina officinalis*). *H. purpurascens* was significantly more abundant on *E. radiata* than the other three algal species at all study sites (see Table 2).

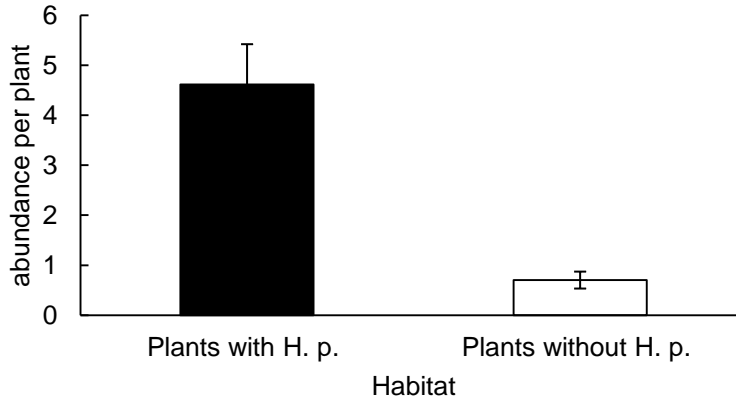
**Table 2.** Analyses of variance testing for sources of spatial variation in the abundance of *Holopneustes purpurascens* among potential host plant species (Sp, *Ecklonia radiata* [Er], *Sargassum vestitum* [Sv], *S. linearifolium* [Sl] and *Corallina officinalis* [Co]), seasons (S, Spring [Sp], Summer [Su], Autumn [Au], Winter [Wi]) and years (Y, 2009/2010, 2010/2011). Balmoral and Long Bay were sampled only in one year each. Numbers in bold indicate a significant difference at  $p < 0.01$ . Lev = Levene's test of equality of error variances. Sid = Sidak test of multiple comparisons. N = 10.

	d.f.	Bare Island			d.f.	Fairlight		
		MS	F	P		MS	F	P
Year (Y)	1.0	1.1	6.4	0.012	1.0	0.3	0.9	0.339
Season (S)	3.0	0.6	3.6	0.014	3.0	0.7	2.5	0.056
Species (Sp)	3.0	4.0	22.7	<b>&lt;0.001</b>	3.0	6.4	22.3	<b>&lt;0.001</b>
Y x S	3.0	0.1	0.5	0.724	3.0	0.3	0.9	0.418
Y x Sp	3.0	0.9	4.8	<b>&lt;0.001</b>	3.0	0.5	1.9	0.123
S x Sp	9.0	0.4	2.4	0.012	9.0	0.9	3.2	<b>0.001</b>
Y x S x Sp	9.0	0.1	0.7	0.688	9.0	0.2	0.9	0.537
Residuals	288	0.2			288	0.3		
Lev			16.8	<0.001			13.564	
Sid	Y x Sp				S x Sp			
	09/10: Er > Sv > (Sl = Co)				Sp, Au, Wi: Er = Sv = Sl = Co			
	10/11: Er > (Sv = Sl = Co)							

	d.f.	Balmoral			d.f.	Long Bay		
		MS	F	P		MS	F	P
Season (S)	3	0.0	0.3	0.791	3.0	0.4	2.2	0.092
Species (Sp)	3	0.3	4.6	0.004	3.0	2.0	12.4	<b>&lt;0.001</b>
S x Sp	9	0.0	3.3	0.957	9.0	0.4	2.2	0.026
Residuals	144	0.1			144	0.2		
Lev			6.1	<0.001			12.5	<0.001
Sid					Sp			
					Er > Sl = Sv = Co			

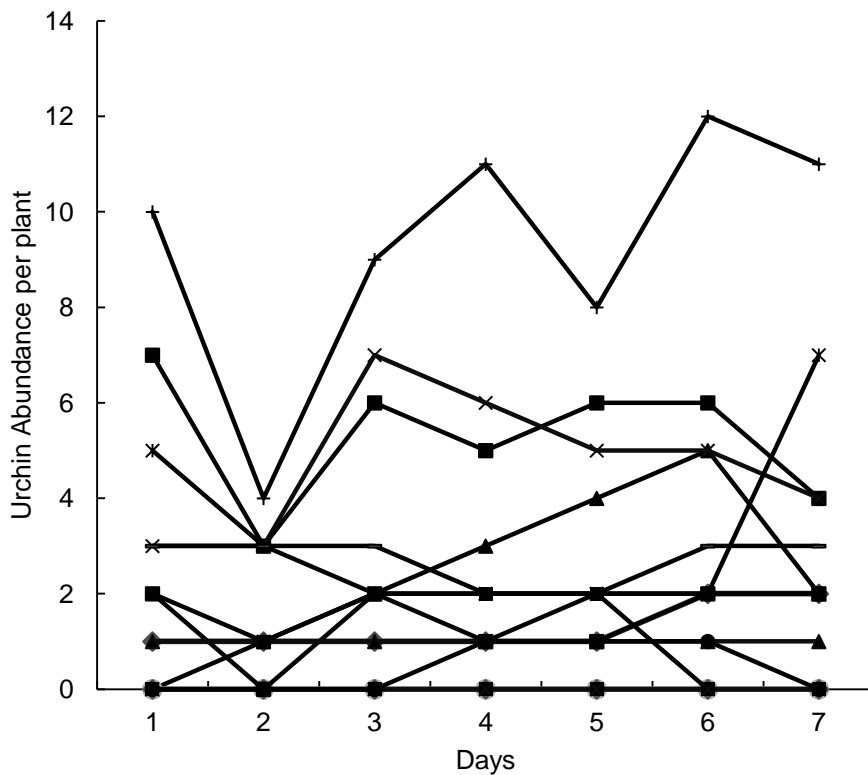
*P. eximius* was not only more abundant on *E. radiata* than on any of the other algal species sampled, it was also significantly more abundant on *E. radiata* plants that contained *H. purpurascens* compared to those that did not (ANOVA:  $F_{237, 1} = 47.00$ ,  $P < 0.00$ , Fig 3).



**Figure 3.** Mean + SE abundance of *Phasianotrochus eximius* on *Ecklonia radiata* with and without *Holopneustes purpurascens*. *P. eximius* was significantly more abundant on *E. radiata* with than without *H. purpurascens* (one-way ANOVA,  $P < 0.001$ ).

#### ***Movement of Holopneustes purpurascens on its host over time***

The abundance of *H. purpurascens* on hosts changed over time (repeated measures ANOVA:  $F_{6, 14} = 35.83$ ,  $P < 0.01$ , Figure 4). As there was no consistent direction to this change (i.e. increase or decrease in numbers), both loss and immigration appeared to occur. Plants that started with higher numbers of urchins than others consistently maintained higher numbers of urchins throughout the experiment (Figure 4).

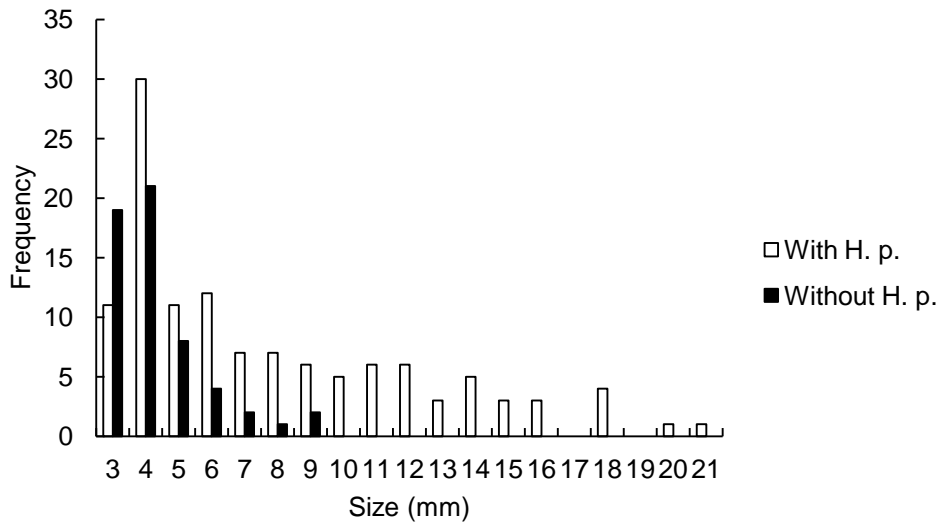


**Figure 4.** Abundance of *Holopneustes purpurascens* on individually tagged *Ecklonia radiata* over a period of 7 days. There was a significant difference in the number of *H. purpurascens* on individual plants over time (Repeated measures ANOVA,  $P < 0.001$ ,  $N = 15$ ).

### *Size-frequency distribution of Phasianotrochus eximius on kelp with and without*

#### ***Holopneustes purpurascens***

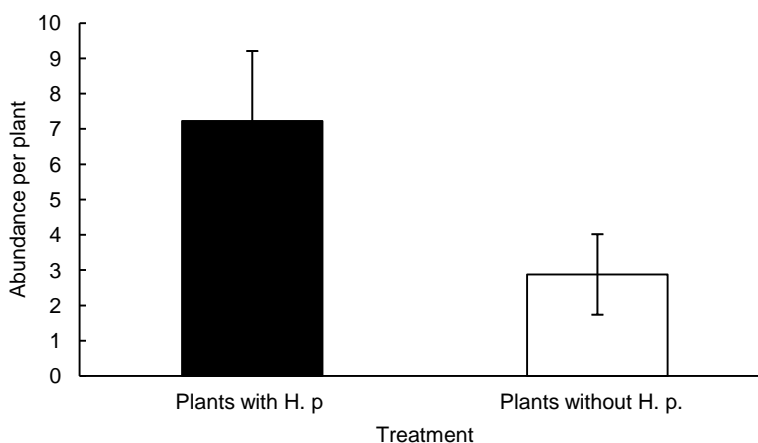
There was a significant difference in the size frequency distribution of *P. eximius* between habitats (Kolmogrov-Smirnov test:  $P < 0.01$ , Figure 5). *P. eximius* greater than 9mm in length were only observed on *E. radiata* with *H. purpurascens*, however smaller *P. eximius* were found across both habitats with *H. purpurascens* and without *H. purpurascens* (Figure 5).



**Figure 5.** Size frequency distribution of *Phasianotrochus eximius* on kelp with *Holopneustes purpurascens* (With H. P.) and without *Holopneustes purpurascens* (Without H. p). There was a significant difference in the size frequency distribution of *P. eximius* between habitats (Kolmogorov-Smirnov test:  $P < 0.01$ ). *P. eximius* greater than 9mm in length were only observed on *E. radiata* with *H. purpurascens*.

#### ***Post-recruitment movement of Phasianotrochus eximius towards Ecklonia radiata with Holopneustes purpurascens***

A greater abundance of *P. eximius* repopulated to plants with *H. purpurascens* compared to plants without *H. purpurascens* ( $\chi^2 = 14.97$ ,  $df = 1$ ,  $P\text{-value} < 0.001$ , Figure 6).



**Figure 6.** Post recruitment recolonisation abundance of *Phasianotrochus eximius* on *Ecklonia radiata* with and without *Holopneustes purpurascens* (H. p.) (*H. purpurascens*, chi-squared:  $P < 0.001$ ).

The average natural density of *P. eximius* per *E. radiata* with *H. purpurascens*, determined from demographic abundance collections (see methods), was exceeded in this experiment by  $2.6 \pm 1.82$  SE. Nevertheless, there was no significant difference between the density of *P. eximius* that repopulated caged plants with *H. purpurascens* to the density of *P. eximius* on uncaged plants with *H. purpurascens* ( $T_{16, 16} = 0.39$ ,  $P\text{-value} = 0.541$ ).

### ***Post-recruitment movement of Holopneustes purpurascens towards Ecklonia radiata with Phasianotrochus eximius***

In no-choice controls performed during the day, in which urchins were offered *E. radiata* without gastropods at either end of the tank only 2 individuals out of the 30 tested selected a piece of kelp. The two urchins differed from one another in the direction they moved, such that there was no evidence for selection in the absence of choice ( $\chi^2 = 0$ , d.f. = 1,  $P = 1$ ).

When *H. purpurascens* were offered *E. radiata* versus *E. radiata* with *P. eximius* during the day, no urchins out of the 30 individuals tested made a choice. When *H. purpurascens* was given a choice between *E. radiata* with versus without *P. eximius* at night, 12 out of 30 urchins made a choice, however there was no significant difference in the choice made with six urchins choosing *E. radiata* with *P. eximius* and six urchins choosing *E. radiata* without *P. eximius* ( $\chi^2 = 1.2$ , d.f. = 1,  $P = 0.273$ ).

### **Discussion**

The objective of this study was to determine the nature of the relationship between the kelp *E. radiata*, the canopy-dwelling sea urchin *H. purpurascens* and the marine gastropod *P. eximius*. Through a combination of field and laboratory experiments and surveys, it was demonstrated that the relationship between *E. radiata* and *P. eximius* was the net outcome of a positive indirect effect, whereby *E. radiata* facilitates *H. purpurascens*, and the urchin in turn facilitates *P. eximius*. This relationship was hierarchical, as the urchin did not respond to

the abundance of the gastropod. Hence, the relationship fitted the definition of a facilitation cascade (see Altieri et al. 2007, Bishop et al. 2012).

At each of the four field sites surveyed, *P. eximius* and *H. purpurascens* had a tightly overlapping distribution, with each species more abundant on the kelp *E. radiata* than the other habitat-forming alga examined (*Sargassum vestitum*, *Sargassum linearifolium*, *Corallina officinalis*). *P. eximius* was more abundant on *E. radiata* occupied by *H. purpurascens* than on plants without the sea urchin. During winter, however, when urchin densities decreased to low abundance, *P. eximius* was sometimes found away from urchins, nestled in the centre of *E. radiata* plants (Bell personal observation). Hence, the relationship appeared facultative, not obligate, and varied in strength over time. The abundance of *H. purpurascens* on individual *E. radiata* also changed over shorter time scales, indicating movement within and between plants. Urchin movement occurs at night, when key predators are less active (Williamson et al. in review).

Greater numbers of *P. eximius* recolonised kelp with than without *H. purpurascens*, but the urchin did not respond to the presence of the gastropod. This, in combination with previous research, suggests that the relationship between the kelp, urchin and gastropod is a hierarchical facilitation cascade (*sensu* Thomsen et al. 2010, Angelini et al. 2011). Following recruitment to the foliose red algae *Delisea pulchra* or coralline turfing algae (Swanson et al. 2004, 2006), *H. purpurascens* displays a chemically-mediated ontogenetic shift in diet and moves from *D. pulchra* to *E. radiata* (Williamson 2004). The gastropod, post recruitment, is facilitated by the urchin, perhaps due to its modification of the kelp habitat by forming a nest. This study did not determine the mechanism by which *H. purpurascens* facilitated *P. eximius*. *H. purpurascens* may provide *P. eximius* with refuge from environmental stressors and predation (Williamson 2004). Alternatively or additionally, *P. eximius* may eat the faeces of *H. purpurascens*.

Larger *P. eximius* were only found on host plants with *H. purpurascens*, whereas smaller gastropods were found on both *E. radiata* with and without the urchin. This suggests that small *P. eximius* recruit to the kelp, irrespective of whether it houses an urchin, but that subsequent processes restrict larger gastropods to kelps with urchins. This pattern was at least in part due to post-settlement movement of large *P. eximius* onto kelp with the urchin. When we removed all *P. eximius* from *E. radiata*, there was greater recolonisation of plants with the urchin than of plants from which it had been excluded. This movement may be in response to a cue produced by the urchin or, alternatively, *P. eximius* may move around randomly until they find a plant with the urchin. It is, however, also possible that greater rates of growth and/or survivorship of *P. eximius* on *E. radiata* with than without the urchin may also contribute to the pattern.

In summary, this study has provided one of the first demonstrations of a facilitation cascade in a subtidal environment. In subtidal environments, where predation is often intense and waves and currents produce abiotically stressful conditions, positive interactions among species may be expected to play a particularly important role in organisation of ecological communities (Bertness 1994, Jones 1994, Hacker 1997, Jones 1997, Stachowicz 2001). Studies are now required to ascertain the mechanism by which the interaction is maintained.

## **Acknowledgements**

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**CHAPTER 3: THE ROLE OF GASTROPOD BEHAVIOUR IN MEDIATING THE  
ESTABLISHMENT AND MAINTENANCE OF A HABITAT CASCADE IN A  
TEMPERATE AUSTRALIAN KELP FOREST**

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## **Abstract**

Habitat cascades are important in structuring ecological communities, but little is known about how these arise and are maintained. We examined the role of habitat selection and predator avoidance by adult *Phasianotrochus eximius* in maintaining its close association with the sea urchin *Holopneustes purpurascens*, which in turn enmeshes itself in the fronds of the common kelp *Ecklonia radiata* throughout temperate parts of Australia. First we assessed whether *P. eximius* responds to cues originating from the kelp, urchin, or crushed conspecifics. Secondly, we assessed whether any responses were visual or olfactory in nature. We partitioned the influence of olfactory and visual signaling by (1) placing stimuli in plastic bags so that gastropods could see them but not receive effluent from them; and (2) exposing the gastropods to effluent from the stimuli, in the absence of visual cues. We demonstrated that *P. eximius* uses visual cues to locate urchins on kelp, but active selection of this habitat is relatively weak, with most gastropods failing to respond to stimuli. No avoidance response was invoked by the threat of predation. Instead *P. eximius* displayed a type of “freezing behavior” in response to crushed conspecifics. Overall our study suggests that behavioural habitat selection by adults may be partially responsible for the development and maintenance of this association. Nevertheless, it is likely that reduced rates of mortality of the gastropod when associated with the urchin also contribute.

**Keywords:** Facilitative, cascade, host, cue, visual, chemical, olfactory

## Introduction

There is increasing recognition of the importance of habitat cascades in maintaining community structure in a diversity of aquatic and terrestrial ecosystems (Bruno et al, 2003, Thomsen et al, 2010, Altieri et al., 2007, Gribben et al., 2009, Thomsen et al., 2010, Angelini et al., 2011, Bishop et al., 2013). These relationships are classified as the net effect of a series of indirect effects whereby a basal habitat-forming species facilitates an intermediary habitat-forming species, and the intermediary habitat-forming species in turn facilitates a focal species (Thomsen et al., 2010, Angelini et al., 2011). Despite the prevalence of these associations, the mechanisms by which they exist remain poorly understood.

The association of a focal species with a habitat-forming species may arise through a number of mechanisms. First, the focal species may be able to detect the habitat-forming species and actively seek it out as habitat. Second, the focal species may not actively choose the habitat forming species *per se*, but may suffer reduced mortality when not associated with it, such that the abundance of the two becomes correlated. Third, the abundances and/or survival of both the focal species and habitat forming species may be positively correlated with the same environmental variable(s). These relationships may be obligate, if survival of the focal species is not possible in their absence. Alternatively the relationships may be facultative, where the focal species, although preferring to reside in the intermediary habitat, is able to survive outside of the association. In obligate mutualistic associations, host recognition is vital for the survival of the participants involved (Ambrosio and Brooks, 2011). However, in facultative relationships the need for host detection is less critical and often the proficiency of host recognition is weaker (Ambrosio and Brooks, 2011). In the sea, detection of both facilitators and predators often occurs by visual and/or waterborne chemical cues (Ambrosio and Brooks, 2011, Mathis and Vincent, 2000, McCormick and Manassa, 2008).

Along the south east coast of Australia, a positive association between the gastropod *Phasianatrochus eximius* (Perry, 1811) and the kelp *Ecklonia radiata* (C. Agardh), is the



outcome of a habitat cascade mediated by a third species, the sea urchin *Holopenustes purpurascens* (Agassiz, 1872) (Bell et al., unpublished, Chapter 2). The short-spined urchin, *H. purpurascens*, uses its tube feet to wrap the lamina of the kelp around its body (Steinberg, 1995). The focal organism, the gastropod *P. eximius*, in turn, resides with *H. purpurascens* in the shelter it builds within the fronds of the kelp (Rogers et al., 1998). The relationship is considered facultative, as *P. eximius* can survive in different types of habitats, but inhabits *E. radiata* plants with *H. purpurascens* in greater abundances (Bell et al., unpublished, Chapter 2).

*P. eximius* is capable of finding its host habitat (*E. radiata*) and facilitator species (*H. purpurascens*) in the field (Bell et al., unpublished, Chapter 2). Hence, active habitat selection seems at least partially responsible for the association. The method by which *P. eximius* detects its host is, however, unknown. *P. eximius* may be positively responding to the urchin alone, the urchin grazing on the kelp, or the kelp alone. Investigating the response of *P. eximius* to its host habitat, facilitator species and/or conspecific derived cues provides us with information on its means of detecting facilitation via chemical and or visual based cues and gives insight into the strength of this association (Schönrogge et al., 2008).

*P. eximius* may detect its host using visual and/or olfactory cues. *P. eximius* is known to have an osphradium, a defining feature of all gastropods, which gives it its chemosensory function (Kohn 1983). It also possesses a pair of eyes at the base of its sensory tentacles, giving it the ability to use vision as an alternative sensory mode. Here, we address the questions: (1) does *P. eximius* respond to cues originating from the kelp, urchin and/or the threat of predation; and (2) are these cues visual or olfactory? We hypothesize that if *H. purpurascens* is responding directly to the urchin, the stimulus will be olfactory rather than as urchins are likely to be visually obscured by the kelp laminae in which they are wrapped.

## Methods

### *Collection of animals and holding conditions*

Urchins *Holopneustes purpurascens*, gastropods *Phasianotrochus eximius* and kelp *Ecklonia radiata* for use in experiments were collected from depths of 3 to 7 m at Fairlight, (33° 80' 00" S, 151° 27' 54" E) Sydney, Australia under SCUBA. Collections were made on plants where both *P. Eximius* and *H. purpurascens* occurred in association. Individuals were immediately transported back to the Macquarie University seawater facility where they were held in the 30,000L recirculating system until commencement of experiments, usually within 24 hrs of collection. Seawater within the facility was maintained at a salinity of 34-36‰ and a temperature of 18°C. These conditions matched those experienced by the organisms in the field. Seawater flow within the tank was 60L h<sup>-1</sup>. Urchins and gastropods were fed *E. radiata* *ad libitum* until the start of the experiment.

### *Does P. eximius respond to host habitats, and/or crushed conspecifics?*

An initial set of choice trials were conducted to assess whether *P. eximius* responds (1) positively to its hosts, *H. purpurascens* and *E. radiata*, or (2) negatively to the threat of damage. The specific predators of the snail were unknown, however, fish and crabs are common in subtidal rocky reef environments within NSW, and these types of predators tend to crush their prey (Andrew and O'Neill 2000). Therefore the threat of predation was measured by testing the response of *P. eximius* to crushed conspecifics rather than to a predator itself. A single gastropod (8-23Mm length) was placed in the centre of a 40 L tank of 14cm long x 14cm wide x 20cm deep cm dimensions, filled with filtered seawater (0.22 µm) and in which one of the three stimuli was provided at one end, and the other end left empty. The gastropod was thus 7cm away and equidistant from either end. One average-sized *H. purpurascens* was used as the stimuli in trial (1) along with a small piece of kelp about 200g in wet weight. For trial (2) a total of six adult snails were crushed and placed on one side of

the tank. Over a 30 min period, we assessed whether the gastropods made a choice to move towards the stimulus, stayed put, did not move, or moved away from the stimulus. A gastropod was recorded as moving towards the stimulus if it touched it in the experimental period. It was recorded as moving away from the stimulus if it moved more than 7cm or more in the opposite direction to the stimulus. After a choice had been made the trial was immediately terminated. The gastropods were given 30 minutes to make a decision and if after 30 minutes the gastropod had not moved either towards or away from the stimulus it was recorded as having not made a choice. These methods were based on the results of a pilot study whereby the snail was given a range of distances and a range of times in which to respond to a cue (*H. purpurascens* on *E. radiata*). It was determined that if a snail did not make a choice after 30 minutes it was unlikely to make a choice at all. Distance did not appear to make a difference to the choices made by snails. The side of the tank on which the stimulus was provided was alternated so as to avoid any experimental bias of gastropods for one end of the tanks. There was no flow of seawater within this experimental set up. A total of between 30 and 60 trials were conducted for each treatment combination. Each *P. eximius* was used only once and the water was changed between trials.

For each treatment, we recorded the proportion of gastropods that moved in response to the stimulus. Among gastropods that moved, we tested the hypotheses that: (1) a greater proportion would move towards than away from the urchin and kelp; and (2) a greater proportion would move away from than towards damaged conspecifics using  $\chi^2$  goodness of fit tests.

### ***Are P. eximius responding to visual cues?***

To assess whether the gastropods were responding visually to the stimuli provided in the first set of experiments, we performed a second experiment in which each of the stimuli were enclosed in a clear plastic Ziplock<sup>TM</sup> bag. These enabled the gastropods to see the stimulus,

but not receive any chemical cue from it, thereby isolating the visual cue. To control for any effect of the plastic bags on the gastropods, movement of gastropods towards bagged organisms was compared to movement towards plastic bags containing only seawater. Hence, the experimental trials were as follows: (1) *H. purpurascens* and *E. radiata* (bagged) versus seawater (bagged); (2) Crushed *P. eximius* (bagged) versus seawater (bagged). For the host stimulus only, we included an additional treatment whereby bagged *H. purpurascens* and *E. radiata* were offered against unbagged *H. purpurascens* and *E. radiata*. This treatment was used to confirm the efficacy of the plastic bags in removing chemical cues. The first treatment that the gastropod touched was recorded as the treatment in which the gastropod had chosen and once a decision had been made the trial was terminated. The gastropods were given 30 minutes to make a decision and if after 30 minutes the gastropod had not touched a treatment after this allocated time it was recorded as having made no choice. Among gastropods that made a decision, we tested for differences in the proportion selecting each of the two treatments using  $\chi^2$  goodness of fit tests.

#### ***Are P. eximius responding to olfactory cues?***

To assess whether *P. eximius* responds to chemical cues emitted by its facilitator (*H. purpurascens*), its host habitat (*E. radiata*), live conspecifics or various combinations of the three, we conducted laboratory trials that permitted *P. eximius* to distinguish between treatments without the ability to see them. Six treatment combinations or trials were assessed: (1) *E. radiata* versus filtered seawater; (2) *H. purpurascens* versus filtered seawater; (3) *H. purpurascens* and *E. radiata* versus filtered seawater; (4) *P. eximius* and *H. purpurascens* versus filtered seawater; (5) *P. eximius* and *E. radiata* versus filtered seawater; and (6) *H. purpurascens*, *E. radiata* and *P. eximius* versus filtered seawater. This experiment did not consider cues from crushed conspecifics because responses to these in previous experiments were weak.

The experiment utilized a Y-maze, based on the design of Davenport (1950), two arms of which gravitationally received seawater from two 2L sub-aquaria, which in turn were fed by source 2L aquaria in which the various treatments were placed. The Y-maze was constructed of clear Perspex, was 2 cm in diameter, and had three 11 cm round arms connected at 60° apart. The third arm drained into a common aquarium which in turn drained into a bucket, placed at a lower level to the rest of the apparatus. The Y-maze received a flow of water from sub-aquaria at a rate of 400 mL min<sup>-1</sup>. Treatments were placed in the source aquaria for 30 min before flow commenced to allow for any olfactory cue to be released into the water. Prior to running the experiment the apparatus was tested using red food colouring to confirm that equal portions of water from the two separate aquaria were being delivered into the Y tube in a steady laminar flow.

At the start of each trial, an individual *P. eximius* was placed inside the Y-maze, halfway between the common aquarium and the intersection of the Y-maze, and its behaviour was initially observed over 5 min. Those gastropods that had failed to move to the intersection of the Y-maze within this initial 5 min observation period were considered to have not made a response to a cue and were recorded as having made no choice. The observation period was determined from pilot studies which indicated that if a gastropod had not moved after 5 minutes it was unlikely to move at all. If a response was apparent within 5 minutes, individuals were left for a further 25 minutes (30 minute in total) to make a choice. A choice was defined as movement to at least half way along one of the arms of the maze. Thirty replicates were done for each treatment combination, and treatments were swapped to the opposite side after 15 replicates. The apparatus was thoroughly flushed between trials to remove any odors from previous trials. Further, a control of filtered seawater versus filtered seawater was used to confirm that in the absence of chemical cues, *P. eximius* displayed no persistent choice of either the left or the right arm of the Y-maze.

Among gastropods that moved, significant differences in the proportion selecting each of the two treatments per trial were assessed using  $\chi^2$  goodness of fit tests.

## Results

### *Does P. eximius respond to habitats and/or crushed conspecifics?*

There was no significant difference in the number of gastropods that made a choice towards or away from *H. purpurascens* and *E. radiata* compared to making no choice ( $\chi^2 = 0.6$ , P-value = 0.44). However, of the 55% of gastropods displaying definitive movement towards or away from the combined treatment of *H. purpurascens* and *E. radiata*, a significantly greater proportion chose to move towards than away from the habitat forming species (Table 1). By contrast, there was a significant greater proportion of gastropods that made a choice towards or away from damaged conspecifics compared to making no choice ( $\chi^2 = 4.8$ , P-value = 0.03). However, among the 73% of gastropods moving in response to damaged conspecifics, there was no significant difference in the proportion moving towards the stimulus versus away from it (Table 1).

### *Are P. eximius responding to visual cues?*

There was no significant difference in the number of gastropods that made a choice towards or away from bagged host species, *H. purpurascens* and *E. radiata* compared to making no choice ( $\chi^2 = 0.54$ , P-value). However, of the 56 % of gastropods making a choice between the bagged host species, *H. purpurascens* and *E. radiata*, and bagged seawater, a significantly greater proportion chose the host species (Table 2). In addition, there was no significant difference in the number of gastropods that made a choice towards or away from bagged or unbagged host species ( $\chi^2 = 0.14$ , P-value = 0.72). However, by contrast, when bagged and unbagged host species were simultaneously offered to gastropods, a similar proportion of the 47% of gastropods that moved selected the bagged and unbagged treatments (Table 1). When

gastropods were offered crushed conspecifics with bagged seawater 33% of gastropods made a choice ( $\chi^2 = 3.34$ , P-value = 0.07). There was no evidence of choice of one treatment over the other (Table 2).

### ***Are P. eximius responding to olfactory cues?***

In the absence of chemical cues, there was no significant difference in the number of gastropods that made a choice versus making no choice ( $\chi^2 = 3.33$ , P-value = 0.68). Of the gastropods that made a choice there was no significant difference in the number of gastropods that chose one side of the Y-maze over the other (Table 3). Hence, any choices made by gastropods in subsequent trials could be interpreted as a response to chemical cues and not experimental artefacts.

When offered a choice between effluent from *H. purpurascens* *E. radiata* or *P. eximius*, versus filtered seawater, between 47% and 67% of gastropods made a choice between treatments. However there was no significant difference in the number of gastropods that made a choice versus making no choice (*H.purpurascens* versus FSW  $\chi^2 = 0.13$ , P-value = 0.72; *E. radiata* versus FSW  $\chi^2 = 0$ , P-value = 1; *P. eximius* versus FSW  $\chi^2 = 0.54$ , P-value = 0.47).

When offered a choice between *E. radiata* and filtered seawater a significantly greater proportion of gastropods moved towards *E. radiata* (Table 3). By contrast, when gastropods were offered a choice between *H. purpurascens* and filtered seawater a significantly greater proportion of gastropods moved away from the scent towards the filtered seawater. When gastropods were offered a choice between conspecifics and filtered seawater there was no significant difference in the choice made (Table 3). In addition there was no significant choice made by gastropods when offered combinations of host habitat, facilitator and/or conspecifics versus filtered seawater (Table 3).

**Table 1.** The results of choice experiments, examining the behavioral response by *P. eximius* to host species, and to crushed conspecifics. Assay = number of individuals in each assay. N = number of individuals that made no response. A = number of individuals that made a choice of treatment A. B = number of individuals that made a choice of treatment B. FSW = filtered seawater.

Choice					Results			
Trial	A	B	Assay	N	A	B	$\chi^2$	P-value
1	<i>H. purpurascens</i> and <i>E. radiata</i>	FSW	60	27	32	1	29.12	<0.01
2	Crushed <i>P. eximius</i>	FSW	30	9	11	10	0.05	0.83

**Table 2.** The response by *P. eximius* to visual cues alone. All treatments were sealed in a clear plastic bag to isolate chemical cues. Assay = number of individuals in each assay. N = number of individuals that made no response. A = number of individuals that made a choice of treatment A. B = number of individuals that made a choice of treatment B. FSW = filtered seawater.

Choice					Results			
Trial	A	B	Assay	N	A	B	$\chi^2$	P-value
1	<i>H. purpurascens</i> and <i>E. radiata</i>	FSW	30	13	15	2	9.94	<0.01
2	Crushed <i>P. eximius</i>	FSW	30	20	5	5	0	1.00
3	<i>H. purpurascens</i> and <i>E. radiata</i> bagged	<i>H. purpurascens</i> and <i>E. radiata</i>	30	16	7	7	0	1.00



**Table 3.** The response by *P. eximius* to olfactory cues alone. Assay = number of individuals in each assay. N = number of individuals that made no response. A = number of individuals that made a choice of treatment A. B = number of individuals that made a choice of treatment B. FSW = filtered seawater.

Choice					Results			
Trial	A	B	Assay	N	A	B	$\chi^2$	P-value
1	FSW (right)	FSW (left)	30	20	3	5	0.80	0.37
2	<i>E. radiata</i>	FSW	30	15	11	4	0.40	0.07
3	<i>H. purpurascens</i>	FSW	30	16	3	11	4.57	0.03
4	<i>P. eximius</i>	FSW	30	13	7	10	0.53	0.47
5	<i>H. purpurascens</i> and <i>E. radiata</i>	FSW	30	19	6	5	0.09	0.47
6	<i>P. eximius</i> and <i>H. purpurascens</i>	FSW	30	28	0	2	2.00	0.76
7	<i>P. eximius</i> and <i>E. radiata</i>	FSW	30	22	2	6	2.00	0.16
8	<i>P. eximius</i> , <i>E. radiata</i> and <i>H. purpurascens</i>	FSW	30	19	7	4	0.82	0.37

## Discussion

Given the strong association between the gastropod, *P. eximius*, and the kelp-dwelling urchin, *H. purpurascens*, we expected that the gastropod would display a strong behavioural response to the presence of one or both of these species. Locating host species is a fundamental stage in the life history of symbiotic species (Williamson et al., 2012; Lechinni, 2011; Hay, 2009, Thiel and Baeza, 2001), with the strength of host habitat recognition capabilities often reflective of the strength of the association (Williamson et al., 2012). To the contrary we found that *P. eximius* displayed only a weak behavioural response to the presence of either the kelp or the urchin. Of the individuals that responded to stimuli it appeared that visual cues were more important in locating the kelp and the urchin. Furthermore, the association of the gastropod with the kelp and urchin did not appear to represent a behavioural response to the threat of damage. No response to crushed conspecifics was observed.

When presented with the kelp and urchin together, such as would be encountered in the field, only 55% of gastropods responded positively to the stimulus. Instead, over 45% of gastropods did not move over the 30 min experimental period. This may have been because the tank used in these experiments was too small, causing the scent to saturate the water so no direction could be detected. Follow-up experiments suggested that the stimulus for movement was primarily visual as opposed to olfactory, as gastropods continued to respond to these two species when effluent was removed. By contrast, olfactory experiments revealed that the chemical cues from the urchin were, if anything, a deterrent to the gastropod and the positive response of the gastropod to the kelp was very weak. Moreover, when olfactory cues were presented together, the gastropod did not respond.

Gastropods vary in their visual capabilities, with eye structures ranging from small simple eyes to well developed camera-type eyes (Seyer et al., 1998). Studies on the vision of marine gastropods such as the common periwinkle (*Littorina littoria*) and the conch (*Strombus raninus*) showed eye lenses with the ability to resolve fine image detail (Seyer et al., 1992).

Other gastropods however, such as the prosobranch *Ampularia* sp., have relatively poor vision, which appears to be suitable for only simple visual behavioral tasks (Seyer et al., 1998). *P. eximius* appears to use vision to carry out relatively difficult tasks of locating a suitable host plant (*E. radiata*) containing its facilitator species, *H. purpurascens*, which is wrapped within the fronds of the plant. In order to carry out this task using visual cues alone, it is likely that this species of gastropod has a complex eye structure with visual capabilities similar to those found in *L. littoria* and *S. raninus*. From this study we know that *P. eximius* is able to recognize its preferable habitat from a distance of 7 cm, however the visual competence of this gastropod to distances greater 7.5cm has not been tested. Given that *P. eximius* is a generalist species, preferentially inhabiting *E. radiata* with *H. purpurascens* but also inhabiting other species of habitat forming algae, it is likely that the behavioural strategy employed by *P. eximius* is to move at random between host species, until sufficiently proximal its host and facilitator whereby it can visually hone in on its preferable habitat (Bell et al., unpublished, Chapter 2).

*P. eximius* did not succeed in displaying a strong response to the urchin and kelp, and responses to crushed conspecifics were similarly weak. When exposed to both visual and chemical stimuli from crushed conspecifics, most gastropods remained still as opposed to moving away from the stimulus. Hence, the association of the gastropod with the urchin and kelp could not be explained as a behavioural response to the threat of damage. Studies have shown that such “freezing behavior” is a common strategy adopted by many species when confronted with danger (Chelini et al., 2009, Caro and Girling, 2005, Misslin, 2003). Freezing behaviour increases an individual’s chance of survival by reducing the likelihood of it being detected or recognized as prey by the predator (Misslin, 2003). This is logical in this situation given the cryptic colouration of the shell of *P. eximius* against the *E. radiata* habitat it usually inhabits. By adopting the freezing behaviour in the presence of crushed conspecifics (which may be indicative of a predator) *P. eximius* is more likely to stay camouflaged against the

kelp background and less likely to be predated on by predatory species that rely mainly on vision to detect prey items.

This study considered only the response of adult *P. eximius* to cues from its hosts, *H. purpurascens* and *E. radiata*. It remains possible that the association is set up earlier in the life history of the gastropod and although adults do not respond behaviorally to the presence of the kelp and urchin, larvae or juveniles do. Another possibility is that *P. eximius* does not simply respond to just one cue (either visual or olfactory), but responds to a multitude of weak cues originating from a range of mechanisms. If so, this would imply that *P. eximius* is relatively resilient in its association with *H. purpurascens* and *E. radiata*. Alternatively, *P. eximius* is not selective in terms of habitat at all, but experiences reduced mortality when associated with *E. radiata* and *H. purpurascens* as opposed to other substrates. Whilst these species exist on exposed coasts and semi protected environments and are able to move around in these conditions, there is a possibility that the simulated flow in this experiment has resulted in an experimental artefact. Previous research has shown that adult *P. eximius* that are associated with *E. radiata* and *H. purpurascens* are larger in size than individuals found on other habitats (Bell et al., unpublished, Chapter 2), suggesting that individuals within this association are able to survive for longer and this is likely a result of the refuge provided by *H. purpurascens* within the fronds of *E. radiata*.

In summary, this study has shown that visual cues play only a weak role in facilitating the establishment of the facultative association between the gastropod *P. eximius* and the urchin *H. purpurascens* and olfactory cues from the host species are unimportant. This study adds to growing evidence that although host recognition is essential for obligate relationships (Williamson et al., 2012, Ambrossio and Brooks, 2011), it may only play a small part in the establishment of facultative relationships. Instead facultative relationships may be driven by enhanced growth or survival of the focal species in the presence of the second. Studies are

now needed that compare how the survivorship and growth rate of the gastropods, *P. eximius* differs inside and outside of the association.

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## **CHAPTER 4: NUTRITIONAL BENEFITS OF A GASTROPOD COHABITING WITH A KELP-DWELLING SEA URCHIN: A CASE OF COPROPHAGY?**

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## Abstract

The role of nutritional quality and shelter in host plant selection was investigated for the herbivorous marine gastropod *Phasianotrochus eximius*. This species preferentially inhabits the common kelp *Ecklonia radiata* and is closely associated with the canopy dwelling sea urchin *Holopneustes purpurascens*. Both *H. purpurascens* and *P. eximius* are found wrapped within the fronds of the kelp, within a shelter built by the sea urchin. This shelter potentially provides the sea urchin and gastropod with protection from predation and physical forces. A 24 hour feeding preference experiment on three main food types gave evidence that *P. eximius* are coprophagous, preferring to consume urchin excrement over kelp with or without epiphytes. Isotopic ratio analysis on these three food types showed urchin excrement was significantly higher in nitrogen, thus having a higher nutrient value than fresh kelp with or without epiphytes. A long term consumption experiment whereby gastropods were fed one of the three main food types for a period of 8 weeks indicated that gastropods that consume urchin excrement performed better, having greater reproductive development. Scanning electron microscopy revealed that *P. eximius* has a “broom-like” radula making it unlikely to be able to rasp at the leathery kelp and more likely to consume soft food types such as the urchin urchin excrement. This study gives evidence for the mechanism by which this positive indirect association exists.

**Keywords:** *Phasianotrochus eximius*, *Holopneustes purpurascens*, *Ecklonia radiata*, herbivory, mesograzers, nutrients, plant herbivore interaction, fitness

## Introduction

While host plant selection by herbivores can be driven by several factors; food and shelter are generally considered the two most important (Puttman 1986, Bostrom and Mattila 1999). The preference to inhabit a particular plant is often made on a costs and benefits basis, whereby traits of the host plant are weighed up against the specific needs of the individual within its environment (Bostrom and Mattila 1999, Poore and Steinberg 1999). For example, when predation pressure is low, a herbivore may choose to graze on a particular host plant, or a variety of host plants that provide higher nutritional value over a plant with higher refuge value. By contrast, when predation pressure is high, the herbivore may be more likely to inhabit a host plant that provides sufficient shelter, regardless of the nutritional value of the host (Puttman, 1986, Duffy and Hay 1991, Ohgushi 2005, Thomsen et al. 2010).

Marine primary producers are typically nutrient poor, thus herbivorous marine invertebrates tend to be generalists (Hay 1991), consuming a mixture of algal species in order to achieve a nutritionally complete diet (Mattson 1980, Cruz-Rivera and Hay 2000b). Diet mixing is beneficial for consumer performance, especially for mobile species that have the ability to search and acquire different types of algae (Cruz-Rivera and Hay 2000b). If predation pressure, competition or physical stressors are high often herbivores are forced to stay closely associated with the host plant that provides the best protection (Cruz-Rivera and Hay 2000b).

Macroalgae provide food and habitat to a variety of mesoherbivores including isopods, amphipods, and small gastropods and echinoderms (Pavia et al. 1999). The fronds of macroalgae give shelter to inhabitants, protecting them from wave action, tides and currents, as well as from predation from fish and other predators (Brawley 1992). Studies have shown a close positive correlation in the abundance and diversity of mesoherbivores and the degree of habitat complexity of macroalgae (Dean and Connell 1987, Hacker and Steneck 1990, Pavia et al. 1999, Langellotto and Denno, 2004, Moran 2010).

The feeding capabilities of mesoherbivores play an important role in plant-herbivore interactions (Steneck and Watling 1982). Gastropods, in particular, have a diverse range of feeding constraints that are based on structural differences in their feeding apparatus (Steneck and Watling 1982). Some gastropods have robust radulae surrounded by strong buccal muscles that enable feeding on large leathery kelp and crustose algae (Steneck and Watling 1982). Others have radulae that act more like brooms that are only capable of sweeping the surface layer of the substratum for microscopic and filamentous algae (Steneck and Watling 1982). This diversity in herbivore feeding capabilities not only affects plant-herbivore interactions but also herbivore-herbivore interactions, allowing different species to coexist on the same host plant due to specialisation of different niche areas within the plant (Steneck and Watling 1982).

This study investigates the importance of food and shelter in host plant selection by the gastropod *Phasianotrochus eximius*. *P. eximius* inhabits the common kelp *Ecklonia radiata*, in close association with the canopy dwelling sea urchin *Holopneustes purpurascens*. Both *P. eximius* and *H. purpurascens* occur wrapped within the lamina of the *E. radiata* of which the sea urchin consumes (Williamson and Steinberg 2012, Bell et al. unpublished). This refuge that the urchin builds is thought to protect the urchin from visual predators (Williamson et al. 2004), and may also serve as a refuge from predation for the gastropod (Bell et al. in review).

## **Methods**

### ***Study site and collection of organisms***

Collections of *Ecklonia radiata* with attached *Phasianotrochus eximius* and *Holopneustes purpurascens* for the various experiments were made from shallow subtidal rocky reef habitat at Long Bay (33° 96' 50" S, 151° 25' 37" E) or Fairlight Beach (33°51'23"S, 151°14'35"E) in Sydney, Australia. This site is representative of the habitat in which *P. eximius*, *H. purpurascens* and *E. radiata* are commonly found and comprises a mosaic of algal species,

including *Ecklonia radiata*, *Sargassum vestitum*, *Sargassum linearifolium* and *Corallina officinalis*. Organisms were transported to the seawater facility at Macquarie University in sealed collection containers with aerated seawater.

### ***Preference on different diets***

To determine whether individual *P. eximius* display a preference among the three most abundant food resources (clean *E. radiata*, fouled *E. radiata* and *H. purpurascens* excrement) in its preferred natural habitat, a 24 hour feeding choice experiment was done. Small samples of clean kelp, fouled kelp and *H. purpurascens* were collected from the field, and excrement collected from the *H. purpurascens* within 48 hours of urchin collection. *H. purpurascens* excrement was obtained by placing several individuals in an aerated container, starving them for 24 hours then feeding them *ad libitum* a mixture of clean and fouled *E. radiata* for approximately 24 hours. *H. purpurascens* excrement collected at the bottom of the container was then separated from the water by first removing urchins and kelp and carefully sieving the excrement from the remaining seawater using 100 µm mesh.

Feeding preferences of *P. eximius* were assessed in a laboratory trial, where ten individuals were offered the three diets and the rate of consumption on each diet assessed over 24 hours (n=10). A laboratory preference experiment was considered appropriate because these gastropods live in close association with their symbiont (*H. purpurascens*) within the fronds of their host *E. radiata* and are almost continuously exposed to each of the different food types tested here in field conditions. Ten *P. eximius* were collected, measured and individuals were placed in separate 500mL glass beakers filled with gently aerated clean seawater.

Individual *P. eximius*, with an average length of  $13.00 \pm 0.89$  (mean  $\pm$  SE) and width of  $6.95 \pm 0.41$ , were offered all three food types at an average wet weight of 1.06g ( $\pm 0.04$  SE) urchin excrement, 1.66g ( $\pm 0.09$  SE) clean kelp and 2.04g ( $\pm 0.11$  SE) fouled kelp arranged in separate areas of the beaker. The wet weight (following gentle blotting with a paper towel) of

each food type was recorded prior to being placed at the bottom of each beaker and, to assess consumption, after 24 hours. So as to separate mass loss due to consumption from mass loss due to food degradation, a further ten ‘control’ beakers containing the three potential food types, but without gastropods, were simultaneously established. Urchin excrement was extracted from each beaker using a 100µm sieve, and then scraped onto paper towel before being blotted and weighed. Individual treatment replicates were randomly paired with individual controls to calculate the amount of each food type consumed by each of the 10 replicate gastropods. The amount of each food type consumed by each gastropod was calculated as:

$$(H_i \times C_f/C_i) - H_f.$$

Where  $H_i$  = initial treatment weight,  $H_f$  == final treatment weight,  $C_f$  = final control weight,  $C_i$  = initial control weight.

This equation enabled determination of the true amount of each diet consumed by the snails by taking into account any change in diet weight due to factors other than consumption.

Analysing data from choice feeding experiments are inherently difficult due to the lack of independence between food types (Peterson and Renaud 1989). The obtained consumption values for each diet were analysed using the non-parametric Friedman test as per Roa (1992). The factor tested was diet type and this was blocked by each replicate.

### ***Performance on different diets***

The longer term consequences (i.e. growth, survival and reproductive fitness) - coined “performance” - of *P. eximius* being fed on diets of clean kelp, fouled kelp and urchin excrement were determined over a period of eight weeks. Sixty adult *P. eximius* were randomly assigned to one of the three diets (20 per treatment) before being measured for shell length, shell width and body wet weight (following towel drying). Each *P. eximius* (mean

shell length =  $9.94 \pm 0.36$  mm  $\pm$  SE, N = 60) along with its corresponding diet was then placed in a separate 100ml plastic floating container with 0.2 mm aperture mesh walls to prevent both the snail from escaping and the diet from floating away. The floating containers were then transferred into a large 2000L tank in Macquarie University's Seawater Facility (SWF). Seawater within the facility was maintained at the same conditions (i.e. temperature, pH, light levels and salinity levels) as those at the site of collection. Seawater flow within the tank was  $60\text{ L h}^{-1}$ . *P. eximius* were fed *ad libitum* their allocated treatment, with algal diets being replaced approximately twice a week. The method with which urchin excrement was collected is described in the section 'preference on different diets'. The excrement diet was replaced every two days. Survivorship, growth (length of shell to nearest mm) and wet weight (blotted dry) of individual *P. eximius* were measured weekly. Mortality of *P. eximius* was determined by examining the body of the snail weekly for a lack of movement and changes from its normal colour to white (indicating that it was in the initial process of decay).

To determine whether the type of diet had a significant effect on the growth of *P. eximius*, differences in weight, shell length and shell width from the start of the experiment to the end of the experiment were assessed for all individuals that survived the eight week duration. Data for differences in length and width did not meet the assumptions of normality and were therefore analysed using the Kruskal-Wallis test. Differences in weight were analysed using a one-way ANOVA. A one-way ANOVA on  $\log_{10}$  transformed data showed no significant difference in the shell length of snails between treatments at the commencement of the experiment ( $F_{2,59} = 0.76$ ,  $p = 0.472$ ). Hence, starting body size did not need to be included as a covariate in analyses.

The differences in the survival of *P. eximius* on the three diets over time was analysed using Cox Proportional Hazard Model.

A comparison of the reproductive status (as a measure of performance) of individuals within each diet treatment at the end of the eight week experimental period, to those collected from the field (at the same place that the initial collection had been made) both before and after the experiment, was made. However, due to the high level of mortality in the first experiment, the eight week experiment was repeated a second time and the surviving gastropods from both experiments were used in order to determine performance across a representative number of individuals. The total number of gastropods used to determine performance on different diets was 32 with 21 gastropods collected from the field before and 27 collected from the field for comparison after the experiment. Here we hypothesise that individuals which consume a diet of greater nutritional value will have greater gonad development. Individuals that survived the entire length of the experiment (within a diet treatment) and those collected from the field at the start and at the end of the experiment (n=20)) were dissected immediately after collection or immediately at the conclusion of the eight week experiment. Their gonad was excised after removal of the dorsal shell and individuals were sexed (if possible) before being fixed in 10% buffered formalin.

Histology of the gonads was assessed for the cellular events of gametogenesis (Vasconcelos et al. 2008). For histological analyses, part of the gonad tissue was dehydrated in graded ethanols and embedded in paraffin. Sections (7  $\mu$ m) were stained with haematoxylin and eosin and mounted on a glass slide with coverslip. Specimens were analysed under an optical microscope connected to an imaging system. Individual gonads from both males and females were categorized into one of six gametogenic stages of development using the maturation scale described in Table 1, adapted from Vasconcelos et al. (2008). Histological sections were observed at x 20 magnification. When more than one gametogenic stage was identified within one histological section, the individual was allocated to the stage that made up the majority of the section. All slides were staged with no prior knowledge of which treatment the tissue had originated from.

**Table 1.** Scale of maturation for the assessment of gametogenic development of the gastropod *Phasianotrochus eximius* (Adapted from Vasconcelos et al. 2008).

Maturation stage	Description of obvious features of the gonad
Immature	Gonad shows some internal structure. Small groups of primary germ cells are dispersed in a loose matrix of connective tissue.
Pre-active	Gonad has less separate follicles embedded in connective tissue. The first gametogenic stages appear near the thicker follicle wall.
Active	Gonad increasingly grows in size until the follicles become closely packed in a dense connective tissue. Males have a few spermatozoa in the lumen of some follicles. Females have small and half-grown oocytes at the periphery of the follicles.
Ripe	Gonad significantly grows in size and the follicles are more closely together in compressed groups. The connective tissue disappears. In males, the follicles are swollen and contain spermatozoa. In females, the follicles are crowded with completely developed oocytes.
Partially Spent	Gonad increasingly decreases in size because spawning is in progress and the lumen of the follicle becomes emptier and surrounded by some connective tissue. Most follicles still contain spermatozoa or ripe oocytes.
Spent	Gonad further decreases in size because all follicles have released gametes. An earlier stage of large empty follicles to a later stage of small empty follicles dispersed in a loose matrix of fully developed connective tissue is apparent.

### *Nutritional value of diets*

To test whether preference or performance on a diet by *P. eximius* was correlated to their nutritional quality, the percentage carbon and nitrogen, as well as carbon to nitrogen ratios were determined for clean *E. radiata* (kelp), fouled kelp and *H. purpurascens* excrement. Algal diets were haphazardly collected from five freshly collected replicate plants. *H. purpurascens* excrement was obtained by starving several *H. purpurascens* for 24 hours then feeding them *ad libitum* a mixture of clean and fouled *E. radiata* within a small collection container with aerated water, for approximately 24 hours. *H. purpurascens* excrement was separated from seawater by sieving it through 100 µm mesh.



Samples of all diets were freeze dried and homogenised into a fine powder. Following homogenisation, subsamples of each were placed into capsules of between 1 and 3 mg weight. Samples were analysed using a LECO CHN analyzer at the department of Chemical and Biomolecular Sciences at Macquarie University. The percentage carbon, nitrogen and the ratio of carbon to nitrogen of the three diets were compared using a one-way ANOVA and post hoc tests were carried out using Tukey's test.

### ***Feeding capability of P. eximius***

The ability of a gastropod to consume a particular type of macroalga depends in part on the toughness of the plant relative to the morphology of the radulae of the gastropod (Steneck and Watling 1982). To determine the feeding capability and limitations of *P. eximius*, the radula was examined using scanning electron microscopy. Radulae from five *P. eximius* were removed and fixed for Scanning Electron Microscopy (SEM). Samples were placed in a watch glass and covering with 5ml of distilled water and two 0.07 g hydroxide pellets. Once removed from the hydroxide, samples were placed in an oven at 60°C for four hours, after which any remaining soft tissue was gently removed with forceps under light microscopy. The radulae were then allowed to thoroughly dry before being mounted to stubs using double sided carbon tape, coated in gold (to ensure conductivity) and attached to an SEM base with a small piece of double carbon sided tape.

### ***Survival in different habitats***

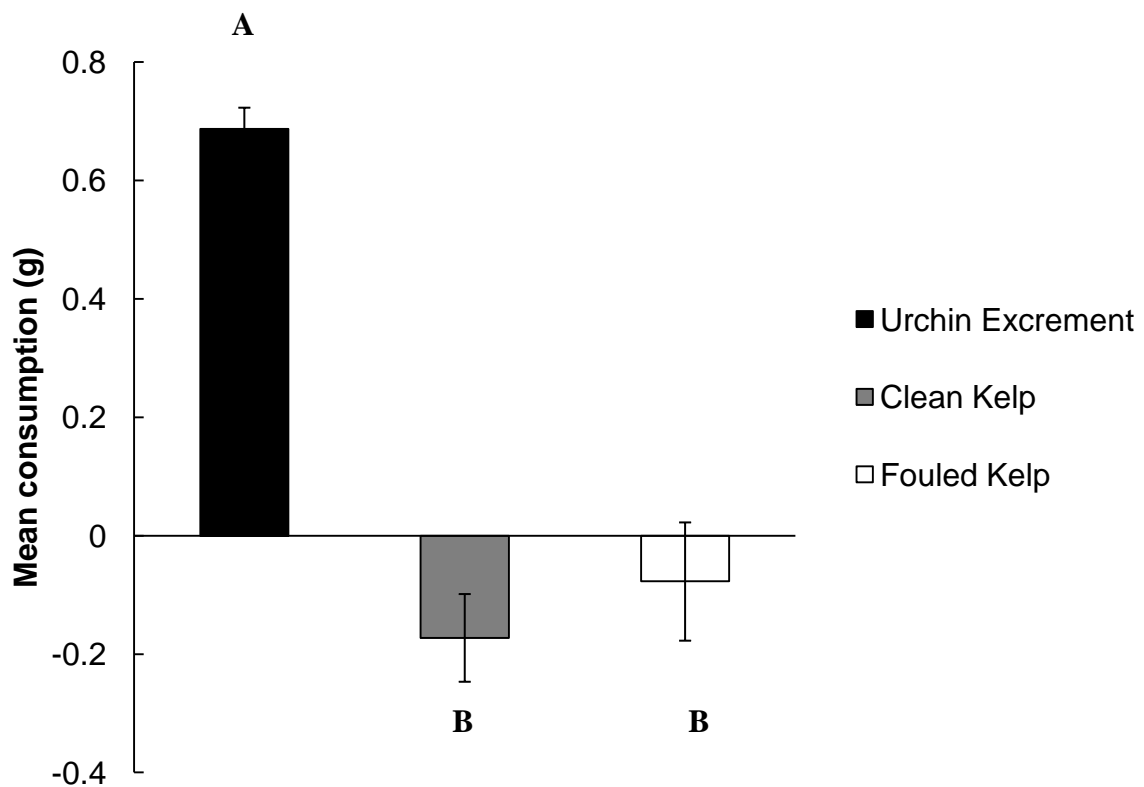
*P. eximius* occurs in *E. radiata* in greater abundances when *H. purpurascens* is present than when *H. purpurascens* is absent (Bell et al. in review). It is likely that this association is, in part, regulated by anti-predator behaviour by *P. eximius* (Bell et al. in review). To determine whether predation on *P. eximius* may be driving the association in addition to any nutritional benefit, a field experiment was therefore run to assess whether short term survival of *P. eximius* on its host plant *E. radiata* with and without its symbiont *H. purpurascens* differed

significantly. Such very short-term tethering experiments have been used successfully to estimate variation in predation rates in other invertebrates (e.g. Stachowicz and Hay, 1999). Thirty *P. eximius* were collected on SCUBA at Fairlight. On land, the shells of individuals were wiped dry and 15Lb monofilament clear coloured fishing line was tethered to the posterior part of the dry shell using a spot of superglue gel and a small square of electrical tape around the superglued area to secure the tether. Twenty *E. radiata* were haphazardly chosen and marked using flagging tape. Each gastropod was then returned to the shallow subtidal and tethered to the middle of a plant using a needle to pierce the base of the stipe of the plant with the fishing line. The length of the fishing line was 40cm, approximately half the height of the plant, allowing gastropods to move to the top and the bottom of the plant easily. Preliminary trials had shown that the tether would remain securely in position on the stipe over time (Bell et al. in review). The tethered gastropod was then exposed to one of two treatments - *E. radiata* plus *H. purpurascens*, or *E. radiata* alone. A control consisting of a rope with a buoy attached to one end of a weight representing an exposed habitat was also used and individual *P. eximius* were tethered to the base of the rope using the same method as above. The survival status (dead or alive) of each tethered gastropod was recorded after 24 hours. The observed and expected survival rates for each treatment within this experiment were analysed using a chi square goodness of fit test. This experiment was repeated and the results combined using Fisher's method of combined probabilities (Underwood, 1997).

## **Results**

### ***Preference on different diets***

When offered a choice of the three diets, *P. eximius* displayed a strong preference for urchin excrement over clean or fouled kelp ( $S = 15.2$ , d.f. = 2,  $P\text{-value} = 0.001$ ). There was no evidence of consumption of clean kelp or fouled kelp by *P. eximius* over the 24 hours (Figure 1).



**Figure 1.** Mean ( $\pm$  standard error) amount of each diet consumed by *Phasianotrochus eximius* during the 24 hour period. Means are corrected for autogenic change.

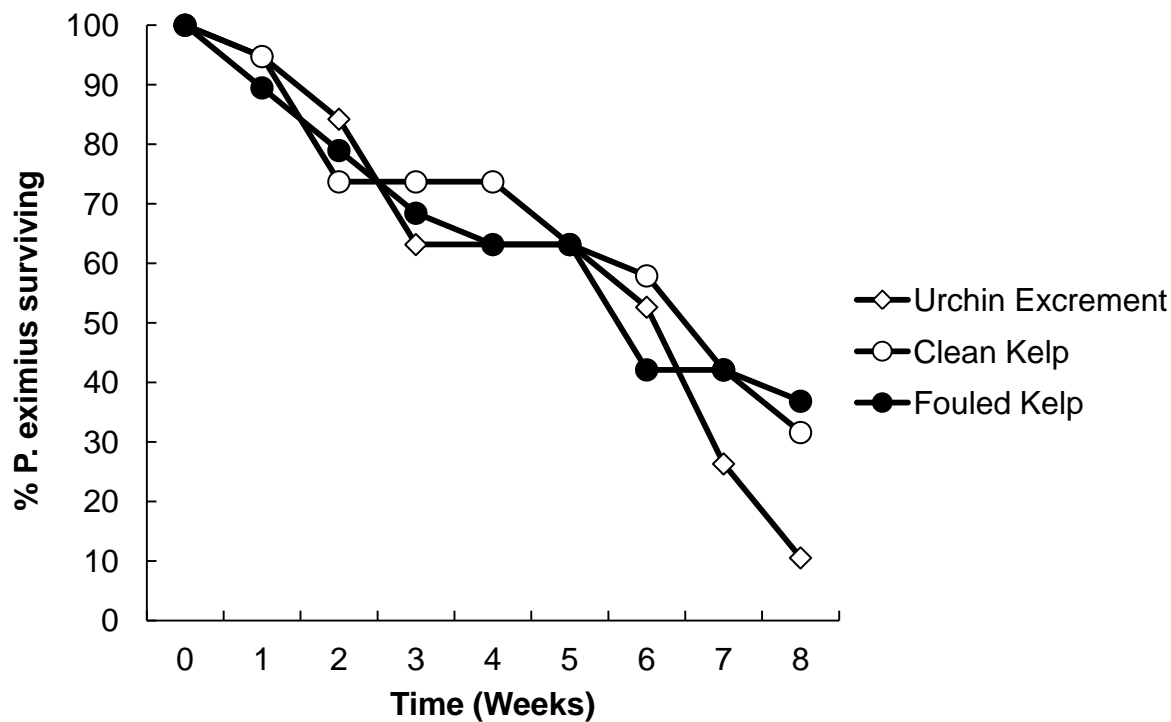
### *Performance on different diets*

There was no difference in the effect of diet over time on survival with survival decreasing in the same manner between treatments over the 8 week period ( $Z = 0.43$ ,  $P\text{-value} = 0.664$ ).

There was no significant difference in the effect of diet on survival in the absence of time ( $Z = -0.45$ ,  $P\text{-value} = 0.655$ ). However, time had a significant effect on survival in the absence of diet ( $Z = -0.392$ ,  $P\text{-value} < 0.00$ ) with mortality increasing over time across all three treatments.

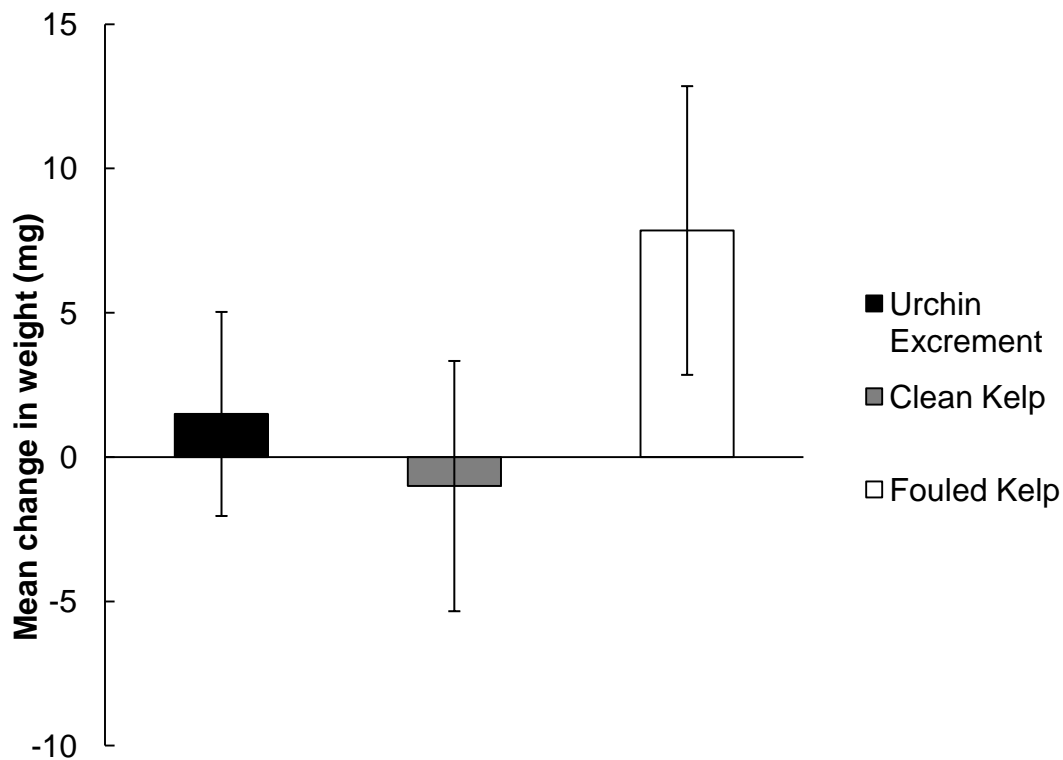
Overall, the survival of gastropods over the 8 week period was low. During the last two weeks, survival within the urchin excrement treatment began to drop quickly in comparison to the clean and fouled kelp treatments, however there was not a significant difference in

mortality between week seven and eight (Figure 2). This result is possibly due to an artefact of the low numbers of individuals left in this treatment and is likely to have resulted due to the death of a comparatively low number of individuals.



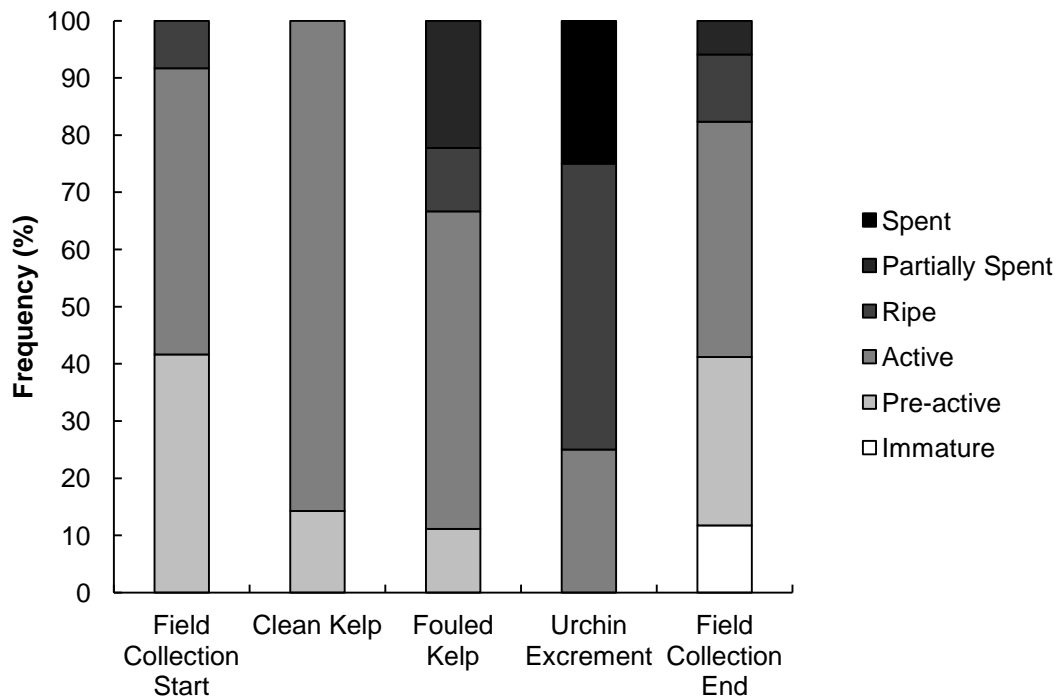
**Figure 2.** Percent survivorship of *P. eximius* fed a diet of clean kelp, fouled kelp or urchin excrement for a period of 8 weeks.

*P. eximius* that survived the 8 week experiment, did not show a significant difference in weight ( $F_{2,12} = 1.14$ ,  $p = 0.353$ ) (Figure 3). A similar pattern was seen for shell length ( $H = 0.77$ , d.f. = 2,  $p = 0.681$ ) and shell width ( $H = 0.93$ , d.f. = 2,  $p = 0.629$ ).



**Figure 3.** Mean ( $\pm$  SE) change in *P. eximius* weight fed a diet of clean kelp, fouled kelp or urchin excrement for a period of 8 weeks.

Diet affected the reproductive status of individuals. In general, male gastropods that were fed urchin excrement had more advanced gonad development compared throughout the experiment with a larger proportion of ripe individuals compared to the other diet treatments and field samples (Figure 4, Table 2). This treatment also had a larger proportion of individuals that were classified as spent, which also differed from the other diet and field samples (Figure 4, Table 2). Male gastropods fed clean kelp or fouled kelp had a similar proportion of individuals classified as being in the active gametogenic stage compared to samples collected from the field both immediately at the start and end the experiment (Figure 4, Table 2). However, both these treatments showed lower occurrences of pre-active individuals and in the clean kelp treatment there was an absence of ripe individuals (Figure 4, Table 2). In addition, the fouled kelp treatment showed a substantial proportion of immature individuals (Figure 4, Table 2).



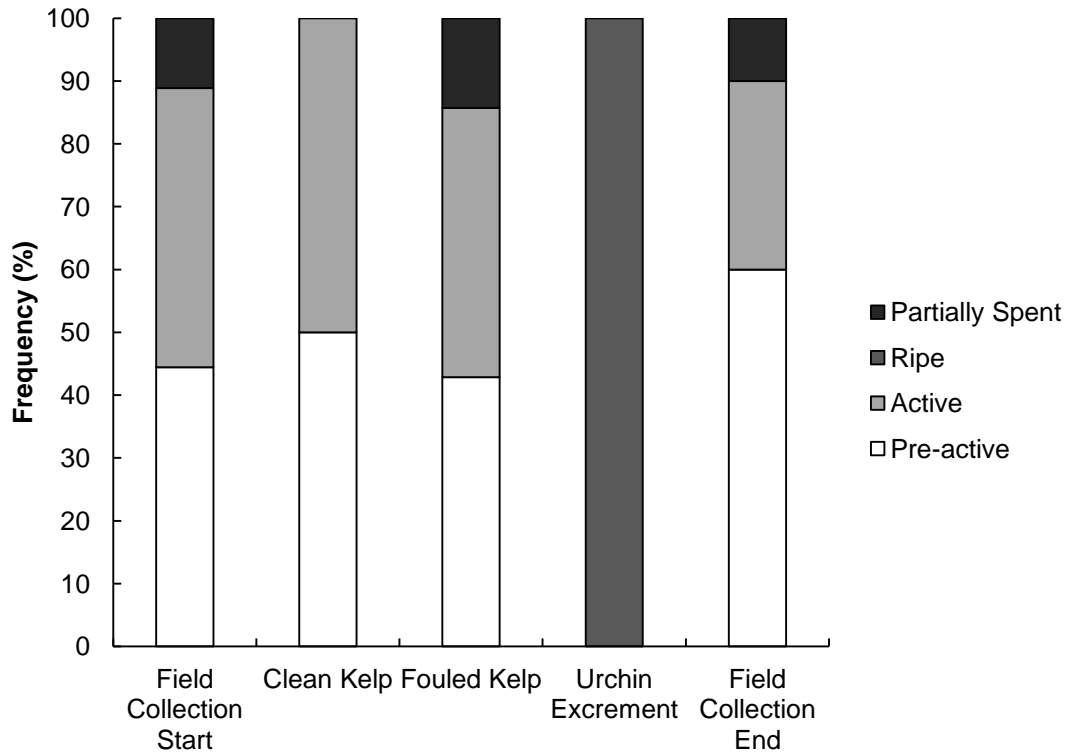
**Figure 4.** Maturity stages of male *P. eximius* after eight weeks of consuming clean kelp, fouled kelp or urchin excrement. The additional Field columns show the maturity stages of individuals collected from the field either at the start or the completion of the experiment (Table 2).

**Table 2.** Number of male *P. eximius* in each maturity stage after eight weeks of consuming one of three diet types compared to the maturity stages of *P. eximius* collected in the field before and after the experiment.

Developmental Stage	Field collection before	Clean Kelp	Fouled Kelp	Urchin Excrement	Field Collection After
immature	0	0	0	0	2
Pre-active	5	1	1	0	5
active	6	6	5	1	7
ripe	1	0	1	2	2
partially spent	0	0	2	0	1
spent	0	0	0	1	0
Total	12	7	9	4	17

Diet had an impact on the maturity status of females. All females fed urchin excrement were classified as ripe at the completion of the experiment. This stage of development was absent in both field samples. There was less of an obvious difference in the gametogenic stages present between diet treatments and field samples. Females fed diets of clean or fouled kelp

showed similar proportions of pre-active, ripe and partially spent individuals, compared to field samples (Figure 5, Table 3). However, there was an absence of partially spent individuals in the clean kelp treatment, which differed from field samples.



**Figure 5.** Percentage occurrence of different developmental stages of gametogenesis in female *P. eximius* fed a diet of either clean kelp, fouled kelp or urchin excrement for a period of 8 weeks. The additional field collection columns show the maturity stages of individuals collected from the field either at the start or the completion of the experiment (Table 3).

**Table 3.** Number of female *P. eximius* in each maturity stage after eight weeks of consuming one of three diet types compared to the maturity stages of *P. eximius* collected in the field before and after the experiment.

Developmental Stage	Field collection before	Clean Kelp	Fouled Kelp	Urchin Excrement	Field Collection After
immature	0	0	0	0	0
Pre-active	4	2	3	0	6
active	4	2	3	0	3
ripe	0	0	0	1	0
partially spent	1	0	1	0	1
spent	0	0	0	0	0
Total	9	4	7	1	10

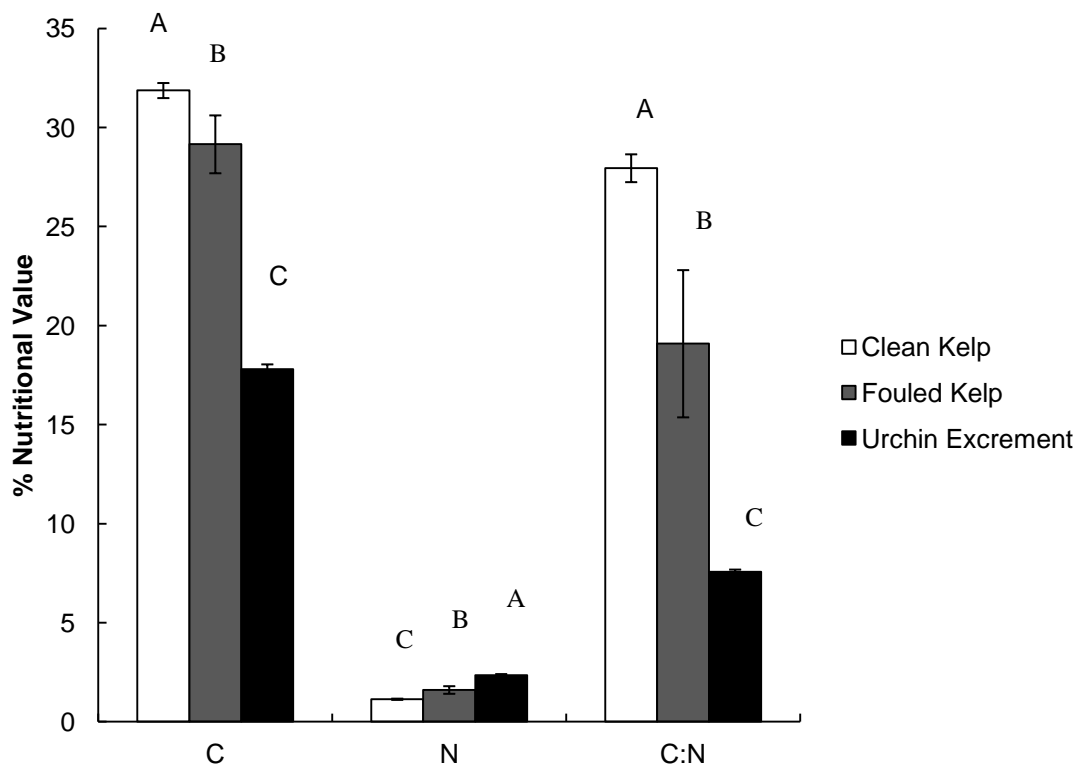
### *Nutritional value of diets*

There was a significant difference in the percentage of carbon between diets ( $F_{2, 11} = 133.88$ ,  $P\text{-value} < 0.01$ ). Tukey's post hoc test showed that clean kelp had the highest level of carbon followed by fouled kelp with urchin excrement having the lowest level of carbon (Figure 6).

The reverse trend was observed with levels of nitrogen between different diets. Again, there was a significant difference between diets ( $F_{2, 11} = 50.73$ ,  $P\text{-value} < 0.01$ ). Tukey's post hoc test showed that urchin excrement had the highest level of nitrogen, followed by fouled kelp and clean kelp had the lowest level of nitrogen (Figure 6).

The redfield stoichiometry ratio (C:N) is a method of determining the carbon and nitrogen fluxes within a system as well as estimating the limiting nutrient (if any) within a marine system (Sterner and Elser 2002). There was a significant difference in the C:N ratio between diets ( $F_{2, 11} = 43.36$ ,  $P\text{-value} < 0.01$ ). Tukey's post hoc test showed that clean kelp had the highest C: N ratio, fouled kelp had the next highest ratio and urchin excrement having the lowest C: N ratio (Figure 6).

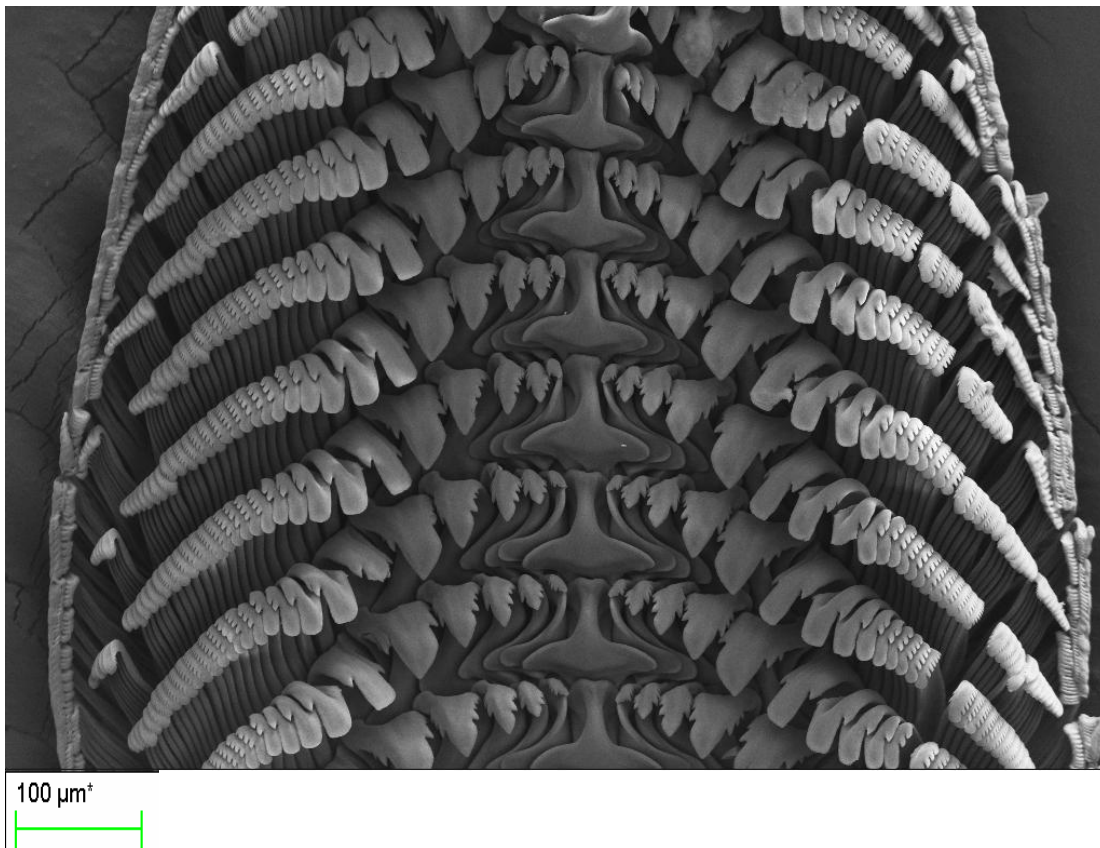




**Figure 6.** Mean (+SE) percentage Carbon (C), Nitrogen (N) and Carbon to Nitrogen Ratio (C: N)  $\pm$  standard error for each of the three diets: clean kelp, fouled kelp and urchin excrement. Separate Analyses were conducted for C, N, and C:N. Bars sharing a letter within C, N, or C: N do not differ significantly in post hoc tests.

### ***Feeding capability of P. eximius***

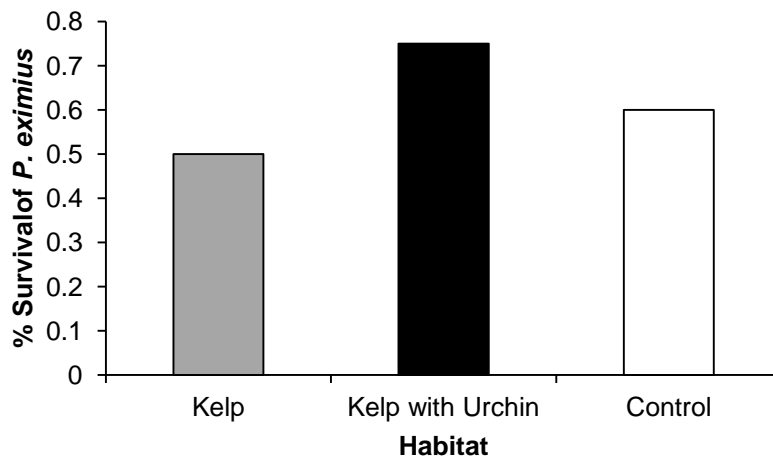
*P. eximius* has a complex structured radula with several rows of teeth and numerous teeth per row (Figure 7). The marginal teeth are long and thin with relatively narrow bases of attachment and have a blunt periphery, whereas, the lateral teeth are shorter and wider than the marginal teeth, with a sharper edge (Figure 7). The rachidian or central tooth is comparatively much shorter and wider than the marginal and lateral teeth (Figure 7) and is unlikely to come into contact with the substratum (Steneck and Watling 1982).



**Figure 7.** SEM of a posterior section of the radula of *P. eximius*.

### *Survival in different habitats*

There was no significant difference in the mortality of *P. eximius* on host plants with *H. purpurascens* compared to those on host plants without *H. purpurascens* or to those in exposed habitats ( $\chi^2 = 6.77$ , d.f. = 4, P-value = 0.15). 50% of *P. eximius* on *E. radiata* without *H. purpurascens* survived the 24 hours period, while 75% of individuals on *E. radiata* plants with *H. purpurascens* survived (Figure 8). This result may have occurred as a consequence of a tethering artefact whereby the tether promoted mortality by causing entanglement in kelp fronds.



**Figure 8.** Percentage survival of *P. eximius* on different habitats.

## Discussion

*Phasianotrochus eximius* is closely associated with the sea urchin *Holopneustes purpurascens* and the common kelp *Ecklonia radiata*. Both *P. eximius* and *H. purpurascens* occur wrapped within the fronds of the kelp within a shelter built by the urchin (Bell et al. in review). This shelter is believed to provide protection from predators or adverse microclimates for the sea urchin (Williamson et al. 2004) and has been hypothesised to serve the same purpose for the gastropod, which also inhabits this shelter (Rogers et al. 1998). However, results from this study show that food preference is a contributing factor to the close association between *P. eximius* and *H. purpurascens* on *E. radiata*. By preferentially inhabiting kelp plants with urchins, *P. eximius* has access to the kelp and epiphytes that grow on the kelp, and also to a third food, the excrement of *H. purpurascens*.

When presented with a choice of clean or fouled *E. radiata* as well as the excrement of *H. purpurascens*, *P. eximius* consumed significantly more of the excrement than the other available diets. When given no choice between these three food types, however, there was no significant difference in the survival of *P. eximius* over an eight week period. Thus, *P. eximius* is able to survive on kelp with and without epiphytes, but appears to prefer to consume urchin excrement.

Nitrogen is scarce within many environments and as a result some organisms have adapted to this scarcity by maximising the use of available nitrogen (White and Sale 1993, Williamson and Rees 1994, Mattson 1980). The excrement of *H. purpurascens* contained a significantly greater amount of nitrogen than diets of clean or fouled kelp. This is interesting as fresh excrement is usually lower in nutrients than the food from which it was derived (White 1993). However, if faeces are allowed to age, microbial transformation increases nitrogen levels as carbon is removed via microbial respiration and nitrogen is taken up from the water column (Mann 1982). Microbial processing may therefore be responsible for the high nitrogen content of *H. purpurascens* excrement.

Coprophagy is an eating behaviour adopted by many animals that have simple digestive systems (such as isopods and some gastropods) (Weiser 1978) and are less able to assimilate the necessary levels of nutrients from plants alone (Mattson 1980). It is common in marine environments (Frankenberg and Smith Jr. 1967), and is of great advantage (Brendelberger 1997) to some aquatic gastropods (Brendelberger 1997). The detritivorous gastropod *Hydrobia ventrosa*, for example, consumes its own excrement to extract available nitrogen from the microbes on the surface of the excrement (Lopez 1978). *P. eximius* may be employing the same strategy in adapting to a coprophagous eating behaviour.

Consumption of the nutrient-rich excrement resulted in greater reproductive fitness for *P. eximius*. Gastropods that were fed a diet of urchin excrement over a period of 8 weeks resulted in individuals with further developed gonads compared to gastropods that were fed clean or fouled kelp. In fact, it appeared to speed up reproductive development as both males and females within this treatment had greater gonad development compared to individuals collected from the field. This could suggest that excrement is not as readily available to gastropods in the field as was in the experiment. The reproductive status of individuals fed 'clean' and 'fouled' kelp were not negatively effected over the study period and closely

resembled the proportion of gametogenic stages found in field samples before and after the experiment.

While there was no significant difference in the growth of *P. eximius* between the three diets over the performance experiment, individuals fed fouled kelp and urchin excrement increased in weight over the eight week period, whilst those fed clean kelp decreased in weight. Kelp plants covered in epiphytes decay at a very rapid rate (Tianjing et al. 1984) and it has been shown that this decay makes the kelp more palatable and easily digestible to marine invertebrates (Norderhaug et al. 2003). Thus *P. eximius* may be advantaged when feeding on both the decaying kelp material and the soft epiphytes thereby leading to the small increases in weight. Further research would need to be done on the rate of decay of *E. radiata* and digestibility of decaying kelp for *P. eximius*, however, to directly assess this.

Dietary constraints for herbivores are closely related to the physiological characteristics of the herbivore itself. For gastropods, the morphological characteristics of the radula are varied and are a principal determinant of the feeding capabilities of species. *P. eximius* has a complex structured radula that fits into the rhipidoglossan functional group, as described by Steneck and Watling (1982). Radulae within this functional group typically have several rows of teeth and numerous muscles in the buccal mass, which help in splaying the teeth when the radula is extended. They are sometimes referred to as brooms as they act in a sweeping motion to pick up food particles. The marginal teeth, which are used for food gathering into the mouth, are long with narrow bases of attachment. Due to the length of the marginal teeth, these types of radulae are unable to exert substantial force onto their substrate and are rarely seen grazing leathery kelp or calcareous algae (Steneck and Watling, 1982). This type of radula has limited grazing capabilities and species with this type of radula generally feed on soft food types (Steneck and Watling 1982). Urchin excrement as a preferred food choice for *P. eximius*, fits

into the feeding limitations of this species as it is soft and easily digestible for an animal with a radula of this type.

Longer term survival of individuals was low over the performance experiment. This may be due to the fact that this species is unable to survive under laboratory conditions for long periods of time. Alternatively it could be because the three diets tested here lacked sufficient nutritional quality to maintain long term survival when consumed alone. The ‘balanced diet hypothesis’ outlines that diets of mixed species often have complementary nutritional qualities (Pennings et al. 1993). A diet of mixed algae improves the growth and overall fitness in many marine herbivores (Aquilino et al 2012, Cruz-Rivera and Hay 2000). The “toxin minimisation hypothesis” (Pennings et al. 1993), which suggests that the consumption of a mixture of plant secondary metabolites is less harmful to herbivores than the consumption of a single type of secondary metabolite, could also explain these results. Phlorotannin levels are quite high in *E. radiata* (Steinberg 1989; Jennings and Steinberg 1997) and *P. eximius* may be avoiding consuming excessive levels of this secondary metabolite by consuming a mixed diet in the wild. As the species composition of fouled kelp is unknown, and thus the range and quantity of secondary metabolites associated with this diet, *P. eximius* may have also been affected by associated harmful chemistry in this diet as well.

It is well known that predation by visual predators has a strong influence on habitat choice by small invertebrates. Macroalgae not only provides food to mesoherbivores but also shelter from predation (Bostrom and Matilla 1999, Duffy and Hay 1991, Watanabe 1984). When predation pressure is high mesoherbivores minimise movement from host plants to avoid predation and sometimes compensate for the lack of diversity in diet by consuming a greater quantity of the available diet (Cruz-Rivera and Hay 2000). Previous research has shown that *P. eximius* is strongly associated with *H. purpurascens* and does not appear to move frequently between host plants (Bell et al. in review). Short-term experiments were therefore

designed to monitor survival of *P. eximius* on kelp plants with *H. purpurascens* compared to individuals on kelp plants without *H. purpurascens* and in exposed habitats, to test for the potential negative effects of predation in exposed environments. Interestingly, however, our results revealed no significant difference in mortality between exposed versus sheltered environments.

In conclusion, the close association between *P. eximius*, *H. purpurascens* and *E. radiata* appears to be driven by the adaptation by *P. eximius* towards a coprophageous feeding behaviour, which provides superior nutritional value compared to clean and fouled kelp. This adaptation has significant benefits on the reproductive performance of *P. eximius*. It also fits into the physiological constraints determined by the feeding apparatus of this species. Whilst being sheltered by the fronds wrapped around *H. purpurascens*, is likely to protect *P. eximius* from predators and adverse microclimates (Bell et al. in review), the results presented here suggest it is not the primary factor attracting *P. eximius* to *H. purpurascens*.

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## **CHAPTER 5: THE SEA URCHIN *EVECHINUS CHLOROTICUS* AS HABITAT FOR THE ISOPOD *EXOSPHAEROMA ECHINENSIS***

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## Abstract

In New Zealand, the sphaeromatid isopod *Exosphaeroma echinensis* lives on sea urchins, including the common *Evechinus chloroticus*; however, little is known of the ecology of this association. This study investigates the nature of the relationship between *E. echinensis* and *E. chloroticus*. In order to first determine the abundance and distribution of these species urchins were collected from three sites near Leigh in northeastern New Zealand, and sealed in bags insitu before being taken back to the laboratory, where the number of inhabiting isopods on each urchin was determined. *E. echinensis* occurred on 60-87% of individual *E. chloroticus* at mean densities of 2-5 isopods/urchin. When given a choice between the three main habitat types in its environment, *E. echinensis* preferred to inhabit *E. chloroticus* as opposed to rocks and exposed environments. The method by which *E. echinensis* finds its host was then considered, by conducting a series of laboratory experiments where the isopod was given both visual and olfactory cues originating from *E. echinensis*. *E. echinensis* were found to use visual rather than olfactory cues to locate their host. A short term feeding choice experiment was conducted whereby isopods were offered equal amounts of the main food types within its environment. *E. echinensis* appeared to prefer to consume the kelp *Ecklonia radiata*, over the excrement of *E. chloroticus*. This study highlights for the first time the nature of the relationship between *E. echinensis* and *E. chloroticus*. These two species are likely to be commensally associated, whereby the isopod benefits and the urchin neither benefits nor is harmed. Fish predation on small benthic crustaceans such as *E. echinensis* is intense, so it is likely that by living between the urchin's spines the isopod derives both shelter from predation and food in the form of kelp. Further studies are needed to quantify this hypothesis.

**Keywords:** Commensalism, rocky reef, host-recognition, echinoid, isopod, New Zealand,

## Introduction

Habitat complexity and its effect on the abundance and diversity of species within communities has been the focus of many recent marine studies (Stachowicz 2001; Bruno 2003; Gribben et al. 2009; Thomsen 2010; Angelini 2011). Foundation species such as kelps, corals and seagrasses offer habitat to other species by providing spatially complex matrices that ameliorate biological and physical stressors and are thus important for maintaining community structure (Bertness 1999; Stachowicz 2001). Such habitat forming species are often primary producers, but for many small marine animals such as isopods, amphipods and crabs, facilitation of habitat often occurs through associations with other marine macroinvertebrates such as sea urchins (Thiel et al. 2001; Marin et al. 2005; De Bruyn et al. 2009b; Williamson et al. 2012). Predation pressure is recognised as the main factor driving the evolution of such associations; however food, mating systems, and physical factors are also considered important (Thiel et al. 2001; Baeza et al. 2003; Taylor and Steinberg, 2005). The sphaeromatid isopod *Exosphaeroma echinensis* lives on rocky reefs in New Zealand, where it has only been recorded from the surfaces of sea urchins, the most common of which is the echinometrid *Evechinus chloroticus* (Hurley and Jansen, 1977). As *E. echinensis* is small and poorly defended, the spines of *E. chloroticus* are thought to provide the isopod with a physical refuge from predation and adverse abiotic factors (Dix 1970). *E. echinensis* is considered to have a commensal relationship with *E. chloroticus* as it commonly lies cryptically along the length of the spines of the sea urchin (Dix 1970), with its brown body and white uropods matching the colour of the urchin's spines (JEB, personal observation). Here we investigate the nature of the association between the isopod *Exosphaeroma echinensis* and the sea urchin *Evechinus chloroticus*. A series of field and laboratory experiments were conducted to answer the following questions: (1) What proportion of *E. chloroticus* have *E. echinensis* inhabiting their spines? (2) Of the urchins with isopods, what is the density and size distribution of *E. echinensis* on *E. chloroticus*? (3) Do *E. echinensis*

preferentially inhabit *E. chloroticus* over other available microhabitats? (4) How much movement of *E. echinensis* is there between individual *E. chloroticus* in the field? (5) Do *E. echinensis* use visual and or olfactory cues to locate *E. chloroticus*? And finally (6) Does *E. echinensis* display a preference among two sources of foods occurring within its habitat on *E. chloroticus*?

## **Materials and Methods**

### ***Study sites***

*E. chloroticus* and *E. echinensis* were collected from Matheson Bay (36.3048° S, 174.7977° E), Kempts Beach (36.2642° S, 174.7272° E) and Panetiki Island (36.2833° S, 174.8167° E) off the coast of Leigh, New Zealand, during December 2011.

### ***Population structure***

To quantify the density of *E. echinensis* per *E. chloroticus*, thirty *E. chloroticus* were haphazardly selected from depths of 5 -10m at each of the above three sites (N = 90). Each urchin was immediately sealed in a clear plastic bag and transported to the laboratory. All isopods were carefully removed from individual urchins using forceps with the aid of flowing freshwater. Isopods were measured for length (mm) to determine the overall length frequency and most abundant size class of *E. echinensis* inhabiting *E. chloroticus*.

### ***Variation in microhabitat preference***

To test whether *E. echinensis* preferentially inhabits *E. chloroticus* over other potential habitats, the position of 30 *E. echinensis* in a rocky habitat environment with *E. chloroticus* in a laboratory setting was examined for a total of 48 hours. All *E. echinensis* were placed in a 30L glass fish tank with filtered seawater (500µm), along with two regular adult sized *E. chloroticus* and two round rocks of similar size to the urchins (100mm diameter) that were collected from the habitat in which *E. echinensis* and *E. chloroticus* exists. Urchins and rocks



were arranged haphazardly throughout the base of the tank. The position of each isopod within the glass container was monitored every six hours and recorded as either on the oral or aboral surface of an urchin, on or under a rock, or on the exposed glass wall of the tank. Observations were made from the outside of the glass tank that was positioned on a stand and could be viewed from underneath to avoid disturbing the habitat. A repeated measures ANOVA was used to test for any significant difference in habitat choice over time. A Tukeys post hoc test was carried out for significant effects.

### ***The use of vision in host detection***

The ability for *E. echinensis* to visually recognize its host from a distance is likely to be an advantageous predation-avoidance mechanism. The role of vision by the isopod *E. echinensis* in the detection of the urchin *E. chloroticus* over other potential hosts (i.e. a rock) was determined by a series of visual tests in a 10 L tank. Here, the 10 L tank was filled with filtered seawater (200 µm) and different treatments were placed at opposite corners of the tank (14 cm apart). ‘Bagged’ (i.e., sealed in a clear plastic bag) treatments could be seen but emitted no chemical cues and were tested against those that were ‘unbagged’, which could also be seen but emitted chemical cues. The choices presented in this experiment were: (1) one unbagged urchin versus an urchin sized rock that was unbagged (to represent an alternate host); (2) One bagged urchin versus one unbagged urchin; (3) one bagged urchin versus bagged filtered seawater (FSW). If isopods did not discriminate between bagged and unbagged treatments they were inferred to be using visual cues alone. If isopods chose unbagged urchins over bagged urchins they were considered to be using olfactory cues or a combination of olfactory and visual cues. If there was no preference then isopods were considered to be using a combination of olfactory and visual cues or visual cues alone. At the start of each test one isopod was placed in the centre of a tank (7 cm from either treatment) and the time taken for the individual to touch a treatment over five minutes was

recorded. Tests were scored as “positive” if the isopod touched a treatment within five minutes, or as ‘negative’ if the isopod failed to touch a treatment within five minutes. A new isopod was used for each replicate and thirty replicates were carried out per treatment combination. Treatments were swapped to opposite sides after 15 replicates to avoid any potential bias towards one side of the tank.

For individuals that scored a positive, preferences were analysed using a chi-square test to for any preferences in the treatment chosen compared to an expected 50:50 choice frequency.

### ***The use of olfaction in host detection***

A laboratory experiment was carried out to test the role of olfaction, or recognition of a waterborne chemical cue, in host detection by *E. echinensis*. The system used to test this, allowed individual *E. echinensis* to distinguish between treatments using seawater flowing over the various treatments (and thus presumably containing any chemical cue emitted from that treatment) in the absence of visual cues. Individual isopods were introduced into a common aquaria from which water from two separate aquaria (one treatment per aquaria), was gravitationally fed into a Y tube. At the intersection of the Y tube equal amounts of the two sources of water come together in laminar flow into the common aquaria (Davenport, 1950). The flow of water through the Y tube was regulated by a tap at the top of each treatment to ensure that equal amounts of each source of water flows at a predetermined rate into the Y tube.

Treatment combinations (one in each treatment aquaria) that were used in this experiment included: (1) *E. chloroticus* versus *E. chloroticus* (positive control) and (2) filtered seawater versus *E. chloroticus*. Thirty replicates were conducted per treatment, with urchins replaced after each replicate and treatments swapped from the left to the right aquaria and *vice versa* after 15 replicates to account for any potential biases by the isopods towards a particular side of the aquaria or towards a particular urchin.

Isopods were collected using a suction sampler and transferred to the seawater facility at the Leigh Marine Laboratory, where they were held in a large container of filtered seawater without food for 24 hours. At the start of each trial, a new isopod was carefully placed in the center of the common aquaria. The time the isopod took to move from the starting point to the intersection of the Y tube was recorded, as well as the time the isopod took to make a choice between treatments (i.e. from the intersection of the Y tube to over half way down one arm of the Y tube towards one particular treatment). Individuals that did not move from the starting point after 30 minutes were recorded as having had no response.

Results were analysed using chi-square tests, which assessed which active choice the individuals made (i.e., which treatment) compared to a 50:50 frequency.

### ***Diet preference***

Urchin excrement is a potential source of food for *E. echinensis* that may be more easily digested than the leathery laminae of the kelp, as shown in other similar associations (Bell et al., in preparation). A short-term preference experiment was done in the laboratory to determine whether isopods showed a clear preference for diets of either fresh *E. radiata* or excrement produced by *E. chloroticus* that had consumed *E. radiata*. Consumption rates of the two diets were also assessed for the duration of the experiment.

The experiment ran in separate 500ml containers containing slightly aerated filtered seawater (500µm) and each diet. Diets of  $31.93 \pm 1.23\text{mg}$  (mean  $\pm$  SE) of kelp and  $30.23 \pm 1.78\text{mg}$  (mean  $\pm$  SE) of urchin excrement were put opposite to each other in the containers.

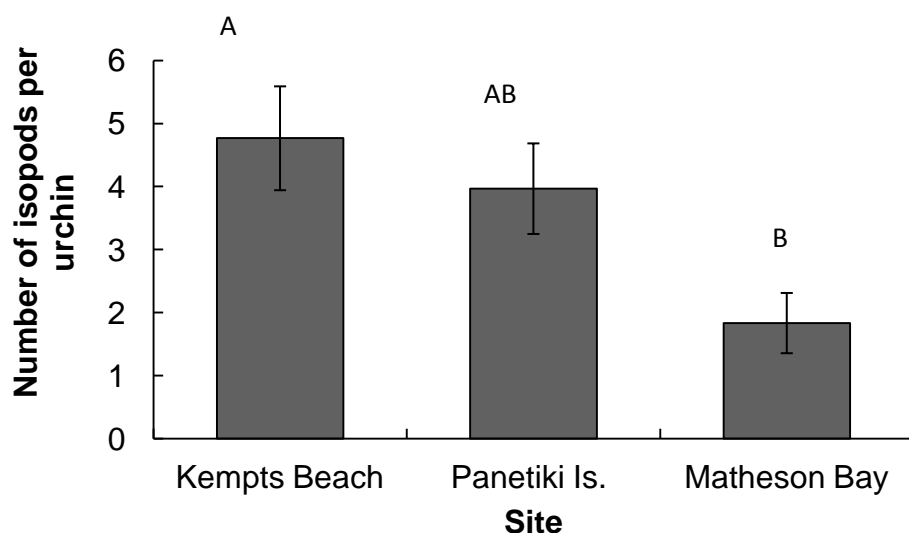
Individual isopods of mean length  $5.20 \pm 0.33$  ( $\pm$  SE) were added to each container and left for 48 h under ambient light and temperature conditions. Thirty replicate containers, each containing a choice of diet and a separate individual, were run. At the end of the experiment, the remaining food was weighed and compared to the original amount of food provided as well as to the autogenic control using the formula  $H_i \times C_f / C_i - H_f$ . Where  $H_i$  = initial treatment

weight,  $H_f$  = final treatment weight,  $C_f$  = final control weight, and  $C_i$  = initial control weight. Consumption values for each diet were analysed using the non-parametric Wilcoxon signed rank test. The factor tested was diet type and this was blocked by each replicate.

## Results

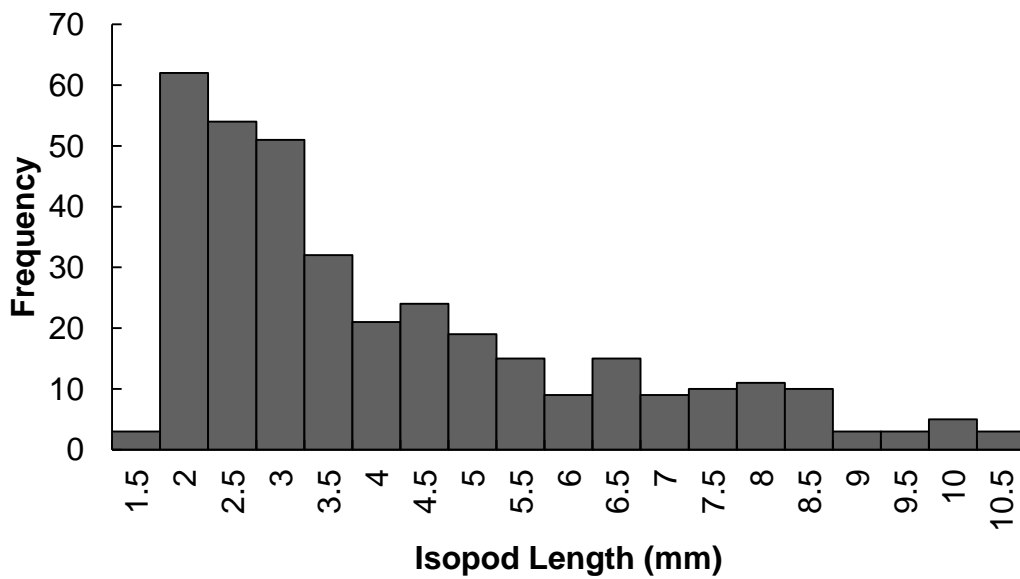
### *Population structure*

A total of 317 *E. echinensis* were collected from urchins from the three sites. Isopods inhabited 86.7% of urchins at Panetiki Is., 83.3% at Kempts Beach, and 60% at Matheson Bay. The highest average abundance of isopods per urchin was observed at Kempts Beach, followed by Panetiki Island and Matheson Bay. Abundances did not differ significantly between Kempts Beach and Panetiki Island, or between Panetiki Island and Matheson Bay, but significantly fewer isopods were observed per urchin at Matheson Bay compared to Kempts Beach ( $F_{2, 87} = 5.01$ ,  $P\text{-value} = 0.009$ ,  $N = 90$ , Figure 1).



**Figure 1.** Density of *E. echinensis* (mean  $\pm$  1 SE,  $N = 30$ ) inhabiting *E. chloroticus* at three sites in northern New Zealand. Bars with the same letter are not statistically different from each other. Columns sharing the same letter were not significantly different in Tukey's post hoc test.

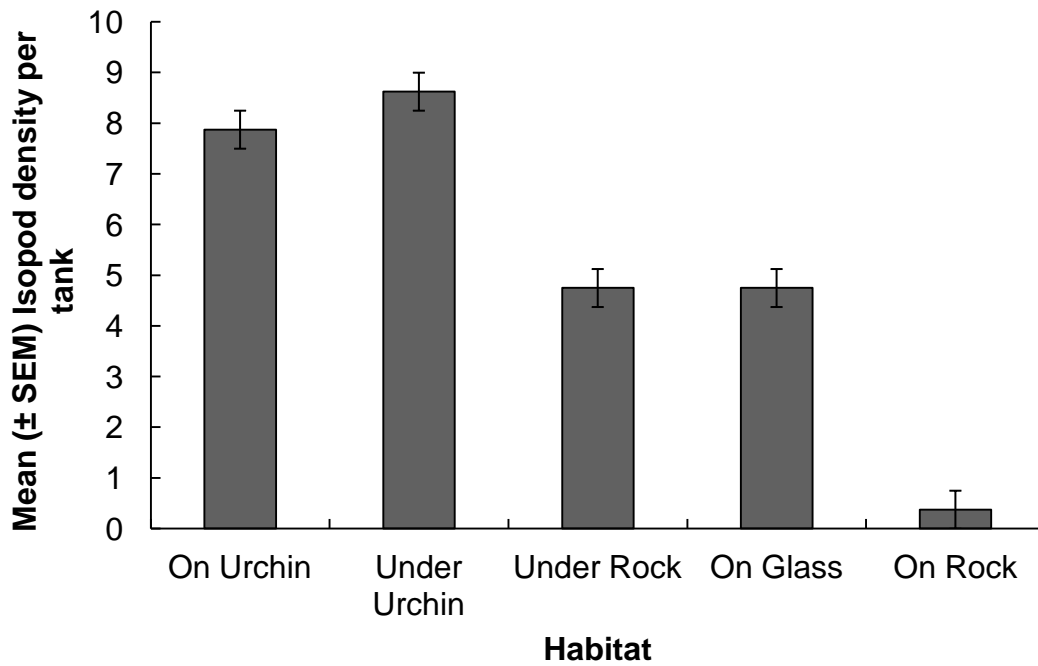
Adult isopods ranged from 1.5 to 10.5 mm in length. The most abundant size class for isopods was 2 mm in length and the least abundant size classes were 1.5 mm and 10.5 mm.



**Figure 2.** Isopod length (mm) frequency distribution at all sites combined (N = 359).

### ***Variation in microhabitat preference***

Overall, significantly more isopods inhabited the oral and aboral sides of urchins compared to on or under rocks, or on the glass ( $F_{4,35} = 4.47$ ,  $P = 0.005$ ,  $N = 39$ ). The greatest isopod density within the tank was on the oral side of urchins ( $8.6 \pm 0.9$  mean  $\pm$  SE) (Figure 3). Isopod density on the aboral side of urchins was  $7.8 \pm 0.89$  (mean  $\pm$  SE) (Figure 3). The lowest isopod density occurred on rocks of the tank where there was a mean of  $0.38 \pm 0.38$  (mean  $\pm$  SE) (Figure 3).



**Figure 3.** Variation in microhabitat preference for isopods (n=30) within a tank measured over 48 hours.

### *The use of vision in host detection*

Isopods used vision to detect host habitat. Significantly more isopods moved towards the urchin, in preference to seawater alone (Table 1,  $\chi^2 = 14.8$ ,  $df = 1$ ). Isopods did not show a preference, when given a choice between an urchin and a rock (Table 1,  $\chi^2 = 2.1$ ,  $df = 1$ ). In addition, isopods did not show a preference between unbagged versus bagged urchins (Table 1,  $\chi^2 = 1.8$ ,  $df = 1$ ).

**Table 1.** Visual host attraction of *Exosphaeroma echinensis* to the sea urchin *Echinensis chloroticus* and to rock against each other and against control signals of seawater

Choice			Results				P - value
Trial	A	B	Assay	N	A	B	
1	Urchin	rock	43	4	24	15	0.35
2	urchin bagged	Urchin unbagged	30	3	17	10	0.41
3	Urchin bagged	Seawater bagged	30	3	23	3	0.00

Choices A and B are the two proposed visual signals; Assay shows the number of replicates; N shows the number of individuals that showed no response; Results A and B show the number of isopods that actively chose A or B respectively. P – Value shows the probability significance of an isopod choosing one signal over another. All results were compared to a theoretical ratio of 50:50.

### ***The use of olfaction in host detection***

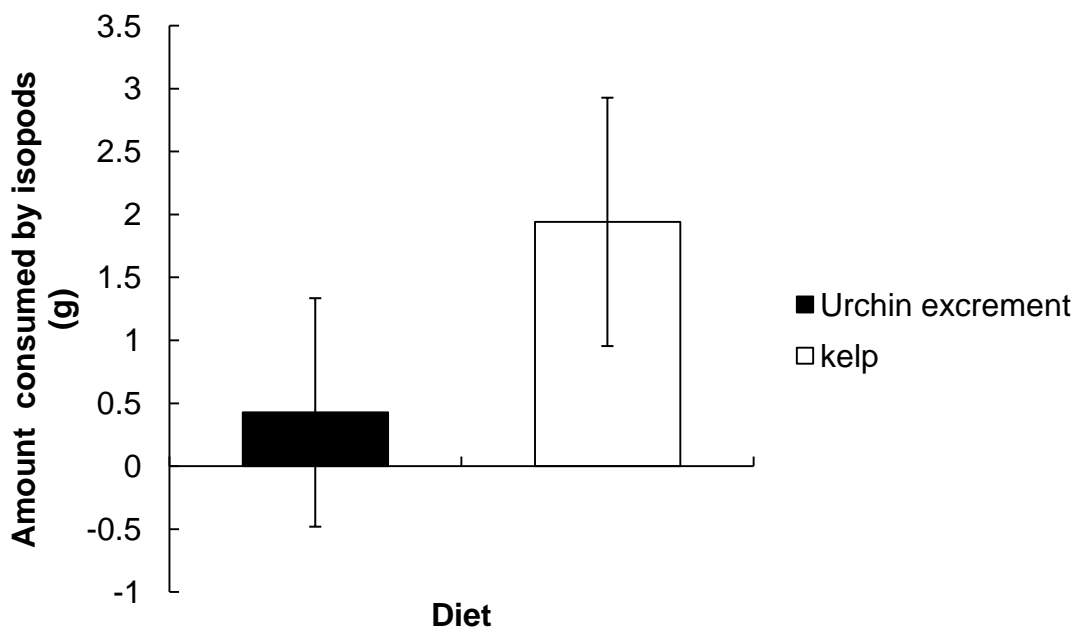
Olfaction did not appear to be the most important cue in host selection for the isopods. Of the isopods that made a choice, there was no significant difference in the choices made by isopods (urchin versus seawater) when compared to a 50:50 ratio (Table 2).

**Table 2.** Olfaction in host detection of *Exosphaeroma echinensis* to the sea urchin *Echinensis chloroticus* and against control signals of filtered seawater (FSW)

Choice			Results				P - value
Trial	A	B	Assay	N	A	B	
1	Urchin	FSW	30	12	13	5	0.16

### *Diet preference*

Isopods consumed more kelp than urchin excrement after 48 hours, however the difference in the amount consumed between the two treatments was not significant (Figure 4,  $W = 192$ ,  $P\text{-value} = 0.41$ ,  $N = 30$ ). There was substantial variation in consumption rates between replicates within a treatment and, although isopods were frequently observed consuming the kelp, individuals were rarely seen consuming the urchin excrement (Bell, personal observation).



**Figure 4.** Mass loss (mg) of each diet type due to consumption by isopods after 48 hours, when given a choice between kelp and urchin excrement. Data are the mean ( $\pm$  SE).

### **Discussion**

In this study, *E. echinensis* inhabited 60-87% of *E. chloroticus* individuals. The length-frequency distribution of *E. echinensis* on *E. chloroticus* was positively skewed, with a greater number of smaller individuals inhabiting these urchins than larger individuals.

Isopods responded more strongly to visual cues than olfactory cues to locate a host urchin. However, research has shown that the mechanisms governing the physical transport of



olfactory signals within the water column such as bottom roughness and turbidity, can strongly influence the ability of a marine species to pick up a particular cue and this may have been a contributing factor in the lack of response to olfactory cues by *E. echinensis* (Weisburg and Zimmer-Faust, 1993).

Laboratory habitat experiments revealed that isopods preferably inhabited urchins over rocky habitat. Food and shelter are considered the main driving factors associated with habitat choice in small crustaceans, and finding habitat that can provide sufficient refuge as well as adequate nutrition improves the fitness of the individual (Boström & Mattila 1999, Lasley-Rasher, 2011).

For certain species the need for shelter varies throughout the day. Visual predators such as fish are usually more active during the day and isopods that are directly threatened by predatory fish often avoid detection by hiding throughout daylight hours (Lasley-Rasher, 2011, Bostrom & Mattila, 1999, Stebbins 1988). Isopods often come out from hiding at night to feed, however, when the threat of predation has reduced (Stebbins 1988). Isopods that were monitored in the laboratory were not observed to actively move between host and habitat throughout the day or at night. It is likely that these isopods have the capacity to move to a host very quickly and efficiently, but once on an appropriate host, mobility dramatically decreases. This may be due to the fact that the risk of predation or adverse physical forces is high and does not decrease at night, thus causing this species to remain sheltered and closely associated with its host throughout both the day and at night (MacDiarmid et al. 1991, Buschmann 1990). Alternatively, it may be because the isopods had no reason to move whilst in laboratory conditions for reasons such as lack of food sources, or alternative hosts. From these results we then asked the question - what do these isopods eat while living mainly on a sea urchin?

By inhabiting *E. chloroticus*, *E. echinensis* is not only likely to gain protection from predators and/or physical forces, but is also likely to steal and feed on pieces of *Ecklonia radiata* that

the urchin is also feeding on. *E. radiata* is a known source of food for *E. chloroticus* (Dix, 1970) and our results showed that *E. echinensis* also consumes *E. radiata*. Moreover, the urchin's excrement may provide an additional food source for *E. echinensis*. Urchin excrement is an easily accessible and more easily digestible source of food than the thick laminae of *E. radiata*. Further studies should investigate the nutritional value and digestibility of urchin excrement versus algae for this isopod.

Together these results suggest that the isopod is using the urchin as shelter and is also possibly stealing and consuming small amounts of the kelp that the urchin consumes. It appears that *E. echinensis* has adapted to its environmental pressures by associating with *E. chloroticus* and thus attaining suitable protection from predation and/or environmental stressors, as well as a reliable source of food. Further studies should investigate questions such as the movement of these urchins and isopods over varying time scales. In addition, assessing the role of predation and host health in regulating isopod density, and how isopods react to visual and chemical signals from different types of predators in the presence and absence of urchins would elucidate a greater understanding of the processes driving this association. Finally, evidence for isopods "stealing" kelp from around the oral region of urchins by monitoring isopod behavior would give further verification as to the benefits of this association for *E. echinensis*.

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## **CHAPTER 6: THE ROLE OF VISUAL AND CHEMICAL CUES IN HOST DETECTION BY THE SYMBIOTIC SHRIMP GNATHOPHYLLIODES MINERI**

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## Abstract

The use of visual and chemical cues in host detection is regarded as important but relatively unknown for many symbionts. The small circumtropical caridean shrimp *Gnathophylloides mineri* forms a close symbiotic association with sea urchins, particularly *Tripneustes gratilla*, in many parts of the world. *G. mineri* is known to occur in temperate eastern Australia but the breadth of host use and selection of hosts amongst different species of sea urchins is relatively unknown. The abundance of *G. mineri* on three co-occurring species of sea urchins, *T. gratilla*, *Heliocidaris tuberculata* and *Pseudoboletia indiana* were measured in eastern Australia. These species of sea urchins were chosen because of either a known prior association with this shrimp elsewhere in the world, or due to their abundance in the area in temperate Australia where the shrimp occurs. Field collections showed that the association between shrimp and sea urchin appears extremely host-specific, with *G. mineri* only observed on *T. gratilla*, at an average density of two shrimp per sea urchin. Moreover, the majority of symbionts occurred on the underside (oral region) of *T. gratilla*. A visual laboratory experiment showed that *G. mineri* would actively move towards *T. gratilla* in preference to the other potential host species in the absence of chemical signals. Using a Y-maze, we tested the reaction of *G. mineri* to the absence of visual signals but the presence of chemical signals originating from their host, and from other species of potential host sea urchins. Seawater alone was used as a negative control. We demonstrated that *G. mineri* could detect *T. gratilla* from a distance, the same host that the shrimp are associated with in the field, using visual and chemical cues. Moreover, *G. mineri* react quickly to visual and chemical host signals by moving towards *T. gratilla* in preference to other species of sea urchins or in the absence of a signal. The relative importance of visual versus chemical cues is, however, unknown.

**Keywords:** symbiosis, crypsis, olfactory, sea urchin, Y-maze, *Tripneustes gratilla*

## Introduction

Symbiotic species often have highly specific host recognition processes to facilitate colonization and fidelity (Combes, 2001). Host detection in symbiotic (i.e., commensal, parasitic and mutualistic) associations can occur through detection of either visual and/or chemical signals emitted by the host. Signal-mediated detection of the host removes the need for direct contact, and is a valuable adaptive mechanism for locating specific hosts (Prokopy and Owens, 1983; Lewis and Tumlinson, 1988; Thiel et al., 2003; Derby and Sorensen, 2008). Once on the host, many external symbionts use behavioural and morphological adaptations to avoid detection by predators (Endler, 2006). This is particularly common among shallow water marine crustaceans (Gray, 1961; Patton et al., 1985; Gherardi, 1991; Gherardi and Calloni, 1993; Takeda et al., 1997; Ng and Jeng, 1999; Baeza and Thiel, 2000; Thiel et al., 2003; Balakirev et al., 2008; De Bruyn et al., 2009; 2010; 2011).

*Gnathophylloides mineri* (Schmitt 1933) is a small caridean shrimp with a circumtropical distribution, occurring in the Caribbean and in the Pacific and Indian oceans (Lewis, 1956; Castro, 1971; Bruce, 1974; 1988; Abele, 1975; Patton et al., 1985; Chase and Bruce, 1993; Okuno and Tanaka, 2001; Maciá and Robinson, 2009). *G. mineri* typically occurs in close association with various species of local sea urchins, where it lives amongst the urchin's spines. Although capable of filter feeding, generally it feeds on the host's thin epithelial layer, which covers the test (Patton et al., 1985; Okuno and Tanaka, 2001; Maciá and Robinson, 2009). Part of the distributional success of *G. mineri* is thought to be due to the highly adaptive nature of the symbiotic associations it forms with many different species of sea urchins.

*G. mineri* is most commonly associated with the sea urchins *Tripneustes ventricosus* and *T. gratilla* (Okuno and Tanaka, 2001). Both species of *Tripneustes* have either brown/purple or black tests and short white spines. *G. mineri* possesses complementary dark longitudinal

stripes down its lower carapace that match the colour of the epithelium of its host. The shrimp also has thin longitudinal lightly coloured lines on its dorsal carapace that match the colour of the sea urchin's spines along with claws that are of a similar width and colour to the spines. Moreover, *G. mineri*'s antennae never protrude beyond sea urchin's spines (Patton, 1985). Such cryptic coloration and behaviour is thought to allow *G. mineri* to blend in with its host, making it difficult for visually dependent predators to detect (Main, 1987; Marciá and Robinson, 2009).

Interestingly, however, not all *G. mineri* are cryptically camouflaged with their host sea urchin. Those inhabiting the white sea urchin *Pseudoboletia indiana* in Hawaii do not resemble the colour of their host (Castro, 1971). Marciá and Robinson (2009) found that, in addition to inhabiting *T. ventricosus*, *G. mineri* were abundant on the sympatric sea urchin *Lytechinus variegatus* despite contrasting starkly with the colour of the spines and test of this species. Marciá and Robinson (2009) conclude that this mismatch of crypsis was a result of the extensive covering of debris that *L. variegatus* deposit on its test. Attached debris would give the shrimp substantially more protection from predation, thus lessening the importance of crypsis. Another hypothesis, however, is that the ability of *G. mineri* to visually discriminate between appropriate and less appropriate hosts is impaired due to the heavy covering of debris on *L. variegatus*. This hypothesis assumes that the detection of a suitable host is essentially driven by visual cues and that chemical cues from the host have no significant role in the process. Although other crustacean symbionts use chemical and/or visual cues to locate sea urchins (Ache and Davenport, 1972; Gherardi and Calloni, 1993; Cournoyer and Cohen, 2011; De Bruyn et al., 2011), it is unknown which cues *G. mineri* use for host detection.

*Gnathophyllodes mineri* occurs cryptically on the spines of *Tripneustes gratilla* (Linnaeus, 1758) on the eastern coast of Australia, towards the sea urchin's southern latitudinal limit of

distribution (Williamson *et al.*, personal observation). The morphological and behavioural adaptations observed in Australia are similar to those that have been observed between *G. mineri* and other species of *Tripneustes* in other countries, suggesting that *G. mineri* may actively choose to associate with this genus of sea urchin, and *T. gratilla* in particular in Australian waters. As such, the shrimp must be able to distinguish this species from other similar sized species in the area, most likely through visual and/or chemical cues. To test this hypothesis, the following questions were asked: (1) what is the abundance of *G. mineri* on *T. gratilla* versus other morphologically similar sea urchins that overlap in distribution and life history strategies to *T. gratilla*; (2) where on the sea urchin's test are the shrimps usually located; (3) does *G. mineri* display preferential movement towards *T. gratilla* over other species of potential host sea urchins using visual cues in the absence of chemical cues; (4) does *G. mineri* preferentially move towards *T. gratilla* over other potential host sea urchins using chemical cues in the absence of visual cues?

## **Materials and Methods**

### ***Collection of animals and holding conditions***

Three species of sea urchins, *Tripneustes gratilla*, *Heliocidaris tuberculata* (Lamarck, 1816) and *Pseudoboletia indiana* (Michelin, 1862) were assessed as potential hosts.

*Gnathophylloides mineri* occurs on *T. gratilla* and *P. indiana* in other parts of the world whereas *H. tuberculata* is the most common morphologically similar species that is consistently abundant in temperate eastern Australia (Miskelly 2002). Individuals were opportunistically collected using SCUBA from depths of 5 to 25m from either from two sites that are representative of areas that *G. mineri* has been observed: Fish Rock (30°56'25''S, 153°06'05''E) at South West Rocks, and Camp Cove or Fairlight in Sydney (33°51'23''S, 151°14'35''E), Australia. The number of individuals collected varied between sea urchin species and were based on the relative abundance of that species. Both sites maintained a

similar relative abundance of the three species of urchins and contained *G. mineri* at the time of collection (Williamson and Väitilingon, personal observation).

Individuals were immediately bagged individually and transported back to the laboratory in Sydney where the number and location of *G. mineri* on each individual was assessed (see below). After counting, urchins and their symbionts were individually held in the 30,000L recirculating seawater facility at Macquarie University until used for experiments. Tanks within the facility were illuminated from above by Gro-Lux fluorescent bulbs ( $9.4 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ ), with day-length corresponding to the external environment. Seawater was maintained at a salinity of 34-36 ‰, at field temperatures (18-19°C), and with an average flow of seawater into each tank at 60L hour<sup>-1</sup>. Animals were fed *ad libitum* a mixture of the kelp *Ecklonia radiata* and the brown alga *Sargassum linearifolium*, the common species of algae present in the collection habitats.

#### ***Abundance and location of Gnathophylloides mineri on sea urchins***

Approximately 24 hours after collection, the external features of each sea urchin were carefully examined for the number of resident *Gnathophylloides mineri* and their location. *G. mineri* was recorded as being either on the oral (bottom) or aboral (top) surface of a sea urchin, and whether they inhabited the ambulacral or inter-ambulacral regions on each surface. These habitats on the urchin are roughly equal as the urchin is radially symmetrical. Data were analysed using chi-square tests comparing the observed abundance and location frequencies to an *a priori* expected frequency of 50:50. No shrimps were observed moving around or leaving their host at any time during this handling period. As with Maciá and Robinson's (2009) study, each *G. mineri* clung tenaciously to a spine of the host thus making it highly probable that most, if not all, of the shrimps remained on the same host from collection to examination. The dimensions of each sea urchin were recorded. Algae that the

sea urchin was consuming at the time, along with the container that the individual inhabited, were also searched for *G. mineri*.

### ***Use of vision in host detection by Gnathophylloides mineri***

The role of vision in the detection of *Tripneustes gratilla* over other potential host sea urchins by *G. mineri* was determined by a series of visual tests in a 40 L tank as per VandenSpiegel *et al.* (1998). Here, the 40 L tank was filled with filtered seawater (0.22 µm) and two identical clear plastic Ziplock™ bags were also filled with filtered seawater (400ml) and placed at opposite ends of the tank, 14 cm apart. These bags held the various treatments used in the visual tests and allowed the test shrimp to see a particular treatment but not to be able to detect it through chemical means. Treatment combinations were: (1) *T. gratilla* versus filtered seawater, (2) *T. gratilla* versus *Heliocidaris tuberculata*, and (3) *H. tuberculata* versus filtered seawater. Responses were tested against a control of filtered seawater versus filtered seawater.

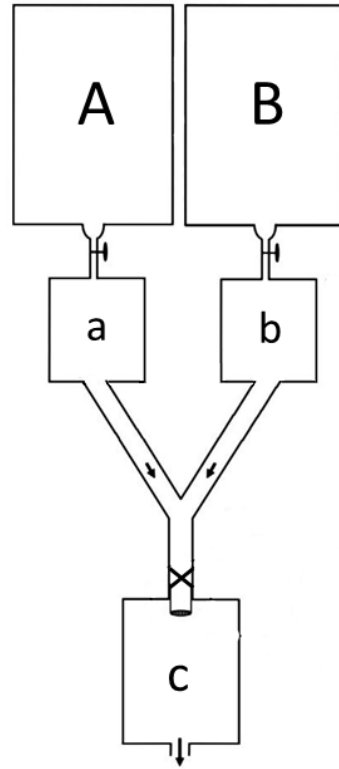
At the start of each test one *G. mineri* was placed in the centre of the tank (7 cm from either treatment) and the time taken for the individual to move towards a treatment over five minutes was recorded. Tests were scored as “positive” if *G. mineri* touched a treatment bag within five minutes, and that treatment was recorded. Tests were scored as negative if the shrimp failed to touch a treatment bag within five minutes. Tests were repeated 50 times. Each *G. mineri* was used only once and water was changed between each trial. Cues were also replaced at each experiment. Plastic bags holding the treatments were checked at the conclusion of each test to ensure that there was no possibility of leaking chemical cues.

Results were analysed using chi-square tests comparing the observed preference frequencies to an *a priori* expected frequency of 50:50. Two different chi-square tests were employed. The first compared *G. mineri* that scored a positive result to those that scored a negative result, or failed to touch a treatment. If an individual scored a positive in the first test, a

second chi-square test was done to distinguish which treatment was chosen. Response times for shrimp stimulated to move were compared across treatments using a one-factor ANOVA. Data were checked for normality prior to analyses.

### ***Use of chemical cues in host detection by *Gnathophylloides mineri****

A laboratory experiment was done to test the role of olfaction, or recognition of a chemical cue, in host detection by *G. mineri* using a system similar to Davenport's "Y-maze" (Davenport, 1950; Fig.1). This system allowed individual shrimp to distinguish between treatments using seawater flowing over the various treatments (and thus containing any chemical cue emitted from those treatments) without the shrimp being able to see any of the treatments. The Y-maze, constructed of clear Perspex (1 cm diameter), consisted of an 11 cm stem connected to two 11 cm arms of the Y at 60° apart. The arms terminated at a 45° elbow. Treatments were introduced into the Y via two separate 2L aquaria (one treatment per aquarium, Fig. 1 A and B), which were gravitationally fed from two separate sources of filtered water and connected with clear plastic tubing. A constant flow of water through the Y-maze (measured at the point where the shrimp was initially placed) was set at 250 ml min<sup>-1</sup> and was regulated by a tap at the base of a common aquarium that the Y-maze drained into (Fig 1). Treatments were left for 30 minutes to allow for a consistent amount of any chemical cues to be released into the water and a new urchin was used for each replicate and assay. Trials prior to the experiment using red food dye in either aquaria showed that flow was steady and laminar and that seawater flowing along the stem consisted of equal portions of water from both aquaria; one on either side of the stem. Thus shrimp placed at the end of the stem could distinguish between water on the left hand side originating from the left aquarium and right hand side water originating from the right aquarium. All tubing and apparatus connecting the aquaria and the Y-maze in the system was thoroughly flushed out with fresh filtered seawater between replicates to remove any odours from either the treatments or test individual from the previous trial.



**Figure 1.** Schematic diagram of the Y-maze used to test the behaviour of the symbiotic shrimp

*Gnathophyllodes mineri* in response to chemical stimuli. ‘X’ marks the start position of the animal. ‘A’ and ‘B’ indicate the 2L aquaria that held the treatments; ‘a’ and ‘b’ are the source aquaria that received seawater gravitationally fed from ‘A’ and ‘B’ respectively; and ‘C’ is the aquarium used to evacuate any remaining seawater at a constant flow.

Treatment combinations used were: (1) *T. gratilla* versus *T. gratilla*; (2) *Tripneustes gratilla* versus filtered seawater; (3) *T. gratilla* versus *Heliocidaris tuberculata*; (4) *T. gratilla* versus *Pseudoboletia indiana*; (5) *H. tuberculata* versus filtered seawater; (6) *P. Indiana* versus filtered seawater; and (7) *P. indiana* versus *P. indiana*. Forty replicates were done for each treatment combination, with the exception of those containing *P. indiana* as this species was not always as readily available in the field as the others. Treatments were swapped from the left to the right arm and *vice versa* after half the replicates. Responses were tested against a control of filtered seawater versus filtered seawater to account for any preference for either the left or the right arm of the Y-maze (Putnam, 1962).

At the start of each trial, one *P. mineri* was carefully placed halfway between the common aquarium and the intersection of the Y-maze. The amount of time it took the shrimp to move



from the starting point to the intersection of the Y-maze (Time A) was recorded, along with the time it took for the shrimp to move from the intersection of the Y-maze to halfway up either arm of the maze (Time B). Individuals that recorded a Time B were considered to have made an active choice towards that particular treatment. Those that did not record either Time A or B (i.e., did not move far enough into the Y-maze) within 20 minutes were recorded as having made no choice.

Results were analysed using two chi-square tests. The first test assessed individuals that made an active choice (recorded times for A and B) versus those that made no choice compared to an *a priori* expected frequency of 50:50. The second chi square test assessed which active choice the individuals made (i.e., which treatment) for those that had recorded times for both A and B. To account for any bias towards the left or right arm of the Y-maze, treatments in the second chi-square test were compared to the frequency obtained from the control (filtered seawater versus filtered seawater) rather than a frequency of 50:50. Response times for shrimp stimulated to move were compared across treatments using a one-factor ANOVA. Data were checked for normality prior to analyses.

## **Results**

### ***Abundance and location of Gnathophylloides mineri on sea urchins***

A total of 216 *Tripneustes gratilla*, 68 *Heliocidaris tuberculata* and 19 *Pseudoboletia indiana* were collected and surveyed for *G. mineri*. Of these 73% of the *T. gratilla* contained at least one *G. mineri*, whereas no shrimp were found on either of the other species. *T. gratilla* individuals that hosted *G. mineri* carried an average of 1.86 ( $\pm 0.22$  SE) shrimp. The average size of *T. gratilla* containing *G. mineri* was  $7.68 \pm 0.06$  cm (diameter) and  $4.48 \pm 0.042$  cm (height).

Measurements of the position of *G. mineri* on each *T. gratilla* revealed a clear trend. Significantly more *G. mineri* preferred the oral surface (80%) of the hosts to the aboral

surface (20%) ( $\chi^2 = 9.0$ , 1 df,  $P = 0.003$ ). There was no significant difference between the number of shrimp on the oral ambulacral surface (36%) and the oral interambulacral surface (44%) of *T. gratilla* ( $\chi^2 = 0.2$ , 1 df,  $P = 0.655$ ). Although not significant, more shrimp inhabited the aboral ambulacral surface (16%) than the aboral interambulacral surface (4%) ( $\chi^2 = 1.8$ , 1 df,  $P = 0.178$ ). *G. mineri* was typically observed in an outward facing position along the spines of the host.

#### ***Use of vision in host detection by Gnathophylloides mineri***

The first control series of filtered seawater versus filtered seawater showed that very few shrimp were stimulated to move and those that did move did not actively favour one side of the aquaria over another (Table 1a). These results eliminated the possibility of *G. mineri* favoring one treatment over the other for unknown experimental factors. In the next two series, the shrimp were actively attracted to their host urchin *Tripneustes gratilla*, in preference to either seawater or to another co-occurring species of sea urchin, *Heliocidaris tuberculata* (Table 1b,c). When *T. gratilla* was not present and the shrimp had to choose between a non host species and seawater alone, there was no significant difference between the number of shrimp that were stimulated to move compared to those that remained stationary. Individuals that actively moved, however, chose to move towards *H. tuberculata* in preference to seawater alone (Table 1d). There was no significant difference between response times for each treatment across all series for shrimp that were stimulated to move (one-factor ANOVA,  $F = 1.451$ ,  $P = 0.238$ ; Table 1a-d).

**Table 1.** Visual host attraction of *Gnathophyllodes mineri* to the sea urchins *Tripneustes gratilla* and *Heliocidaris tuberculata* against each other and against control signals of filtered seawater (FSW).

	Time (sec)		Choice		Assay	Results			Chi-square (p)	
	Minimum	Maximum	A	B		N	A	B	(1)	(2)
a	32	140	FSW	FSW	50	44	3	3	< 0.001	1.0
b	2	104	<i>T. gratilla</i>	FSW	50	5	42	3	< 0.001	< 0.001
c	1	245	<i>T. gratilla</i>	<i>H. tuberculata</i>	50	5	37	8	< 0.001	< 0.001
d	3	110	<i>H. tuberculata</i>	FSW	50	17	30	3	0.124	< 0.001

Time (sec) shows the minimum and maximum reaction times. Choice A and B are the two proposed visual signals; Assay shows the number of replicates; N shows the number of individuals that showed no response; Results A and B show the number of shrimp that actively chose choice A or B respectively.

Chi-square (1) shows the probability significance of a shrimp making an active choice towards a signal; Chi-square (2) shows the probability significance of a shrimp choosing one signal over another. All results were compared to a theoretical ratio of 50:50

### ***Use of chemical cues in host detection by Gnathophyllodes mineri***

As with the visual experiment, the negative control series (seawater versus seawater) showed that relatively few shrimp were stimulated to make a choice and that, of those that did move to the intersection of the Y-maze, there was no significant difference in the arm of the tube that they chose (Table 2a). A positive control series (*T. gratilla* versus *T. gratilla*) showed that the majority of shrimp actively moved towards the host species, but that one arm of the Y-maze was not favoured over the other (Table 2b). These results show that *G. mineri* were able to perceive a cue and move towards it within the Y-maze and that there was no obvious asymmetry in the experimental design.

Shrimp were stimulated to move when *T. gratilla* was presented as an option against another signal, and actively moved towards their host species regardless of the type of other stimuli present (Table 2c-h). Only roughly half of the shrimp presented with either *H. tuberculata*

versus seawater as signals were stimulated to move. Of those that did move, significantly more individuals actively moved towards *H. tuberculata* (Table i-j). Shrimps presented with a choice of *P. indiana* or seawater were not particularly stimulated to move or to actively choose one signal over another, thus acting in a similar manner to shrimp exposed to the seawater versus seawater controls (Table 2 k-l). In some of these cases, more individuals chose not to move at all than to make a choice. This was unexpected but a further series that was run testing *P. indiana* versus *P. indiana* resulted in a similar result where individuals were not stimulated to move or to make a choice either, thus supporting the results above (Table 2m). Interestingly, however, a similar number of choices were made by individuals when presented with a choice of *P. indiana* or *T. gratilla* (Table 2h).

The reaction times between treatments where shrimp were stimulated to move varied amongst individuals (see min/max times in Table 2). There was, however, no significant difference in reaction time between treatments for individuals that moved from the starting point of the Y-maze (Time A; one-factor ANOVA,  $F = 1.423$ ,  $P = 0.240$ ; Table 2a-m), or for individuals that moved from the intersection to halfway up an arm of the Y-maze (Time B; one-factor ANOVA,  $F = 0.894$ ,  $P = 0.447$ ; Table 2a-m).

**Table 2.** Chemical host attraction of *Gnathophyllodes mineri* to the sea urchins *Tripneustes gratilla*, *Heliocidaris tuberculata* and *Pseudoboletia indiana* against each other and against control signals of filtered seawater (FSW)

	Min/Max time (sec)		Choice		Results				Chi-square (p)	
	Time A	Time B	A	B	Assay	N	A	B	(1)	(2)
a	1/663	4/1200	FSW	FSW	20	11	5	4	0.655	0.739
b	1/11	3/346	<i>T. gratilla</i>	<i>T. gratilla</i>	20	1	9	10	< 0.001	0.504
c	1/69	2/1126	<i>T. gratilla</i>	FSW	20	1	18	1	< 0.001	<0.001
d	1/129	5/1200	FSW	<i>T. gratilla</i>	20	7	2	11	0.18	0.004
e	2/58	3/1200	<i>T. gratilla</i>	<i>H. tuberculata</i>	20	1	17	2	<0.001	<0.001
f	1/142	13/424	<i>H. tuberculata</i>	<i>T. gratilla</i>	20	0	4	16	< 0.001	0.002
g	2/135	1/894	<i>T. gratilla</i>	<i>P. indiana</i>	17	5	11	1	0.09	0.001
h	1/154	3/432	<i>P. indiana</i>	<i>T. gratilla</i>	15	7	4	4	0.796	0.776
i	1/30	3/1200	<i>H. tuberculata</i>	FSW	20	11	2	7	0.655	0.048
j	1/65	5/1200	FSW	<i>H. tuberculata</i>	20	10	6	4	1	0.751
k	2/187	2/12	<i>P. indiana</i>	FSW	13	8	1	4	0.405	0.116
l	2/13	3/35	FSW	<i>P. indiana</i>	7	3	3	1	0.705	0.421
m	2/381	3/157	<i>P. indiana</i>	<i>P. indiana</i>	10	7	2	1	0.206	0.685

Time (sec) shows the minimum and maximum reaction times for individuals that moved from the starting point to the intersection in the Y-maze (Time A), and those that moved from the intersection to halfway up an arm of the Y-maze, thus making an active choice (Time B). Choice A and B are the two proposed visual signals; Assay shows the number of replicates; N shows the number of individuals that showed no response; Results A and B show the number of shrimp that actively chose choice A or B respectively.

Chi-square (1) shows the probability significance of a shrimp making an active choice towards a signal; Chi-square (2) shows the probability significance of a shrimp choosing one signal over another for individuals that had recorded times for both Time A and B. All results were compared to a theoretical ratio of 50:50

## Discussion

As in many other parts of the world, *Gnathophylloides mineri* forms a close association with *Tripneustes gratilla* in eastern Australia. *G. mineri* specifically inhabited *T. gratilla* despite the presence of other similar species of sea urchins that overlap in distribution with *T. gratilla*. Moreover, *G. mineri* has the ability to use both visual and chemical cues to locate its host from a distance, and rapidly swam against the water current towards its host in preference to other cues. When its host was absent, shrimp either remained stationary or moved at a slower rate towards another non-host sea urchin.

Locating a host is a fundamentally important stage in the life history of symbionts and, as such, the use of several sensory modes increases the likelihood of detecting an appropriate host. The ability to use more than one sensory cue to detect potential hosts or predators is a relatively common strategy for marine organisms (Arvelund et al., 1999; Dalesman and Inchley, 2008; DeBose et al., 2008; Hay, 2009), and symbiotic decapod crustaceans routinely use a combination of visual and chemical cues for host location (Huang et al., 2005; Sikkel et al., 2011). The ectosymbiotic shrimp *Synalpheus demani* uses visual cues for small-scale host detection but uses chemical signals from the host crinoid to increase activity and orientate towards the host from a distance (Huang et al., 2005). The parasitic isopod *Gnathia marleyi* is more successful in locating its host fish in the field when it is able to employ a combination of visual and chemical signals rather than just one signal (Sikkel et al., 2011). There should be strong selection for modes of ability to detect and locate appropriate hosts since failure to find a host or inappropriate host choice increases the risk of predation and lowers reproductive success.

While our experiments showed that *G. mineri* responded rapidly and with accuracy towards its host using two remote signals, they could not determine the relative importance of visual versus chemical cues in the detection of *T. gratilla*. Visual and chemical cues in host detection often occur in terms of hierarchy and sequence: symbionts use chemical means to

locate the general area of an appropriate host, then visual signals in the final stages of host detection (Segura et al., 2007; Debose and Nevitt, 2008). Chemical cues are generally considered reliable mediators of habitat choice for different life history stages in marine specialist invertebrates as they provide a unique signature from the host (Williamson et al., 2000; Williamson and Steinberg in press). If the host itself is either variably abundant or cryptic and thus likely to produce more subtle or unreliable chemical signals, the relative importance of visual or chemical cues may change.

In temperate east Australia, *Tripneustes gratilla* occurs in patchy populations along its southern limit of distribution. At sites where *T. gratilla* occurs more reliably, such as our study site at South West Rocks, the species fluctuates from being highly abundant to relatively scarce from year to year (Williamson, personal observation). It is therefore reasonable to expect that chemical signals from *T. gratilla* should vary in strength, depending on the population density of *T. gratilla* at that time. Thus the host chemical cue should be somewhat unreliable at times for *G. mineri*. In light of this, it is perplexing that no shrimp were found on other similar species of sea urchins that overlapped in distribution with *T. gratilla*. *Heliocidaris tuberculata* is consistently abundant in our study sites (Williamson, personal observation; Wright et al. 2005), albeit in lower densities than *T. gratilla* at times. It generally occurs in hollows in the rocky subtidal, potentially allowing substantial protection for any epibionts; a criterion deemed critically important in host choice for *G. mineri* in other areas (Maciá and Robinson, 2009). Moreover, the primary spines on *H. tuberculata* are, on average, roughly three times as long as those of *T. gratilla* (Miskelly, 2002), potentially offering a further increase in protection from predation (Tegner and Dayton, 1981; Hartney and Grorud, 2002).

Several hypotheses can be posed to explain the absence of *G. mineri* on *H. tuberculata*. As host choice is decided prior to physical contact with potential hosts and assuming that *G.*

*mineri* in eastern Australia do move between individual urchins occasionally as suggested by Patton et al. (1985), all hypotheses indicate some form of adaptive associative learning by the shrimp to distinguish the appropriateness of sea urchins as hosts from a distance. First, a difference in the nutritional content or the constitution of pigment cells within the epithelia of the two species of sea urchins may drive this pattern of host use. Patton et al. (1985) suggests that the pigments of *G. mineri* most likely originate from sequestered host pigments. If this were the case, one would expect that the *T. gratilla* epithelium would be significantly more nutritious and/or contain more camouflaging pigments than other potential hosts for *G. mineri* but has yet to be tested.

An alternative hypothesis is that *T. gratilla* may offer greater protection against predators for *G. mineri* through the morphology of the host's spines. Spines of *H. tuberculata* are oval in cross section and substantially thicker than the spines of *T. gratilla* (Miskelly, 2002), thus potentially making them more difficult for these shrimp to inhabit. Certainly host-specific symbionts in other systems are constrained by the morphology of their host's point of attachment. For example, species of *Geomydoecus*, commonly known as gopher lice, occur in close association with particular species of geomyid rodents (Morand et al., 2000). The lice are extremely specialized and have difficulty surviving on hosts that have larger hair diameters than those of their native host. This is hypothesized to be a result of an increased risk of dislodgement by the lice (Reed et al., 2000).

*G. mineri* showed less interest in *Pseudoboletia indiana* as a host species than *T. gratilla*, despite associating with *P. indiana* in Hawaii (Titgen, 1989) and having similar spines and habitat to *T. gratilla*. As with the Hawaiian species, *P. indiana* in eastern Australia has a white test and spines, and shrimp occurring on this species are not cryptically camouflaged in terms of colour. Marciá and Robinson (2009) suggest that such mismatches with host colour show the relative importance of chemical cues over visual cues for this shrimp. Although no



visual signals with *P. indiana* were tested in this study and this hypothesis cannot therefore be discounted, results in our visual experiment suggest that visual signals are important to the shrimp. *P. indiana* is patchily distributed in low densities along the temperate eastern Australia (Rowe and Gates, 1995), but appears more abundant in Hawaii (Titgen 1989). The difference in the abundance may relate to the historical ability of *G. mineri* to assess the attractiveness of this host species through associative learning in Australia. By remaining stationary in the presence of signals originating from *P. indiana*, the shrimp may merely have been waiting for a familiar signal. The decreased mobility of shrimp in the absence of visual or chemical cues from *T. gratilla* suggest that remaining stationary in the absence of a reliable familiar cue may be a camouflage strategy in itself. These ideas, however, currently remain untested.

There are obvious benefits in this association for *G. mineri*, including nourishment from the epithelium of its host, the provision of a structure that protects it from predators (spines); and the cryptic coloration of *G. mineri* through sequestering ingested pigment cells into their own surface layers. Conversely, the advantages, if any, for *T. gratilla* are much less apparent.

Although no damage to *T. ventricosus* by *G. mineri* has been previously reported due to the ability of the shrimp to move around the test and allow the epithelium to regenerate (Patton et al., 1985), the impact of epithelial grazing may depend on the density of its symbionts.

Opportunistic predation by the melitid amphipod *Elasmopus levis* on the epithelium of the sea urchin *Lytechinus variegatus* can substantially affect the health of the host and even lead to death (Gibbs et al., 2011). Despite the various benefits for *G. mineri*, individuals residing on *T. gratilla* are exposed to various risks. For example, the urchin's pedicellariae, which are used for protection from predators and fouling organisms, may be fatal to the shrimp if attacked (Campbell and Rainbow, 1977; Mebs, 1984; Nakagawa et al., 2003; but see Guenther et al., 2007).

*G. mineri* did not generally occur in large numbers on *T. gratilla*. This may be as a result of interspecific interactions limiting the density of associates on hosts (Schmitt and Holbrook, 2003). Many of those that did occur on *T. gratilla* inhabited the oral surface of its host, or the ambulacral areas where the urchin's primary spines are located. These results are consistent with previous research on both species from other areas of the globe (Patton et al., 1985; Maciá and Robinson, 2009). Moreover, previous authors have noted that if *T. ventricosus* are inverted all *G. mineri* inhabiting the oral surface will migrate towards the aboral surface, which was now underneath (Patton et al., 1985).

A similar study (VandenSpiegel et al., 1998) observed that another shrimp species, *Synalpheus stimpsonii*, regularly occurred in pairs on the underside of its host sea urchin. The abundance and distribution of *G. mineri* on *T. gratilla* has important implications for the reproductive success of the shrimp. There are huge costs for failure to reproduce, hence, and low numbers of shrimp on hosts may suggest issues in locating a mate, particularly if this population has an unequal male to female ratio as documented for other species of caridean shrimps (Nakashima, 1987; Gherardi and Calloni, 1993).

To date, many aspects of this symbiotic relationship are unknown, and further research should focus on questions such as movement of shrimps over varying time scales, the reproductive mode of the shrimp, and the relative protection that *T. gratilla* offers *G. mineri* in comparison with other co-occurring species of sea urchins. In addition, assessing how male and female *G. mineri* successfully find their host through chemosensory capabilities and on the nutritional value of the epithelium as some epibiota select host species on the basis of their quality as a habitat rather than as a source of food (Williamson et al., 2004). Finally, isolation and identification of the particular water-borne chemical cue(s) originating from *T. gratilla* that attracts *G. mineri*, along with assessing the uniqueness of the cue in the habitat, would give an indication of the specificity of the cue as an indicator of chemical host detection.

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## **CHAPTER 7: GENERAL DISCUSSION**

### **Positive versus negative effects of sea urchins**

Sea urchins are major grazers in rocky reef ecosystems and have the ability to drastically modify the biogenic structure of habitats by maintaining ‘barrens’ (areas devoid of macroalgae) (Steneck 2013). To date, most of the ecological literature on sea urchins has focused on the cascading negative effects of increasing sea-urchin abundance, arising from the overharvest of their predators or shifting environmental conditions, which can lead to a loss of structural habitat (Estes 1978, Harold and Reed 1985, Pinnegar et al. 2000, Carter et al. 2007, Terborgh and Estes, 2010, Byrnes et al 2006). However, this thesis highlights that some sea urchins can have positive effects on particular associated species. These positive effects most likely occur at smaller scales compared to the negative effects associated with large-scale herbivory, and often act in complex systems of direct and indirect pathways, making them more difficult to uncover. For example, sea urchins have been found to benefit subtidal mussels by grazing on the kelp that grows on the mussels shell. During storm events, ungrazed kelp on mussel shells can cause enough drag to dislodge the mussel from the substratum (Witman, 1987) and decorating behaviour by an Antarctic sea urchin has positive effects on seaweed that has become detached from the substratum, by “rescuing” it from being washed ashore or carried out of the photic zone (Amsler et al 1999). The results of this study emphasize the important role that positive interactions involving sea urchins can have in structuring species diversity and the need for further research in this area.

### **Associations directly versus indirectly mediated by sea urchins**

A comparison between the three associations investigated in this thesis in addition to other studies of positive urchin interactions, highlights the diverse roles that sea urchins play in both directly and indirectly enhancing biodiversity (Table 1). Sea urchins are well known to harbour a range of small mobile invertebrates that directly inhabit their spines for shelter

(McCloskey 1970, Castro 1971, Patton 1985, Stebbins 1988, Chia et al. 1999, Day and Branch 2002, Theil and Baeza 2003, Doti 2008, Prakash et al 2012). The isopod *Exosphaeroma echinensis* inhabiting the sea urchin *Evechinus chloroticus* as well as the shrimp *Gnathophylloides mineri* on the sea urchin *Tripneustes gratilla* investigated within this thesis find active patterns of habitat choice from their associations with these sea urchins. By inhabiting the spines of these two species of sea urchins, both *G. mineri* and *E. echinensis* are likely to gain protection from predation and other environmental stressors (Patton et al. 1985, Doti et al. 2008, Maciá and Robinson 2009).

In addition to the obvious benefit of shelter for small marine invertebrates, there are often a number of other factors that can act simultaneously to drive the positive associations that urchins have with other species. Results from this study show that *E. echinensis* is also likely to benefit by associating with *E. chloroticus* by having regular access to the same food types that *E. chloroticus* consumes (Chapter 5). By inhabiting the spines of the sea urchin, *E. echinensis* is likely to ‘steal’ the macroalgae and/or epiphytes that the urchin uses to cover itself with for its own consumption, or the food that the urchin itself is feeding on. In addition, many species that associate with sea urchins do so for reproduction. Studies have shown that the shrimp *G. mineri*, which associates with many species of sea urchins including *T. gratilla* (Patton et al 1985), moves within the spines of its urchin host or between hosts in order to find suitable mates (Maciá and Robinson 2012). After copulation some species that inhabit urchin spines use this safe environment as a refuge for juvenile development (Stebbins 1989). The amphipod *Dulichia rhabdoplastis* stays close to its juvenile offspring on urchin spines, guarding them from predation and other adult amphipods (McCloskey 1970). A similar occurrence of juvenile refuge occurs for the abalone *Haliotis midae*, which recruits to the underside of the sea urchin *Parechinus angulosus* for protection as well as for food (Day and Branch 2002).

While there are numerous examples of species that are positively associated with sea urchins directly, examples of positive indirect associations that are mediated by sea urchins are less common (Table 1). This may be due to the inherent difficulty in identifying indirect effects within ecosystems or that they are simply less common (Wootton 2002). This study describes one of the first known examples of a positive indirect effect involving a sea urchin, in the form of a facilitation cascade (Thomsen et al. 2010, Oghushi et al. 2005), whereby the kelp *Ecklonia radiata* facilitates the urchin *Holopneustes purpurascens* by providing it with suitable habitat and food, and the urchin in turn facilitates the gastropod *Phasianotrochus eximius* by providing it with an easily accessible and nutritious food source (Chapters 2, 3 and 4). *P. eximius* has adapted to consume the excrement of *H. purpurascens* whilst living within close proximity to the sea urchin, enmeshed within the fronds of *E. radiata* and this appears to be the main driving factor for this association. However, *P. eximius* is likely to be vulnerable to predation outside of its preferred complex habitat structure, and this may be an additional benefit of associating with *H. purpurascens*. The modified habitat in which both species exist is thought to benefit the sea urchin by providing it with a shelter from abrasion by kelps and other objects ‘whipping’ by in the water as a result of adverse abiotic factors such as wave action, tides and currents as well as from predation (Rogers et al. 1998). Covering behaviour in other species of sea urchins has also been considered an adaptation to avoid surge (James 2000). The sea urchin *Toxopneustes roseus* covers itself in shell fragments and foliose algae in areas of high surge throughout the Gulf of California (James 2000). It is possible that *H. purpurascens* has adapted in a similar way to *T. roseus* by covering itself to mitigate wave action within the exposed environment in which it inhabits (Steinberg 1995). It would not be incorrect, therefore, to hypothesise that *P. eximius* also benefits from inhabiting the shelter built by *H. purpurascens*, however further research is required to verify this.

A similar positive indirect effect occurs between the sea urchin *Parechinus angulosus* and the juvenile abalone that recruit to the underside of their tests (Day and Branch 2002). In addition

to directly providing protection for the abalone, the association indirectly facilitates the provision of food for this species, as it is able to easily feed on drift algae that the urchin captures on its spines for its own consumption (Day and Branch 2002). Chapter 5 hypothesises a similar interaction involving the isopod *E. echinensis*, which exhibits strong host fidelity, and prefers to consume the same species of macroalga that its host *E. chloroticus* consumes and decorates itself with. An interesting comparison is that of the positive association between the isopod *Dulichia rhabdoplastis* and sea urchin *Strongylocentrotus franciscanus*, which also appears to be indirectly mediated (McCloskey 1970). Within this relationship, the isopod builds strings of detritus made from its own fecal pellets that it connects to the spines of the sea urchin (McCloskey 1970). The strings are colonised by a rich layer of diatoms, which the isopod subsequently feeds on (McCloskey 1970). Here, the sea urchin indirectly facilitates the isopod by providing it with a habitat that it in turn uses to capture its prey (McCloskey 1970). This species may also benefit directly by using the spines of the sea urchin as refuge when needed.

There are many examples of species that directly inhabit the spines of sea urchins (Table 1) (Castro 1971, Chia 1999, Prakash 2012) with untested statements on the nature of these relationships. It is often just assumed (not tested) that the main factor driving associations of small mobile invertebrates and sea urchins is protection from predation and environmental stressors. However, it appears that within each of these examples of positive indirect effects involving sea urchins (Table 1), there are both direct effects of shelter and indirect effects via the provision of food or shelter for the associate, illustrating the complex nature of these interactions (Table 1). This thesis demonstrates that, by investigating not only the direct effects of a species interaction but also the indirect effects, often a seemingly simple association can in fact be based on more complex foundations (Chapter 4 and Chapter 5).

## Methods of host location

Locating a host is a fundamental requirement for survival in species associated with sea urchins (Thiel and Baeza, 2001, Hay, 2009, Lechinni, 2011, Williamson et al., 2012). The method of host recognition for many urchin associations appears, however, to have been understudied, with merely descriptions of the associations without evidence of the methods of host location employed by sea urchin associates (Table 1). This thesis explored host recognition capabilities for three sea urchin associations. Choice experiments revealed that *Gnathophylloides mineri* responds strongly to both visual and olfactory cues emitted by its host the sea urchin *Tripneustes gratilla*, and has the ability to swim against the flow of water towards its host cue. Chemical cues within the marine environment are an effective method of recognition because species can respond to unique signals from a relatively large distance (Williamson et al. 2000, Williamson et al. 2012). Visual cues within the marine environment are generally considered to be effective from smaller distances (Debose & Devitt 2008, Segura et al. 2007). Using a combination of sensory cues is common in symbiotic marine organisms as it dramatically increases the effectiveness of host recognition (Dalesman & Inchley 2008, DeBose et al. 2008, Hay 2009). The strength of host recognition capabilities is likely to be a good determinant of the strength of the association between symbiotic species (Williamson et al. 2012). These results reflect the strong association between *G. mineri* and *T. gratilla*. By comparison, the two sea urchin associated species, the gastropod *P. eximius* and the isopod *E. echinensis*, respond strongly to visual cues emitted by the urchin species that they associate with, as opposed to chemical cues (Chapter 3 and 5). This may reflect the relatively weaker relationship that these two species have with their sea urchin associates, in comparison to that between *G. mineri* and *T. gratilla*, as both species (*P. eximius* and *E. echinensis*) are able to survive outside of their preferred habitat with urchin their urchin hosts (Table 1, Chapter 2 and 5). Alternatively, it may reflect an inability to detect chemical cues.

More research needs to be done on the sensory abilities of these species to tease apart these hypotheses.

Table 1: Similarities and differences between sea urchin mediated positive associations throughout temperate rocky reef habitats from different regions

Study	Region	Organisms	Interaction		Nature of the association		Method of urchin recognition		Benefit to associating species		
			Direct	Indirect	Faculative	Obligate	Visual Cue	Olfactory Cue	Food	Shelter	Reproduction
This study	Eastern Australia	Kelp + Urchin +Gastropod	X	X	X		X		X	X	
This study, Patton et al. (1985), Macia and Robinson (2012)	Eastern Australia	Urchin + Shrimp	X		X		X	X		X	X
This study	New Zealand	Urchin + Isopod	X	X	X		X		X	X	
Prakash et al. (2012)	India	Urchin + Crab	X								
Doti (2008)	SW Atlantic	Urchin + Isopod	X		X					X	
Thiel and Baeza (2003)	SE Pacific	Urchin + Crab	X		X					X	X
Day and Branch (2002)	South Africa	Urchin + Abalone	X	X	X				X	X	
Chia et al. (1999)	Eastern Indian Ocean	Urchin + Crab	X								
Stebbins (1989)	NE Pacific	Urchin + Isopod	X		X					X	X
Castro (1971)	NE Pacific	Urchin + Shrimp	X							X	
McCloskey (1970)	NE Pacific	Urchin + Amphipod + Diatom	X	X	X				X	X	

## **Future Research**

Positive interactions have been increasingly recognised for their importance in the structure and functioning of ecosystems (Thomsen et al 2010). However, studies focusing on the role of negative species interactions in shaping ecosystems still far outweigh those focusing on the importance of positive effects. Indirect effects add to the complexity with which ecosystems function and are intrinsically difficult to quantify, requiring long term manipulative experimental design (Ohgushi 2005). Whilst interest in indirect effects has recently grown, there is still a gap in our understanding of the roles that individual indirect effects have and their importance within many systems (Wootton 2002). An understanding of positive interactions, and both the direct and indirect pathways of occurrence, is essential to accurately predict the impact of potential perturbations for successful management of ecosystems. Future research should focus on the entire framework of these ecosystems to capture potentially important cascading effects that might further define species relationships. Experiments should centre on the effects of feeding behaviour and the nutritional benefits of association; the role of predation and the risks within versus outside of association with an urchin; as well as environmental stressors such as wave action and climate change on the survival of associates within and outside of preferred habitats. Finally the reproductive ecology of urchin associates as well as intraspecific interactions on hosts in driving potential facilitation cascades.

## **Conclusion**

Each of the associates examined within this thesis appear to have adapted to their environment by forming important positive relationships with sea urchins for food and/or shelter (McCloskey 1970, Stebbins 1989, Thiel et al. 2003, Doti 2008, Maciá and Robinson 2009). Throughout the past 50 to 100 years human impacts on marine ecosystems (such as overfishing) have resulted in a downturn in the abundance of species that prey on sea urchins

in some areas (Tegner and Dayton 2000). Within such areas this has caused an increase in the abundance of herbivorous species (such as sea urchins), and in turn is likely to have had a positive effect on species that associate with sea urchins (Steneck et al. 2002). Recently, however, direct threats to urchins by humans, such as harvesting for food (Lawrence 2001), creating suboptimal conditions that induce disease (Wang 2013), increased sedimentation (Phillips and Shima 2006) and ocean acidification (Schlegel et al. 2012) on local to regional scales have increased, which in turn will negatively impact on the species with which the urchins facilitate. This issue has been identified as particularly relevant to commercially harvested species that rely on urchins for survival, such as the abalone *H. midae*, which depends on the sea urchin *P. angulosus* throughout its juvenile stage for both food and shelter in South Africa. Depletion of sea urchin stocks in this location has seen a decline in abalone recruits, which has had significant impacts on the abalone industry in this region (Day and Branch 2002). Due to their relatively unstudied nature, many of these types of associations may disappear before we have the opportunity to understand their importance within ecosystem functioning. With a greater level of understanding of the important roles that sea urchins play within various ecosystems, the cascading effects as a result of threats to sea urchins can be managed appropriately, for the purpose of maintaining future biodiversity.

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# APPENDIX A: ROBUST AGE AND GROWTH PARAMETERS FOR THREE HEAVILY EXPLOITED SHARK SPECIES OFF TEMPERATE EASTERN AUSTRALIA (2013) ICES JOURNAL OF MARINE SCIENCE. 164.

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## Age and growth parameters for three heavily exploited shark species off temperate eastern Australia

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The removal of large predatory sharks from the world's oceans poses profound threats to marine community structure and species conservation. Effective management of exploited shark stocks requires a sound understanding of the life histories of target species. Here we provide the first assessment of age and growth for *Carcharhinus brevipinna* in Australian waters, and for *C. obscurus* and *C. plumbeus* in eastern Australian waters, based on interpretations of vertebral growth bands. In doing so, we provide arguably among the most robust growth parameters to date for the abovementioned taxa on the bases of genetic validation and sample size and distribution, but acknowledge equally a range of limitations—most notably those associated with vertebral ageing and our lack of age validation. Comparatively, the three species displayed both contrasts and consistencies in their growth characteristics off Australia's southeast coast. For all three sharks, rates of growth were greatest in the years immediately after birth, males grew more rapidly than females in the juvenile phase, and females were observed to grow larger, live longer and were generally larger at any given age. Longevity and all modelled growth parameters ( $L_{\infty}$ ,  $k$  and  $L_0$ ), however, differed among the three species, and appeared to challenge the findings for conspecific populations in other parts of the world. The validity of these latter comparisons is, however, compromised by a range of confounding factors. Nevertheless, we provide the least conservative  $k$  estimates for *C. obscurus* and *C. plumbeus* of those previously reported, and extend maximum age estimates for *C. brevipinna*. In this way, our results have important implications for the assessment of natural mortality, productivity, and hence resilience to stock depletion, in these species in southeastern Australian waters.

**Keywords:** asymptotic growth models, Carcharhinidae, dusky shark, fishery management, sandbar shark, spinner shark, vertebral ageing.

### Introduction

Apex predators play a fundamental role in regulating species abundance and community structure in ecosystems (Ritchie and Johnson, 2009). The removal of such organisms, via natural or anthropogenic causes, can induce profound and complicated cascading impacts on lower trophic levels—as has been demonstrated in terrestrial (Moreno *et al.*, 2006; Beschta and Ripple, 2009; Wallach *et al.*, 2010) and marine environments (Myers *et al.*, 2007; Baum and Worm, 2009). Biological traits such as slow growth rate, long lifespan, late onset of maturity and low reproductive output render many apex predators vulnerable to rapid population decline and slow rates of recovery (Musick, 1999; Purvis *et al.*, 2000; Webb *et al.*, 2002;

Field *et al.*, 2009). This is exemplified in oceanic species such as sharks, where continued overexploitation has led to the depletion of virgin stocks in many parts of the world (e.g. Baum *et al.*, 2003; Ferretti *et al.*, 2008). While levels of decline are highly debatable (Burgess *et al.*, 2005), there is nevertheless widespread consensus regarding the need for effective shark fishery management and conservation (Barker and Schlüssel, 2005).

Dusky (*Carcharhinus obscurus*), spinner (*Carcharhinus brevipinna*) and sandbar (*Carcharhinus plumbeus*) sharks are three large-medium carcharhinid shark species found throughout much of the world's tropical and warm-temperate coastal and continental shelf waters (Last and Stevens, 2009). Highly sought-after for their

fins (Clarke *et al.*, 2006), all three species are important components of commercial and artisanal catches in multispecies shark fisheries across the globe (e.g. Amorim *et al.*, 1998; Castillo-Géniz *et al.*, 1998; McVean *et al.*, 2006; Henderson *et al.*, 2007; White, 2007; Morgan *et al.*, 2009; Manojkumar *et al.*, 2012). Recreational catches and rates of bycatch in non-target fisheries are also suspected to be substantial but, as for most shark species, they remain largely unquantified (Bonfil, 1994).

*Carcharhinus obscurus*, *C. brevipinna* and *C. plumbeus* are highly vulnerable to overfishing and human-induced habitat alteration due to their life-history traits (e.g. Simpfendorfer *et al.*, 2002; Capapé *et al.*, 2003; Carlson and Baremore, 2005; Dudley *et al.*, 2005; McAuley *et al.*, 2006; Baremore and Hale, 2012), susceptibility to multiple harvest methods, and utilization of inshore nursery habitat for neonate and juvenile development (e.g. Thorpe *et al.*, 2004; Conrath and Musick, 2007; Taylor and Bennett, 2013). Consequently, the sustainability of targeted fishing activities exploiting *C. obscurus* and *C. plumbeus* in particular has been subject to considerable scrutiny in recent years (e.g. Sminkey and Musick, 1996; McAuley *et al.*, 2005, 2007a; Cortés *et al.*, 2006; Romine *et al.*, 2009; Anon., 2011a, b), resulting in global IUCN classifications of “vulnerable” for both species (Musick *et al.*, 2009a, b). Some populations have experienced greater levels of fishing mortality than others. In the Northwest Atlantic, for example, *C. obscurus* is regionally listed as “endangered” (Musick *et al.*, 2009a), and declines of up to 64–99% in *C. obscurus* and *C. plumbeus* stocks are purported (Cortés *et al.*, 2006; Myers *et al.*, 2007; Baum and Blanchard, 2010). Comparatively, *C. brevipinna* is considered of less conservation concern in spite of similar life-history traits, and is globally IUCN listed as “near threatened” (Burgess, 2009).

In Australian waters, the three study species are actively targeted along the eastern, northern and western coastlines, as well as the southern coastline in the case of *C. obscurus*, with capture typically via demersal longlines, demersal and pelagic gillnets, and handlines (Simpfendorfer and Donohue, 1998; Macbeth *et al.*, 2009; Harry *et al.*, 2011a; Tillett *et al.*, 2012; Rogers *et al.*, 2013). Dramatic increases in commercial catches of these species have been reported from Australia over recent decades. For example, a sixfold increase in landings of *C. obscurus* (~100–600 tonnes (t)) and a fourfold increase in landings of *C. plumbeus* (~100–415 t) were reported from Western Australian waters between 1980 and 1990, and 1995 and 2004, respectively (McAuley *et al.*, 2007a, b). Despite extensive management measures having been implemented in this region (Simpfendorfer and Donohue, 1998), underestimation of both species’ vulnerability to fishing mortality has failed to halt unsustainable fishing levels and declining stocks (McAuley *et al.*, 2007a). Off Australia’s southeastern seaboard, a threefold increase in total shark catch (152 to 457 t) was recorded between 2005 and 2007 by the New South Wales Ocean Trap and Line Fishery (NSW OTLF), where *C. plumbeus*, *C. obscurus* and *C. brevipinna* were the three most abundantly caught species, respectively (Macbeth *et al.*, 2009). During this time, shark fishing associated with the NSW OTLF was managed by input controls limiting the number of potential participants but was not subject to restrictions on the volume of catch able to be taken, highlighting the urgent need for assessment of shark exploitation and management arrangements off Australia’s southeast coast.

Effective management of exploited shark populations requires a sound understanding of the life history of target species. For example, robust estimates of age provide a basis for determining other pertinent parameters such as longevity, growth rate, natural

mortality, and hence resilience to various levels of fishing pressure (Goldman, 2004). Cosmopolitan distributions and commercial importance have led to numerous vertebral-ageing studies on *C. obscurus*, *C. brevipinna* and *C. plumbeus*. Age and growth parameters are available for all three species from the Indian Ocean and Northwest Atlantic (Casey *et al.*, 1985; Branstetter, 1987; Casey and Natanson, 1992; Natanson *et al.*, 1995; Sminkey and Musick, 1995; Natanson and Kohler, 1996; Allen and Wintner, 2002; Carlson and Baremore, 2005; McAuley *et al.*, 2006; Hale and Baremore, 2010) as well as from the western Pacific for *C. brevipinna* and *C. plumbeus* (Jounget *et al.*, 2004, 2005), and the central Pacific for *C. plumbeus* (Romine *et al.*, 2006). In Australian waters, validated age and growth studies have been conducted on *C. obscurus* (Simpfendorfer *et al.*, 2002) and *C. plumbeus* (McAuley *et al.*, 2006) off the west coast. While the propensity for vertebrae to underestimate age in large adult sharks is purported (Francis *et al.*, 2007; Andrews *et al.*, 2011), the abovementioned studies revealed all three to be long-lived species, exhibiting generally slow rates of growth and conforming to the patterns outlined by Cortés (2000)—i.e. initially faster growth in males than females, females growing older and to larger sizes than males, and growth rates for both sexes being fastest during the juvenile stage.

Although the growth dynamics of *C. obscurus*, *C. plumbeus* and *C. brevipinna* have been widely documented across much of their respective distribution ranges, many such studies report biologically unrealistic growth parameters. Most notably, estimates of theoretical asymptotic length ( $L_{\infty}$ ) are typically overestimated, translating to underestimates of the growth coefficient ( $k$ ). Inaccuracies such as these have profound implications for demographic analyses and population models, and generally stem from sampling biases. Nonetheless, life-history characteristics have been reported to vary among conspecific shark populations (Lombardi-Carlson *et al.*, 2003; Driggers *et al.*, 2004; Cope 2006; Harry *et al.*, 2011b). Accurate age and growth parameters specific to both geographically and genetically distinct populations, therefore, are critical for informed regional fishery management.

In southeastern Australian waters, life-history information on the three study species (and all exploited carcharhinids for that matter) is currently undefined. The objective of the present study, therefore, was to provide the first detailed assessment of the age and growth of *C. brevipinna* in Australian waters, and of *C. obscurus* and *C. plumbeus* in eastern Australian waters, based on interpretations of vertebral growth bands.

## Methods

### Sample collection and genetic validation

Samples of vertebrae were collected between November 2007 and September 2010 by scientific observers on board commercial shark-fishing vessels operating off Australia’s New South Wales (NSW) coast between Tweed Heads (28°4’S) and Sydney (34°3’S) (Figure 1). All animals were sexed and recorded for total ( $L_T$ ), fork ( $L_F$ ) and precaudal lengths ( $L_{PC}$ ) to the nearest centimetre.

Owing to the morphological similarities among carcharhinids, a small quantity (<2 g) of white muscle tissue was collected from each individual and tested, using mitochondrial DNA, to validate species identity. Vertebrae and data associated with misidentified individuals were excluded from analyses.

Morphometric relationships between  $L_T$ ,  $L_F$  and  $L_{PC}$  were determined using linear regression analyses, with male and female relationships statistically compared using analyses of covariance (ANCOVA) (Table 1).



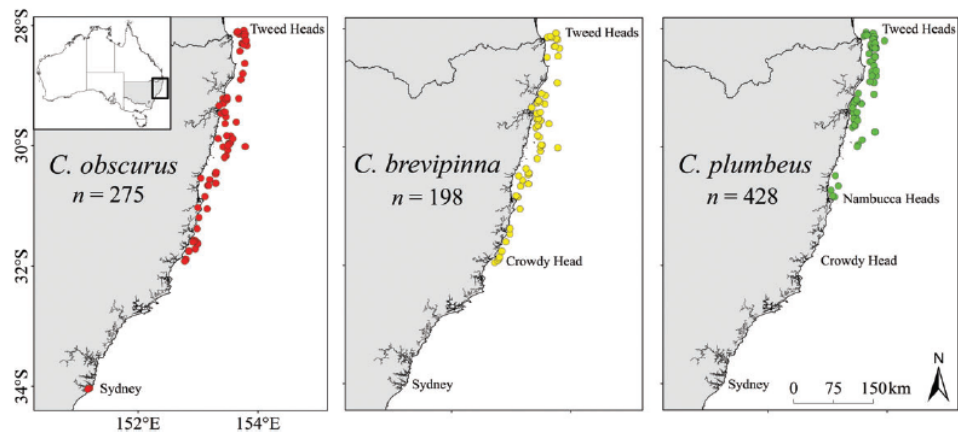


Figure 1. Study area and capture location for individual sharks aged.

Table 1. Morphometric relationships (cm) for *Carcharhinus obscurus*, *C. brevipinna* and *C. plumbeus* in New South Wales waters.

Species	Equation	n	$r^2$	ANCOVA		
				F	d.f.	p
<i>C. obscurus</i>	$L_T = 1.305(L_{PC}) + 8.021$	255	0.99	0.086	253	0.770
	$L_T = 1.203(L_F) + 4.226$	236	0.99	0.004	234	0.951
<i>C. brevipinna</i>	$L_T = 1.286(L_{PC}) + 6.208$	183	0.99	0.668	181	0.415
	$L_T = 1.188(L_F) + 3.519$	191	0.99	1.178	189	0.279
<i>C. plumbeus</i>	$L_T = 1.316(L_{PC}) + 4.566$	424	0.98	0.406	422	0.525
	$L_T = 1.206(L_F) + 2.747$	427	0.98	0.820	425	0.366

ANCOVA revealed no statistical difference between male and female length relationships for any of the species, hence regression equations represent combined sexes. All relationships were linear and highly significant ( $p < 0.001$ ).  $L_T$ ,  $L_F$  and  $L_{PC}$  denote total, fork and precaudal lengths, respectively.

Vertebrae preparation and ageing protocol

A section of 3–5 vertebrae was sampled from the cervical region of the vertebral column (i.e. anterior to the first dorsal fin) of each shark, stored on ice, and frozen upon return to the laboratory. In preparation for ageing, vertebrae samples were thawed, manually cleaned of excess soft tissue, separated into individual centra, and soaked in a 5% sodium hypochlorite solution (bleach) until all remaining soft tissue had been removed. Soak time varied from 15–45 min depending on the size of the centra. Cleaned vertebrae were rinsed thoroughly in tap water and stored in 70% ethanol. One vertebra from each shark was chosen at random, removed from the alcohol and air-dried in preparation for sectioning. Centra were sagittally sectioned through the focus to 0.5–0.6 mm thickness using an Isomet low-speed diamond-blade saw.

To determine the best vertebra preparation method, trials were conducted comparing unstained sections to sections stained with alizarin red and crystal violet. MicroCT scanning was also investigated as an alternative visualization technique (Geraghty et al., 2012). All four methods produced comparable section readability, however neither method noticeably enhanced growth-band clarity relative to the other. For practicality, unstained sections were employed for ageing analysis.

Unstained sagittal sections were fixed to a glass slide with waxed resin, and examined under reflected light on a dark background using an Olympus SZ dissecting microscope fitted with digital camera. Growth bands were counted by two independent readers

(Reader 1 and Reader 2) without prior knowledge of the size, sex or date of capture of the subject. Reader 1 was experienced in shark ageing methods and interpretation, while Reader 2 was relatively inexperienced. Digital images were taken of each vertebral section, and growth bands were independently marked by each of the readers using ImageJ. Archived images of both readers' ageing interpretations permitted accurate review. A growth band was defined as a band-pair, comprising one opaque and one translucent band (Cailliet et al., 2006). For the purpose of this study, the term age count is used to denote estimates of age based on annual band-pair deposition; the latter having been validated for *C. obscurus* (Simpfendorfer et al., 2002) and *C. plumbeus* (McAuley et al., 2006) in Australian waters, but has been assumed here for *C. brevipinna* in the absence of age validation for this species. Age counts were derived by counting fully formed translucent bands along the corpus calcareum occurring after the birth-mark, the latter being denoted by an angle change on the centrum face (Goldman, 2004) (Figure 2). The readability of each vertebral section was scored according to the following definitions: 5, all growth bands well defined and visible; 4, almost all bands visible, clear interpretation possible; 3, most bands visible, interpretation reliable to within  $\pm 1$ ; 2, bands visible, majority difficult to interpret; 1, unreadable. All sections deemed unreadable were excluded from further analyses. Age counts agreed upon between readers were adopted as the final age count for those vertebral sections. For any section where there was disagreement between readers, a final age count was decided upon by the more experienced

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reader (Reader 1) following an interactive review and evaluation of both readers' interpretations.

#### Between-reader bias and precision

A combination of methods was used to evaluate bias and precision in age counts between readers (Cailliet and Goldman, 2004). Bias was investigated using age-bias plots and Bowker's test of symmetry to determine whether observed count differences were systematic or due to random error (Campana et al., 1995; Hoenig et al., 1995; Campana, 2001). Inter-reader precision estimates were calculated using the coefficient of variation (CV) (Chang, 1982) and percent-age agreement (PA) (Goldman, 2004).

#### Growth modelling

The von Bertalanffy growth function (von Bertalanffy, 1983) has been the model most applied for describing growth in elasmobranchs (Cailliet and Goldman, 2004), however studies comparing the performance of multiple models have demonstrated others to be more appropriate in some shark species (Carlson and Baremore, 2005; Natanson et al., 2006; Barreto et al., 2011). Six candidate models, therefore, were fitted to observed length-at-age data for each species. Modified, three-parameter forms of the von Bertalanffy (VB-3), Gompertz (GOM-3) and logistic (LOGI-3) growth models were given by the following equations, where  $L_a$  is observed length at age  $a$  and  $L_0$  (length-at-birth),  $L_\infty$  (theoretical asymptotic length) and  $k$  (growth coefficient) are fitted parameters (Simpfendorfer et al., 2000; Braccini et al., 2007; Thorson and Simpfendorfer, 2009):

$$\text{(VB-3)} \quad L_a = L_0 + (L_\infty - L_0)(1 - e^{-(ka)})$$

$$\text{(GOM-3)} \quad L_a = L_0 \left( e^{\left( \frac{L_\infty}{L_0} \right) (1 - e^{-ka})} \right)$$

$$\text{(LOGI-3)} \quad L_a = \frac{L_\infty \cdot L_0 \cdot e^{(ka)}}{L_\infty + L_0(e^{(ka)} - 1)}$$

Two-parameter versions of the above equations were also computed (VB-2, GOM-2 and LOGI-2) by substituting  $L_0$  for a fixed length-at-birth value. Empirical  $L_0$  values for each species were

estimated to be between the largest observed embryos and the smallest free-swimming individuals encountered during this study: 94 cm  $L_T$  for *C. obscurus*, 80.5 cm  $L_T$  for *C. brevipinna* and 71 cm  $L_T$  for *C. plumbeus* (P. Geraghty, unpublished data). Models were fitted using the method of non-linear least squares in the statistical package R (R Development Core Team, 2010).

A multimodel inference (MMI) information-theoretical approach was used to determine the most appropriate growth model for each species (Burnham and Anderson, 2001; Katsanevakis and Maravelias, 2008; Harry et al., 2011b). Model performance was evaluated using Akaike's information criteria (AIC), with the best-fit model displaying the lowest AIC value. AIC differences were calculated as  $\Delta_i = y_i - x_{\min}$  and used to rank the support of the remaining models ( $i = 1-6$ ) relative to the best model. Models with  $\Delta$  of 0–2 had substantial support; models with  $\Delta$  of 4–7 had considerably less support; models with  $\Delta > 10$  had essentially no support (Burnham and Anderson, 2002). Akaike weights ( $w_i$ ) were calculated as the weight of evidence in favour of a model being the best in the set of candidate models (Burnham and Anderson, 2002). The 95% confidence intervals (CI) around the best-fit parameter estimates were derived from 10 000 resampled datasets.

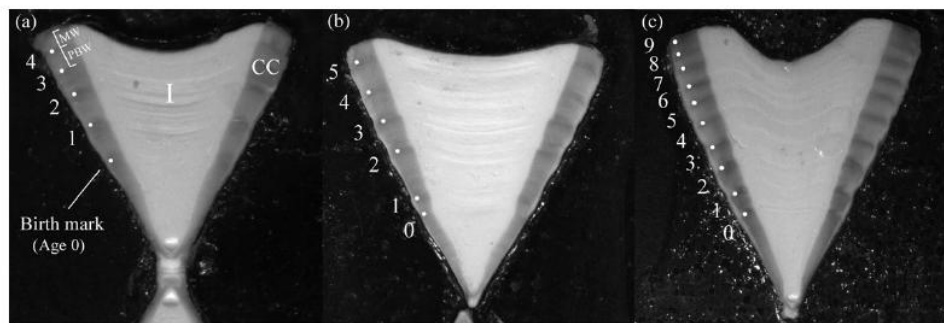
#### Growth-band periodicity

Verification of growth-band periodicity was achieved via marginal increment analysis. Only sections displaying clearly defined, unambiguous growth bands on the centrum outer margin were included. Marginal increment ratios (MIRs) were calculated using the following equation, with means ( $\pm$  SE) subsequently plotted against month:  $MIR = MW/PBW$  (Conrath et al., 2002), where  $MW$  = margin width and  $PBW$  = previous band-pair width (see Figure 2).

## Results

#### *Carcharhinus obscurus*

*Carcharhinus obscurus* was caught along the NSW coast between Tweed Heads and Sydney (Figure 1). Vertebrae from 275 genetically confirmed individuals, ranging in size from 92–386 cm  $L_T$ , were sectioned and read. Specimens sampled for both sexes were predominantly large ( $>270$  cm  $L_T$ ), although some small individuals were also obtained (Figure 3a).



**Figure 2.** Unstained sagittal sections from an (a) 4+ year old, 145 cm total length ( $L_T$ ) male *C. plumbeus*, (b) 5+ year old, 176 cm  $L_T$  female *C. brevipinna*, and (c) 9+ year old, 245 cm  $L_T$  female *C. obscurus*. Fully formed translucent bands occurring after the birth-mark are marked with white dots. All three sections were scored a readability of 5. I = intermedialia, CC = corpus calcareum, MW = margin width, PBW = previous band width.

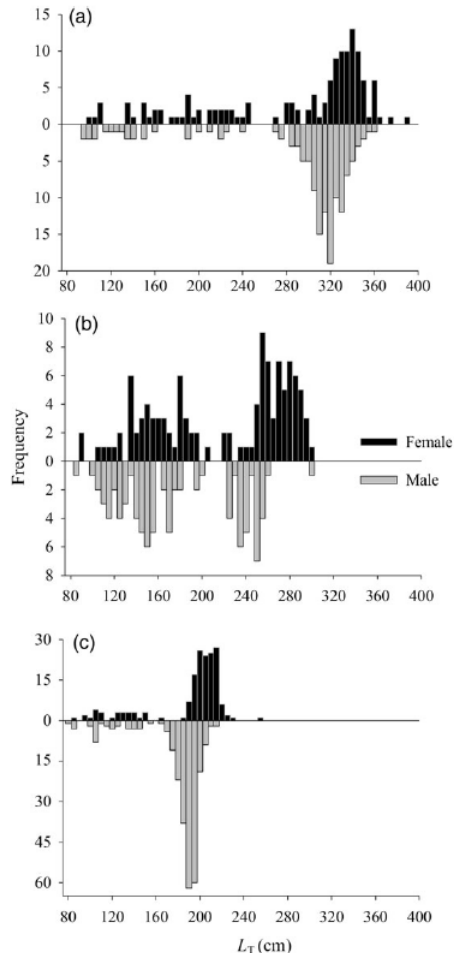
Vertebral growth-band readability was generally high in individuals  $\leq 270$  cm  $L_T$  and comparatively low in individuals  $> 270$  cm  $L_T$  (Figure 4a). Overall mean ( $\pm$  SE) readability was moderate ( $2.6 \pm 0.05$ ). Those sections deemed unreadable (18) were excluded from further analyses. Growth was therefore examined using observed length-at-age data from 257 individuals (126 females and 131 males), with lengths ranging from 99–386 cm  $L_T$  for females and 92–356 cm  $L_T$  for males.

An age-bias plot and Bowker's test of symmetry identified no systematic bias in age counts between Reader 1 and Reader 2 ( $\chi^2 = 80.5$ , d.f. = 68,  $p > 0.05$ ) (Figure 5a). Overall interreader precision

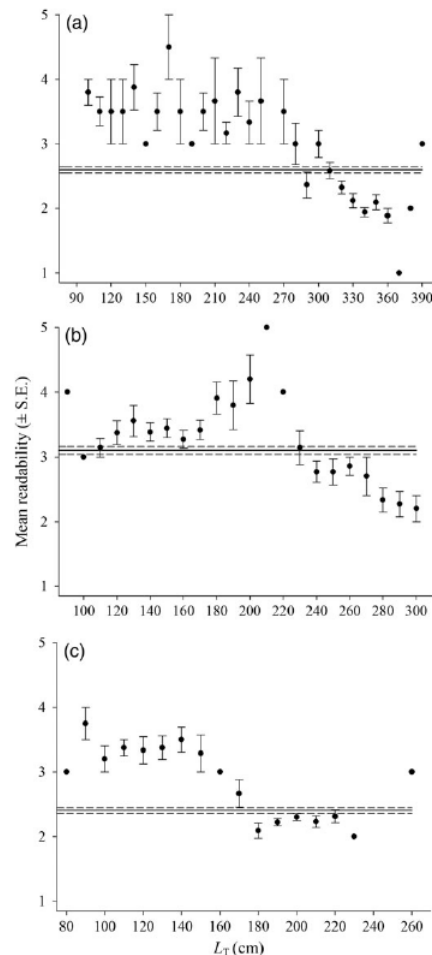
was high ( $CV = 7.48$ ) (Campana, 2001), despite  $PA$  being  $< 30\%$  (Figure 5a, Table S1). Agreement with the final age count was 72.4% for Reader 1 and 37.0% for Reader 2.

Marginal increment analysis provided evidence for annual band-pair deposition commencing in midwinter. Marginal increment ratios peaked in autumn (March–May) and remained high in early winter (June), but were comparatively small in late winter (August) and spring (September–November) (Figure 6a).

All six growth models provided good fits of the observed length-at-age data for both sexes (Figure 7a). Statistically, the three-



**Figure 3.** Length-frequency distributions, demonstrating differences in attainable size, of (a) *C. obscurus* ( $n = 275$ ), (b) *C. brevipinna* ( $n = 198$ ), and (c) *C. plumbeus* ( $n = 428$ ) specimens aged via vertebral analysis.



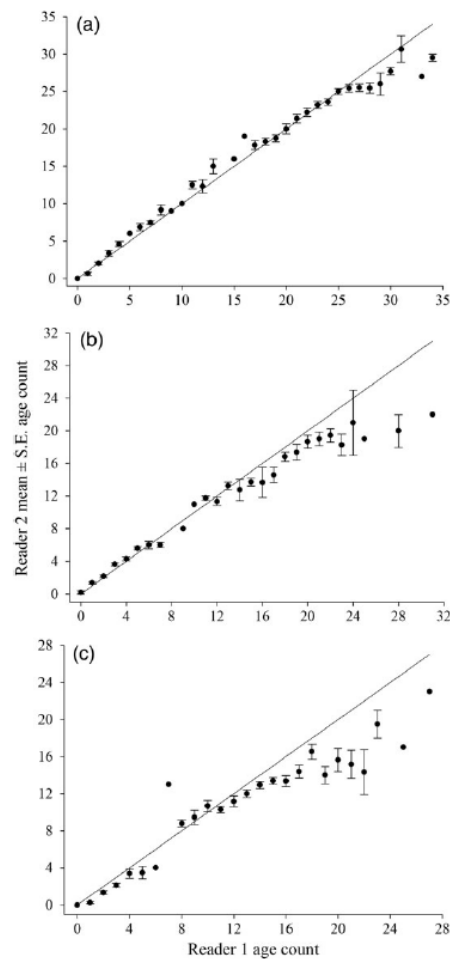
**Figure 4.** Mean readability ( $\pm$  SE) by total length ( $L_T$ ) for (a) *C. obscurus* ( $n = 275$ ), (b) *C. brevipinna* ( $n = 198$ ), and (c) *C. plumbeus* ( $n = 428$ ). Solid and dashed lines represent overall mean readability and upper and lower standard errors, respectively.

parameter von Bertalanffy (VB-3) growth function was the best model for describing female *C. obscurus* growth in NSW waters, with  $L_{\infty}$ ,  $k$  and  $L_0$  estimated at 365.03 cm  $L_T$ , 0.083 and 107.03 cm  $L_T$ , respectively (Table 2a). The two-parameter von Bertalanffy (VB-2) model was considered the best for describing male growth, with  $L_0$  fixed at 94 cm  $L_T$  and  $L_{\infty}$  and  $k$  estimated at 336.28 cm  $L_T$  and 0.108, respectively (Table 2a).

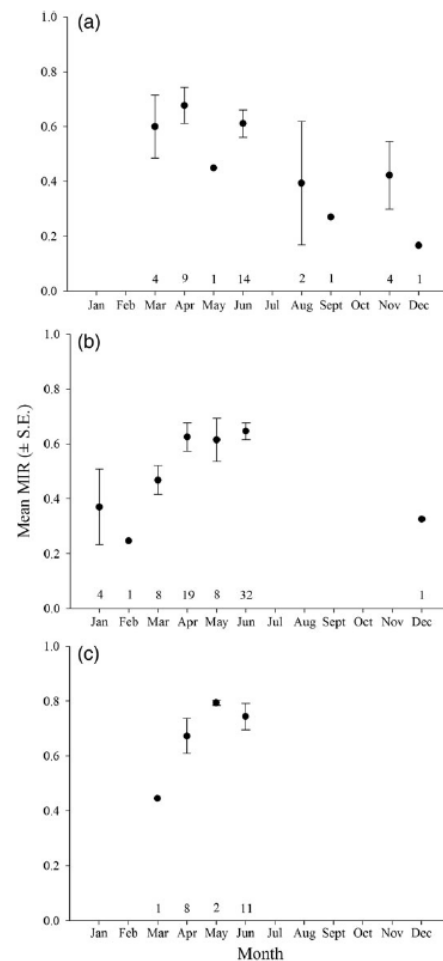
Observed mean length-at-age varied between sexes (Table S2). At most ages, females were larger than males. Predicted length-at-age, however, suggested less contrast between males and females, with

both sexes similar in size for the first 17 years of life (Figure 8, Table S2). Females and males displayed similar longevity, with the oldest observed *C. obscurus* being a 359 cm  $L_T$  female aged at 33 years, and the oldest observed male being a 347 cm  $L_T$  individual aged at 32 years (Figures 7a and 8).

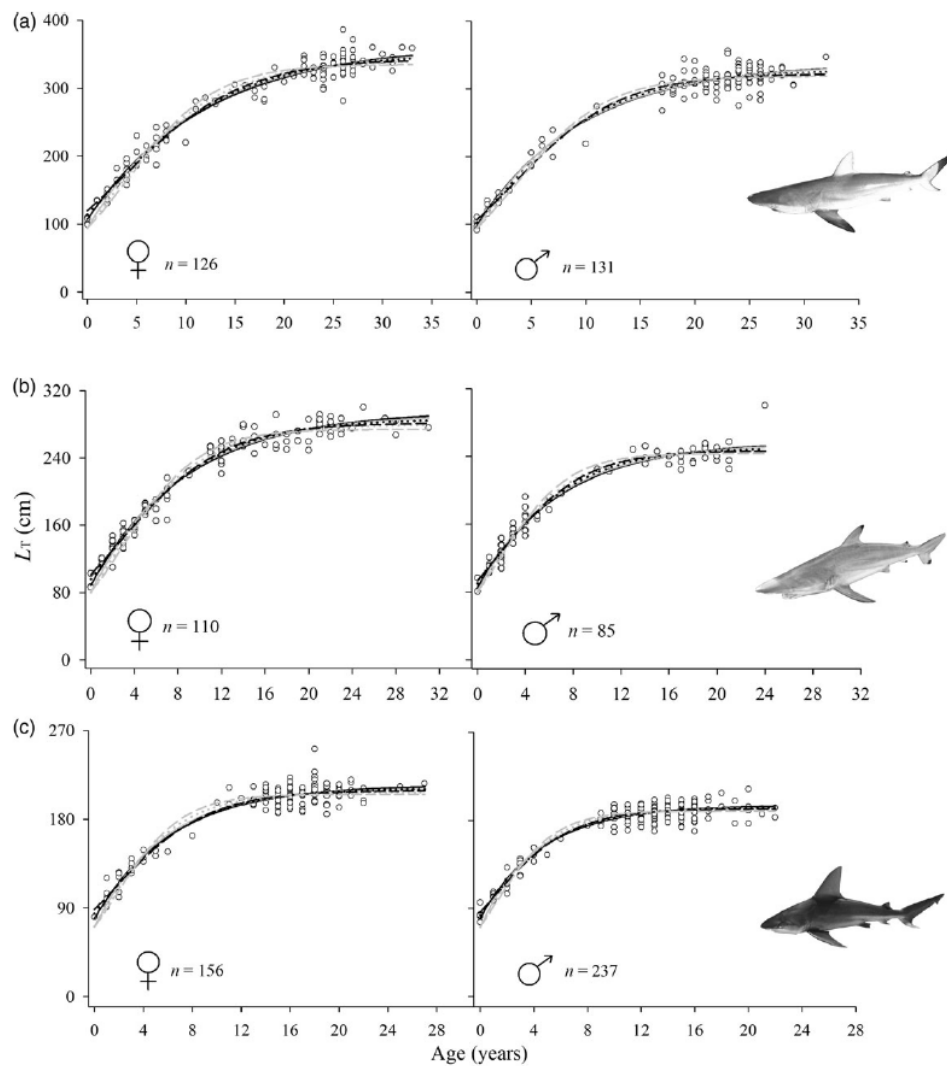
Analysis of modelled yearly growth increments suggested males grow at a faster rate than females for the first eight years of life, after which females grow faster than males (Figure 9, Table S2). For both sexes, growth was greatest in the first year following birth (Figure 9, Table S2).



**Figure 5.** Between-reader age-bias plots of (a) *C. obscurus* ( $n = 257$ ,  $CV = 7.48$ ), (b) *C. brevipinna* ( $n = 195$ ,  $CV = 12.6$ ), and (c) *C. plumbeus* ( $n = 393$ ,  $CV = 19.8$ ) vertebral age counts. One-to-one equivalence lines are shown.



**Figure 6.** Monthly mean marginal increment ratios (MIR,  $\pm$  SE) for (a) *C. obscurus*, (b) *C. brevipinna*, and (c) *C. plumbeus* in NSW waters. Monthly sample sizes are shown.



**Figure 7.** Observed total ( $L_T$ ) length-at-age for (a) *C. obscurus*, (b) *C. brevipinna*, and (c) *C. plumbeus* in NSW waters as determined by vertebral analysis. Fitted candidate growth curves: VB-3 (black line); VB-2 (grey line); GOM-3 (dotted black line); GOM-2 (dotted grey line); LOGI-3 (dashed black line); LOGI-2 (dashed grey line).

#### *Carcharhinus brevipinna*

*Carcharhinus brevipinna* was caught along the NSW coast between Tweed Heads and Crowdy Head (Figure 1). Vertebrae from 198 genetically confirmed *C. brevipinna*, ranging in length from 81–300 cm  $L_T$ , were sectioned and read. Length-frequency distributions exhibited two modes for both sexes (Figure 3b).

Vertebral growth-band readability was high in individuals  $\leq 230$  cm  $L_T$  and lower in larger specimens ( $>230$  cm  $L_T$ ) (Figure 4b). Overall mean ( $\pm$  SE) readability was high ( $3.1 \pm 0.06$ ), although three were deemed unreadable and excluded from further analyses. Growth, therefore, was investigated using observed length-at-age data from 195 individuals (110 females and 85 males),



**Table 2.** Summary of fitted parameter values (with 95% CI) and Akaike's Information Criteria results from six candidate models describing (a) *Carcharhinus obscurus*; (b) *Carcharhinus brevipinna*; and, (c) *Carcharhinus plumbeus* growth in New South Wales waters.

	Model	$L_{\infty}$	$L_0$	$k$	AIC	$\Delta$	$w$	RSE
<b>(a) <i>Carcharhinus obscurus</i></b>								
<b>Females (n = 126)</b>	<b>VB-3</b>	<b>365.03 (354.99, 377.68)</b>	<b>107.03 (97.70, 115.98)</b>	<b>0.083 (0.071, 0.095)</b>	<b>1037.14</b>	<b>0.00</b>	<b>86.41</b>	<b>14.54</b>
	VB-2	357.16 (350.02, 365.23)	94	0.095 (0.086, 0.103)	1042.92	5.78	4.80	14.94
	GOM-3	350.11 (343.50, 358.45)	114.02 (106.06, 121.73)	0.124 (0.109, 0.139)	1041.75	4.60	8.65	14.81
	GOM-2	341.64 (336.73, 346.89)	94	0.155 (0.144, 0.165)	1061.35	24.21	0.00	16.07
	LOGI-3	342.96 (337.62, 349.20)	119.94 (112.34, 127.29)	0.165 (0.148, 0.184)	1050.04	12.90	0.14	15.31
<b>Males (n = 131)</b>	<b>VB-3</b>	<b>338.15 (329.68, 349.89)</b>	<b>98.22 (89.52, 106.69)</b>	<b>0.104 (0.087, 0.121)</b>	<b>1078.36</b>	<b>1.04</b>	<b>20.92</b>	<b>14.56</b>
	VB-2	<b>336.28 (329.19, 345.50)</b>	<b>94</b>	<b>0.108 (0.095, 0.123)</b>	<b>1077.32</b>	<b>0.00</b>	<b>35.28</b>	<b>14.55</b>
	GOM-3	327.52 (322.00, 334.77)	102.75 (94.77, 110.43)	0.153 (0.133, 0.175)	1077.54	0.22	31.59	14.51
	GOM-2	324.63 (320.31, 329.93)	94	0.168 (0.152, 0.185)	1080.28	2.96	8.05	14.72
	LOGI-3	322.23 (317.98, 327.64)	106.84 (98.97, 114.20)	0.205 (0.179, 0.233)	1081.62	4.30	4.10	14.74
	LOGI-2	319.07 (315.64, 322.96)	94	0.241 (0.220, 0.263)	1090.14	12.82	0.06	15.28
<b>(b) <i>Carcharhinus brevipinna</i></b>								
<b>Females (n = 110)</b>	<b>VB-3</b>	<b>296.04 (288.18, 305.36)</b>	<b>89.06 (81.22, 96.39)</b>	<b>0.113 (0.098, 0.127)</b>	<b>858.90</b>	<b>9.67</b>	<b>0.54</b>	<b>11.74</b>
	VB-2	291.70 (285.40, 298.35)	80.5	0.124 (0.115, 0.134)	861.74	12.51	0.13	11.94
	GOM-3	286.57 (280.87, 293.00)	95.97 (89.58, 102.10)	0.162 (0.145, 0.180)	850.76	1.53	31.54	11.31
	GOM-2	280.15 (275.56, 284.78)	80.5	0.198 (0.186, 0.210)	870.78	21.55	0.00	12.44
	<b>LOGI-3</b>	<b>281.63 (276.85, 286.79)</b>	<b>101.43 (95.87, 106.76)</b>	<b>0.212 (0.192, 0.233)</b>	<b>849.23</b>	<b>0.00</b>	<b>67.79</b>	<b>11.23</b>
<b>Males (n = 85)</b>	<b>VB-3</b>	<b>274.12 (269.94, 278.32)</b>	<b>80.5</b>	<b>0.287 (0.271, 0.302)</b>	<b>893.10</b>	<b>4.388</b>	<b>0.00</b>	<b>13.77</b>
	VB-2	257.24 (250.23, 266.52)	85.67 (77.78, 93.92)	0.145 (0.122, 0.170)	651.09	0.44	30.51	10.83
	<b>VB-2</b>	<b>254.67 (249.07, 261.30)</b>	<b>80.5</b>	<b>0.158 (0.145, 0.172)</b>	<b>650.65</b>	<b>0.00</b>	<b>37.93</b>	<b>10.86</b>
	GOM-3	250.31 (245.11, 256.93)	90.31 (83.84, 97.46)	0.210 (0.180, 0.241)	651.65	1.00	23.06	10.86
	GOM-2	247.01 (242.55, 252.21)	80.5	0.248 (0.232, 0.265)	656.93	6.28	1.64	11.27
	LOGI-3	246.91 (242.58, 252.31)	93.98 (87.96, 100.37)	0.277 (0.241, 0.316)	654.08	3.42	6.85	11.02
	LOGI-2	243.66 (239.46, 248.37)	80.5	0.355 (0.336, 0.377)	667.93	17.28	0.01	12.02
<b>(c) <i>Carcharhinus plumbeus</i></b>								
<b>Females (n = 156)</b>	<b>VB-3</b>	<b>214.59 (210.24, 220.75)</b>	<b>79.45 (71.33, 87.33)</b>	<b>0.159 (0.131, 0.189)</b>	<b>1148.92</b>	<b>3.20</b>	<b>9.85</b>	<b>9.46</b>
	VB-2	211.80 (208.87, 215.20)	71	0.182 (0.164, 0.201)	1151.21	5.49	3.13	9.56
	GOM-3	211.27 (207.85, 215.91)	84.60 (77.87, 91.16)	0.206 (0.174, 0.243)	1146.20	0.48	38.26	9.38
	GOM-2	207.54 (205.40, 209.98)	71	0.266 (0.243, 0.290)	1158.86	13.14	0.07	9.80
	<b>LOGI-3</b>	<b>209.27 (206.38, 213.09)</b>	<b>88.42 (82.50, 94.19)</b>	<b>0.253 (0.216, 0.297)</b>	<b>1145.72</b>	<b>0.00</b>	<b>48.70</b>	<b>9.37</b>
<b>Males (n = 237)</b>	<b>LOGI-2</b>	<b>205.47 (203.64, 207.45)</b>	<b>71</b>	<b>0.369 (0.341, 0.401)</b>	<b>1171.18</b>	<b>25.46</b>	<b>0.00</b>	<b>10.20</b>
	<b>VB-3</b>	<b>195.34 (193.15, 197.99)</b>	<b>80.27 (75.58, 84.75)</b>	<b>0.214 (0.191, 0.238)</b>	<b>1607.72</b>	<b>7.51</b>	<b>1.37</b>	<b>7.12</b>
	VB-2	193.50 (191.78, 195.38)	71	0.244 (0.226, 0.264)	1621.22	21.00	0.00	7.34
	GOM-3	193.12 (191.36, 195.12)	83.15 (79.01, 87.05)	0.273 (0.246, 0.302)	1600.96	0.74	40.28	7.02
	GOM-2	190.93 (189.62, 192.31)	71	0.337 (0.314, 0.361)	1629.97	29.75	0.00	7.47
	<b>LOGI-3</b>	<b>191.74 (190.22, 193.50)</b>	<b>85.75 (81.88, 89.44)</b>	<b>0.332 (0.301, 0.367)</b>	<b>1600.22</b>	<b>0.00</b>	<b>58.35</b>	<b>7.00</b>
	LOGI-2	189.43 (188.28, 190.63)	71	0.451 (0.422, 0.482)	1646.96	46.74	0.00	7.75

Parameters are asymptotic total length ( $L_{\infty}$  cm  $L_T$ ), total length-at-birth ( $L_0$  cm  $L_T$ ) [fixed for 2-parameter models at (a) 94 cm  $L_T$  for *C. obscurus*, (b) 80.5 cm  $L_T$  for *C. brevipinna*, and (c) 71 cm  $L_T$  for *C. plumbeus*], and growth coefficient ( $k$ ). Akaike's Information Criteria values (AIC), Akaike differences ( $\Delta$ ) and Akaike weights ( $w$ ) show the relative support for each model. RSE = residual standard error. The "best-fit" model for each sex, as determined by AIC, is bolded.

ranging in length from 86–300 cm  $L_T$  for females and 81–300 cm  $L_T$  for males.

No systematic bias in age counts was identified between Reader 1 and Reader 2 ( $\chi^2 = 69.7$ , d.f. = 55,  $p > 0.05$ ) (Figure 5b). Interreader precision was acceptable ( $CV = 12.6$ ) (Campana, 2001) and overall PA was 36.4% (Figure 5b, Table S1). Agreement with final age count was 80% for Reader 1 and 44.6% for Reader 2.

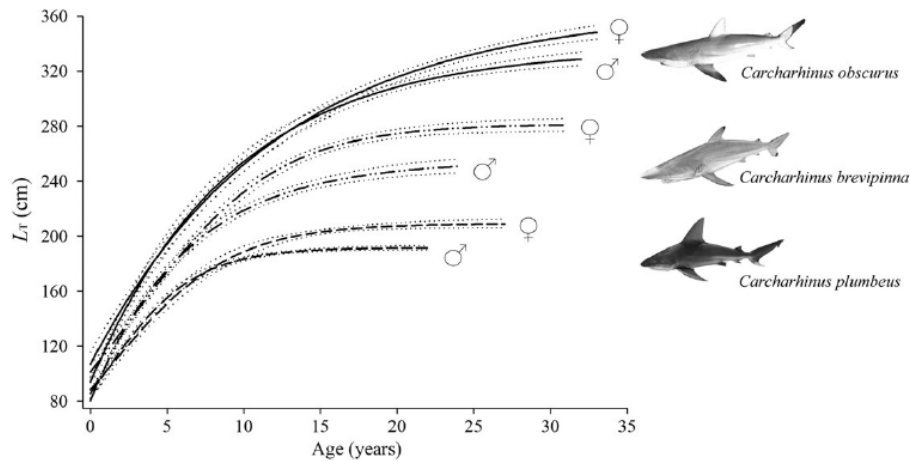
Marginal increment analysis suggested band-pair deposition commencing in midwinter. Marginal increment ratios were lowest in the summer months (December–February), increasing to a maximum value in early winter (June) (Figure 6b).

All growth models provided good fits of the observed length-at-age data for both sexes (Figure 7b). Statistically, the three-parameter logistic (LOGI-3) growth function was the best model for describing female *C. brevipinna* growth in NSW waters, with  $L_{\infty}$ ,  $k$

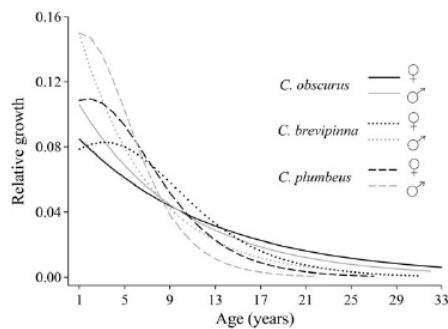
and  $L_0$  estimated at 281.63 cm  $L_T$ , 0.212 and 101.43 cm  $L_T$ , respectively (Table 2b). The VB-2 model was considered the best for describing male growth, with  $L_0$  fixed at 80.5 cm  $L_T$  and  $L_{\infty}$  and  $k$  estimated at 254.67 cm  $L_T$  and 0.158, respectively (Table 2b).

Observed mean, and predicted length-at-age suggested similar sizes for both sexes over the first seven years of life (Figure 8, Table S3). At all subsequent ages, females were considerably larger than males. Longevity varied between sexes, with the oldest observed *C. brevipinna* being a 276 cm  $L_T$  female aged at 31 years and the oldest observed male being a 300 cm  $L_T$  individual aged at 24 years (Figures 7b and 8).

Modelled yearly growth increments indicated males grow at a faster rate than females for the first four years of life, with growth in the first three years being substantially greater in males than females (Figure 9, Table S3). From the age of five onwards,



**Figure 8.** Comparative statistical “best-fit” growth curves, as determined by Akaike’s Information Criteria, for female and male *C. obscurus*, *C. brevipinna* and *C. plumbeus* in NSW waters. Dotted lines indicate 95% CIs based on 10 000 bootstrap iterations.



**Figure 9.** Relative growth (yearly growth increment/total growth) over observed lifespans of *C. obscurus*, *C. brevipinna* and *C. plumbeus* in NSW waters. Total growth ( $L_{\infty}$  minus  $L_0$ ) was calculated from values derived from statistical “best-fit” growth models, as determined by Akaike’s Information Criteria.

females grow at a faster rate than males. Growth was greatest in the first and third years after birth in males and females, respectively (Figure 9, Table S3).

#### *Carcharhinus plumbeus*

*Carcharhinus plumbeus* was caught along the NSW coast between Tweed Heads and Nambucca Heads (Figure 1). Vertebrae from 428 genetically confirmed *C. plumbeus*, ranging in length from 76–251 cm  $L_T$ , were sectioned and read. Specimens were predominantly large (>170 cm  $L_T$ ) individuals for both sexes (Figure 3c).

Vertebral growth-band readability was moderately high in individuals  $\leq 160$  cm  $L_T$ , but generally poor in larger specimens (>160 cm  $L_T$ ) (Figure 4c). Overall mean ( $\pm$  SE) readability was

quite low ( $2.4 \pm 0.04$ ). Following the exclusion of 35 vertebral sections deemed unreadable, growth was examined using observed length-at-age data from 393 individuals (156 females and 237 males), with lengths ranging from 81–251 cm  $L_T$  for females and 76–212 cm  $L_T$  for males.

Between-reader bias in age counts was identified for this species ( $\chi^2 = 165.2$ , d.f. = 97,  $p < 0.001$ ); Reader 2 systematically under-aged vertebrae relative to Reader 1 (Figure 5c). Consequently, overall interreader precision was low ( $CV = 19.8$ ,  $PA = 15.3\%$ ) (Campana, 2001) (Figure 5c, Table S1). Agreement with final age count was 63.4% for Reader 1 and 25.2% for Reader 2.

Marginal increment analysis provided limited information, but was suggestive of increasing ratios throughout the autumn months, peaking in May and remaining high in early winter (June) (Figure 6c). This supports, albeit tentatively, band-pair deposition from midwinter onwards.

All candidate growth models provided good fits of the observed length-at-age data for both sexes (Figure 7c). Statistically, the LOGI-3 growth function was the best model for describing both female and male *C. plumbeus* growth in NSW waters (Table 2c). Asymptotic growth ( $L_{\infty}$ ) and  $L_0$  estimates were larger for females (209.27 and 88.42 cm  $L_T$ ) compared with males (191.74 and 85.75 cm  $L_T$ ). In contrast,  $k$  was higher for males (0.332) than females (0.253) (Table 2c).

Observed mean length-at-age reported similar sizes for both sexes over the first five years of life; predicted length-at-age suggested similar sizes over the first nine years of life (Figure 8, Table S4). At all subsequent ages, females were considerably larger than males. Longevity varied between sexes; the oldest observed *C. plumbeus* being a 216 cm  $L_T$  female aged at 27 years, and the oldest observed males being two individuals measuring 183 and 193 cm  $L_T$  and aged at 22 years (Figures 7c and 8).

Analysis of modelled yearly growth increments indicated that males grow at a faster rate than females for the first four years of life, after which females grow faster than males (Figure 9, Table

S4). Rate of growth was greatest in the first and second years after birth for males and females, respectively (Figure 9, Table S4).

## Discussion

This study marks the first assessment of the age and growth of *C. brevipinna* in Australian waters, and of *C. obscurus* and *C. plumbeus* off Australia's east coast, where all three were demonstrated to be long-lived. The six candidate growth models fitted the observed length-at-age data well for all three species. Nevertheless, growth parameters varied markedly among models. Statistically, female growth was best described by the three-parameter von Bertalanffy function for *C. obscurus*, and by the three-parameter logistic model for *C. brevipinna* and *C. plumbeus*. Male growth was best modelled by the two-parameter von Bertalanffy function for *C. obscurus* and *C. brevipinna*, and by the three-parameter logistic curve for *C. plumbeus*. Based on criteria outlined by [Branstetter \(1987\)](#) and [Musick \(1999\)](#), our best-fit growth coefficients ( $k$  values) suggest that in southeastern Australian waters *C. obscurus* is a slow-growing species, *C. brevipinna* has a slow to moderate rate of growth, and *C. plumbeus* is a moderate to rapidly growing species.

Statistical “best-fit” ranking, however, doesn't necessarily convey biological reality ([Wang and Milton, 2000](#); [Romine et al., 2006](#); [Bubley et al., 2012](#)). Growth-model goodness-of-fit and resultant parameter estimates can be highly influenced by sampling biases, such as those imparted by gear selectivity or historic length-selective fishing mortality ([Thorson and Simpfendorfer, 2009](#); [Harry et al., 2013](#)). In the present study, a general under-representation of small-to-medium individuals resulted in three-parameter models overestimating length-at-birth ( $L_0$ ) for all three species (Table 2). In addition, the von Bertalanffy functions produced the most realistic estimates of theoretical asymptotic length ( $L_\infty$ ), while the logistic and Gompertz models underestimated  $L_\infty$  in all cases (Table 2). Given that  $L_\infty$  and  $k$  are negatively correlated, an underestimate in the former causes an overestimate in the latter. Statistical output, therefore, must be considered in conjunction with observed biological data when determining the most suitable model ([Cailliet et al., 2006](#)).

With this in mind, we propose the two-parameter von Bertalanffy (VB-2) function to be the most appropriate for describing the growth of both sexes of all three species off the southeast coast of Australia. Despite a lack of statistical support in most cases (Table 2), the VB-2 model provided the most biologically accurate fit to each dataset given the incorporation of empirical lengths-at-birth and realistic  $L_\infty$  output, and are referred to henceforth. However, while models with fixed  $L_0$  are highly applicable where small individuals are inadequately sampled, they are limited by a failure to account for variable length-at-birth or rapid early growth ([Neer et al., 2005](#); [Cailliet et al., 2006](#); [Thorson and Simpfendorfer, 2009](#)), and are vulnerable to biased parameter estimates with slight variations in  $L_0$  ([Pardo et al., 2013](#)).

*Carcharhinus obscurus*, *C. brevipinna* and *C. plumbeus* displayed both contrasts and consistencies in their growth characteristics in southeastern Australian waters. With respect to attributes common to all three species: growth rates were greatest in the years immediately after birth and decreased progressively over time, males grew more rapidly than females in the juvenile phase (hence displaying greater  $k$  estimates) after which their growth rate slowed below that of females, and females were observed to grow larger, live longer and were generally larger at any given age. These growth patterns are typical of sharks ([Cortés, 2000](#)) and

corroborate the findings of previous work on these species from other parts of the world (refer to literature cited in Table 3). In addition, vertebral band-pair deposition appeared to occur annually in all three sharks commencing in the midwinter months, although our marginal increment analyses were severely limited in their sample size and monthly cover.

Longevity, however, varied among *C. obscurus*, *C. brevipinna* and *C. plumbeus* in the study area. Maximum observed ages for females and males respectively were 33 and 32 for *C. obscurus*, 27 and 22 for *C. plumbeus*, and 31 and 24 for *C. brevipinna*. In the case of the former two species, these estimates are consistent with those reported from other oceanic regions where comparable methodologies were employed (Table 3). In contrast, our maximum age estimates for *C. brevipinna* are considerably higher than those previously reported for this species (Table 3)—such discrepancies between NSW and other geographic regions, however, may be the result of a range of confounding factors, such as variations in technique of preparation and reading of vertebrae, reader accuracy and precision, as well as sample size and distribution ([Cailliet et al., 1990](#); [Carlson et al., 2006](#)).

The parameters  $L_\infty$  and  $k$ , and hence rates of incremental and relative growth, also varied considerably among the study species in NSW waters. Yearly growth increments were largest in *C. obscurus* and smallest in *C. plumbeus* at any given age (Tables S2–S4)—not an unexpected result given the difference in maximum size attained by these species (Figure 3; [Last and Stevens, 2009](#)). Taking these differences into account, however, the reverse pattern was observed in the juvenile phase, where relative growth rates were highest in *C. plumbeus* and lowest in *C. obscurus* (Figure 9).

Our estimates of  $L_\infty$  and  $k$  did not necessarily agree with previous estimates for the same species in other areas. Similarly, rates of incremental growth were also observed to vary. Comparisons based solely on annual growth increments, however, are of limited value given that maximum attainable size within a species can vary among geographically distinct locations ([Last and Stevens, 2009](#)). We therefore recommend that measures of relative growth, as calculated in our study, be reported in conjunction with incremental growth so that more robust population (and species) comparisons can be drawn.

For *C. brevipinna*, our estimates of  $L_\infty$  and  $k$  are generally within the range of those reported for this species from other oceanic basins (Table 3). In contrast, our parameters for *C. obscurus* and *C. plumbeus* are markedly different from those reported by most other studies; our  $L_\infty$  and  $k$  estimates being comparatively low and high, respectively (Table 3). This implies that juvenile and adolescent *C. obscurus* and *C. plumbeus* are not as slow growing in NSW waters as has been reported in other parts of the world. However, rather than reflecting true conspecific differences, we propose that these discrepancies are driven by differences in sample size and length-distribution—in most cases highlighting the shortcomings of previous studies. All published works describing the growth of *C. obscurus* have grossly overestimated  $L_\infty$  (and hence underestimated  $k$ ) relative to biological reality; the same can be said for *C. plumbeus*, but with notable exceptions (Table 3). These inaccuracies stem from either small sample sizes ([Natanson et al., 1995](#); [Natanson and Kohler, 1996](#)) or a comparative overrepresentation of small individuals, resulting in poorly defined growth curve asymptotes (e.g. [Casey et al., 1985](#); [Sminkey and Musick, 1995](#); [Simpfendorfer et al., 2002](#); [McAuley et al., 2006](#)). While the present study also displayed generally poor balance among size classes, the contrasting bias towards large individuals of *C. obscurus* and *C. plumbeus* translated to pronounced growth asymptotes and hence lower (more realistic)  $L_\infty$  and higher



Table 3. Comparative growth-model parameters based on vertebral analysis.

Species	Oceanic region	Reference	n	Size range	Max. ages (sex)	Model	Female			Male		
							$L_{\infty}$	k	$L_0$	$L_{\infty}$	k	$L_0$
<i>C. obscurus</i>	SE Indian	Simpfendorfer et al. (2002)	305	77.7–333.9	32 (F), 25 (M)	VB (2)	418.6	0.043	92.1 <sup>a</sup>	397.7	0.045	92.1 <sup>a</sup>
	NW Atlantic	Natanson et al. (1995)	120	89.7–356.7	33 (F), 25 (M)	VB (3)	420.2	0.039	102.9	448.9	0.038	95.7
	SW Indian	Natanson and Kohler (1996)	42	99.1–353.6	34 (F)	VB (3)	395.7 <sup>b</sup>	0.047 <sup>b</sup>	–	–	–	–
	SW Pacific	Present study	257	92.0–386.0	33 (F), 32 (M)	VB (2)	357.2	0.095	94.0 <sup>a</sup>	336.3	0.108	94.0 <sup>a</sup>
<i>C. brevipinna</i>	SW Indian	Allen and Wintner (2002)	67	78.4–282.5	17 (F), 19 (M)	VB (3)	307.9	0.100	–	261.1	0.146	–
	NW Atlantic	Branstetter (1987)	15	67.0–208.0	11.3 (F), 8 (M)	VB (3)	214.0 <sup>b</sup>	0.212 <sup>b</sup>	72.2	–	–	–
	NW Atlantic	Carlson and Baremore (2005)	259	57.8–233.7	17.5 (F), 13.5 (M)	VB (3)	270.6	0.080	–	500.5	0.030	–
	NW Atlantic	Carlson and Baremore (2005)	259	57.8–233.7	17.5 (F), 13.5 (M)	VB (2)	242.8	0.110	–	333.0	0.070	64.9 <sup>b</sup>
<i>C. plumbeus</i>	W Pacific	Joung et al. (2005)	208	125.0–304.0	21 (F), 17 (M)	GOM (3)	263.2	0.160	75.2	239.6	0.140	74.9
	SW Pacific	Present study	195	81.0–300.0	31 (F), 24 (M)	VB (3)	288.2	0.151	75.0	257.4	0.203	75.0
	Central Pacific	Romine et al. (2006)	187	46.0–147.0 <sup>c</sup>	23 (F), 19 (M)	VB (2)	291.7	0.124	80.5 <sup>a</sup>	254.7	0.158	80.5 <sup>a</sup>
	Central Pacific	Romine et al. (2006)	187	46.0–147.0 <sup>c</sup>	23 (F), 19 (M)	VB (3)	164.9 <sup>c</sup>	0.080	–	151.1 <sup>c</sup>	0.090	–
	Central Pacific	Romine et al. (2006)	187	46.0–147.0 <sup>c</sup>	23 (F), 19 (M)	VB (2)	152.8 <sup>c</sup>	0.100	47.0 <sup>ac</sup>	138.5 <sup>c</sup>	0.120	47.0 <sup>ac</sup>
	Central Pacific	Romine et al. (2006)	187	46.0–147.0 <sup>c</sup>	23 (F), 19 (M)	VB (3)	143.5 <sup>c</sup>	0.170	47.0 <sup>ac</sup>	130.4 <sup>c</sup>	0.190	47.0 <sup>ac</sup>
<i>C. plumbeus</i>	NW Atlantic	Hale and Baremore (2010)	1194	39.0–202.0 <sup>d</sup>	27 (F), 22 (M)	LOGI (3)	146.4 <sup>c</sup>	0.170	–	134.3 <sup>c</sup>	0.190	–
	NW Atlantic	Hale and Baremore (2010)	1194	39.0–202.0 <sup>d</sup>	27 (F), 22 (M)	VB (3)	181.2 <sup>d</sup>	0.120	–	173.0 <sup>d</sup>	0.150	–
	NW Atlantic	Hale and Baremore (2010)	1194	39.0–202.0 <sup>d</sup>	27 (F), 22 (M)	VB (2)	178.3 <sup>d</sup>	0.140	46.0 <sup>ad</sup>	172.1 <sup>d</sup>	0.150	46.0 <sup>ad</sup>
	NW Atlantic	Casey et al. (1985)	475	~51.9–241.0	21 (F), 15 (M)	VB (3)	360.4	0.040	–	309.6	0.050	–
	W Pacific	Joung et al. (2004)	362	82.0–219.0	20.8 (F), 19.8 (M)	VB (3)	210.0 <sup>b</sup>	0.170 <sup>b</sup>	–	–	–	–
	SE Indian	McAuley et al. (2006)	235	58.7–178.8	25 (F), 19 (M)	VB (2)	279.4	0.039	53.7 <sup>e</sup>	259.3	0.044	53.7 <sup>e</sup>
	NW Atlantic	Sminkey and Musick (1995) <sup>e</sup>	188	67.7–229.8	24 (F), 20 (M)	VB (3)	263.3	0.059	–	245.9	0.059	–
	NW Atlantic	Sminkey and Musick (1995) <sup>f</sup>	412	57.0–215.1	22 (F), 18 (M)	VB (3)	220.5	0.086	–	221.8	0.087	–
	SW Pacific	Present study	393	76.0–251.0	27 (F), 22 (M)	VB (2)	211.8	0.182	71.0 <sup>a</sup>	193.5	0.244	71.0 <sup>a</sup>
	SW Pacific	Present study	393	76.0–251.0	27 (F), 22 (M)	VB (2)	211.8	0.182	71.0 <sup>a</sup>	193.5	0.244	71.0 <sup>a</sup>

$L_{\infty}$  = theoretical asymptotic length, k = growth coefficient,  $L_0$  = length at birth, VB = von Bertalanffy, GOM = Gompertz, LOGI = logistic, number of model parameters in parentheses. All length measurements expressed as total length ( $L_t$ , cm) unless otherwise stated, and converted where appropriate using publication-specific morphometric equations (if provided). Fixed parameter <sup>a</sup>combined sexes, <sup>b</sup>precadual length ( $L_K$ ), <sup>c</sup>fork length ( $L_F$ ), <sup>d</sup>1980–1981, 1991–1992.



(more accurate)  $k$  values. The influence of sample length-distribution on growth parameters is further emphasized by far less variation being observed between NSW waters and other geographic regions where species-specific length-distributions more closely resembled those of the present study (e.g. Allen and Wintner, 2002; Joung et al., 2004, 2005; Hale and Baremore, 2010).

Notwithstanding the abovementioned limitations, differences in growth characteristics between southeastern and western Australian waters should not be ruled out entirely for *C. obscurus*, possibly warranting further investigation. Our predicted annual growth increments for juveniles of this species were markedly larger than those reported by Simpfendorfer (2000) based on tag-recapture data, and a study by Geraghty et al. (unpublished data) demonstrated evidence for genetic differentiation in this species, albeit weak, between the two abovementioned regions.

On the bases of genetic validation and sample size and distribution, we propose the growth-model parameters presented herein to be among the more robust currently available for all three taxa. That said, however, due consideration must be given to the lack of age-validated longevity in the present study. Tag-recapture and bomb radiocarbon data have provided compelling evidence for vertebral-band analysis underestimating age in large adult sharks, including our study species (Casey and Natanson, 1992; Natanson et al., 1995; Francis et al., 2007; Andrews et al., 2011)—purportedly a result of discontinued band-pair deposition coinciding with a cessation of somatic growth, and/or problems with the interpretation of growth bands on the centrum outer edge. This is particularly relevant to the present study in which most sharks aged were large adult individuals. It is worth noting too that various studies have computed maximum theoretical ages based on reported maximum sizes and modelled growth parameters, yielding greatly elevated longevity estimates (e.g. Natanson and Kohler, 1996; McAuley et al., 2006)—however, such calculations are highly speculative and likely of limited value. Nevertheless, by compromising longevity estimates and growth model parameters, age underestimation has far-reaching implications for shark population modelling and assessment—highlighting the need for age validation of older age classes.

Similarly, the influence of section readability on our results also warrants some consideration. In all of the three study species, readability demonstrated a generally decreasing trend as shark size increased. This emphasizes a potential source of inaccuracy in our age counts given that the majority of sharks aged in the present study were large adults.

The results of the present study indicate that *C. obscurus*, *C. brevipinna* and *C. plumbeus* are all long-lived species displaying both contrasts and consistencies in their growth dynamics in temperate eastern Australian waters. While our results appear to challenge findings emanating from other parts of the world, confounding factors render definitive interregion conclusions potentially misleading. Nevertheless, we report the least conservative  $k$  estimates for *C. obscurus* and *C. plumbeus* of the published literature to date, which has profound implications relating to assessments of natural mortality and survival. Using  $k$  as an index of potential stock vulnerability to excessive mortality (Musick, 1999), our results suggest that these two species may in fact be somewhat more resilient to overexploitation (at least in NSW waters) than current population models would assert (Sminkey and Musick, 1996; McAuley et al., 2007a; Romine et al., 2009). This study also extends current estimates of maximum age for *C. brevipinna*—suggestive of greater reproductive potential. While the intrinsic

susceptibilities of the three study species to overfishing are well established (particularly for *C. obscurus* and *C. plumbeus*), our results potentially warrant some level of optimism when considering the resilience of these species to fishing pressure, at least in NSW waters. Given this, the true implications of our findings remain purely speculative in the absence of age validation (particularly of older age classes), reproductive parameters (work currently in progress), and hence demographic analyses, defined from the study region.

### Supplementary data

The following supplementary data is available at *ICES Journal of Marine Science* online:

Table S1. Outlining percentage agreement between Reader 1 and Reader 2 age counts for each of the three study species.

Tables S2–S4. Reporting mean ( $\bar{x}$ ) and predicted ( $P$ ) length-at-age (total length,  $L_T$ , cm), and growth rates (yearly growth increment,  $G$ , cm-yr<sup>-1</sup>), for female and male *C. obscurus*, *C. brevipinna* and *C. plumbeus* in NSW waters.

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APPENDIX B: THE ROLE OF VISUAL AND CHEMICAL CUES IN HOST  
DETECTION BY THE SYMBIOTIC SHRIMP *GNATHOPHYLLOIDES MINERI*  
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The role of visual and chemical cues in host detection by the symbiotic shrimp  
*Gnathophylloides mineri*

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ABSTRACT

The use of visual and chemical cues in host detection is regarded as important but relatively unknown for many symbionts. The small circumtropical caridean shrimp *Gnathophylloides mineri* forms a close symbiotic association with sea urchins, particularly *Tripneustes gratilla*, in many parts of the world. *G. mineri* is known to occur in temperate eastern Australia but the breadth of host use and selection of hosts amongst different species of sea urchins is relatively unknown. The abundance of *G. mineri* on three co-occurring species of sea urchins, *T. gratilla*, *Heliocidaris tuberculata* and *Pseudoboletia indiana* were measured in eastern Australia. These species of sea urchins were chosen because of either a known prior association with this shrimp elsewhere in the world, or due to their abundance in the area in temperate Australia where the shrimp occurs. Field collections showed that the association between shrimp and sea urchin appears extremely host-specific, with *G. mineri* only observed on *T. gratilla*, at an average density of two shrimp per sea urchin. Moreover, the majority of symbionts occurred on the underside (oral region) of *T. gratilla*. A visual laboratory experiment showed that *G. mineri* would actively move towards *T. gratilla* in preference to the other potential host species in the absence of chemical signals. Using a Y-maze, we tested the reaction of *G. mineri* to the absence of visual signals but the presence of chemical signals originating from their host, and from other species of potential host sea urchins. Seawater alone was used as a negative control. We demonstrated that *G. mineri* could detect *T. gratilla* from a distance, the same host that the shrimp are associated with in the field, using visual and chemical cues. Moreover, *G. mineri* react quickly to visual and chemical host signals by moving towards *T. gratilla* in preference to other species of sea urchins or in the absence of a signal. The relative importance of visual versus chemical cues is, however, unknown.

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1. Introduction

Symbiotic species often have highly specific host recognition processes to facilitate colonisation and fidelity (Combes, 2001). Host detection in symbiotic (i.e., commensal, parasitic and mutualistic) associations can occur through detection of either visual and/or chemical signals emitted by the host. Signal-mediated detection of the host removes the need for direct contact, and is a valuable adaptive mechanism for locating specific hosts (Derby and Sorensen, 2008; Lewis and Tumlinson, 1988; Prokopy and Owens, 1983; Thiel et al., 2003). Once on the host, many external symbionts use behavioural and morphological adaptations to avoid detection by predators (Endler, 2006). This is particularly common among shallow water marine crustaceans (Baeza and Thiel, 2000; Balakirev et al., 2008; De Bruyn et al., 2009; 2010; 2011; Gherardi, 1991; Gherardi and Calloni, 1993; Gray, 1961; Ng and Jeng, 1999; Patton et al., 1985; Takeda et al., 1997; Thiel et al., 2003).

*Gnathophylloides mineri* (Schmitt, 1933) is a small caridean shrimp with a circumtropical distribution, occurring in the Caribbean and in the Pacific and Indian oceans (Abele, 1975; Bruce, 1974; 1988; Castro, 1971; Chace and Bruce, 1993; Lewis, 1956; Maciá and Robinson, 2009; Okuno and Tanaka, 2001; Patton et al., 1985). *G. mineri* typically occurs in close association with various species of local sea urchins, where it lives amongst the urchin's spines. Although capable of filter feeding, generally it feeds on the host's thin epithelial layer, which covers the test (Maciá and Robinson, 2009; Okuno and Tanaka, 2001; Patton et al., 1985). Part of the distributional success of *G. mineri* is thought to be due to the highly adaptive nature of the symbiotic associations it forms with many different species of sea urchins.

*G. mineri* is most commonly associated with the sea urchins *Tripneustes ventricosus* and *Tripneustes gratilla* (Okuno and Tanaka, 2001). Both species of *Tripneustes* have either brown/purple or black tests and short white spines. *G. mineri* possesses complementary dark longitudinal stripes down its lower carapace that match the colour of the epithelium of its host. The shrimp also has thin longitudinal lightly coloured lines on its dorsal carapace that match the colour of the sea urchin's spines along with claws that are of a similar width and colour to the spines. Moreover, *G. mineri*'s antennae never protrude beyond

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sea urchin's spines (Patton et al., 1985). Such cryptic coloration and behaviour is thought to allow *G. mineri* to blend in with its host, making it difficult for visually dependent predators to detect (Maciá and Robinson, 2009; Main, 1987).

Interestingly, however, not all *G. mineri* are cryptically camouflaged with their host sea urchin. Those inhabiting the white sea urchin *Pseudoboletia indiana* in Hawaii do not resemble the colour of their host (Castro, 1971). Maciá and Robinson (2009) found that, in addition to inhabiting *T. ventricosus*, *G. mineri* were abundant on the sympatric sea urchin *Lytechinus variegatus* despite contrasting starkly with the colour of the spines and test of this species. Maciá and Robinson (2009) conclude that this mismatch of crypsis was a result of the extensive covering of debris that *L. variegatus* deposit on its test. Attached debris would give the shrimp substantially more protection from predation, thus lessening the importance of crypsis. Another hypothesis, however, is that the ability of *G. mineri* to visually discriminate between appropriate and less appropriate hosts is impaired due to the heavy covering of debris on *L. variegatus*. This hypothesis assumes that the detection of a suitable host is essentially driven by visual cues and that chemical cues from the host have no significant role in the process. Although other crustacean symbionts use chemical and/or visual cues to locate sea urchins (Ache and Davenport, 1972; Cournoyer and Cohen, 2011; De Bruyn et al., 2011; Gherardi and Calloni, 1993), it is unknown which cues *G. mineri* use for host detection.

*G. mineri* occurs cryptically on the spines of *T. gratilla* (Linnaeus, 1758) on the eastern coast of Australia, towards the sea urchin's southern latitudinal limit of distribution (Williamson et al., personal observation). The morphological and behavioural adaptations observed in Australia are similar to those that have been observed between *G. mineri* and other species of *Tripneustes* in other countries, suggesting that *G. mineri* may actively choose to associate with this genus of sea urchin, and *T. gratilla* in particular in Australian waters. As such, the shrimp must be able to distinguish this species from other similar sized species in the area, most likely through visual and/or chemical cues. To test this hypothesis, the following questions were asked: (1) what is the abundance of *G. mineri* on *T. gratilla* versus other morphologically similar sea urchins that overlap in distribution and life history strategies to *T. gratilla*; (2) where on the sea urchin's test are the shrimps usually located; (3) does *G. mineri* display preferential movement towards *T. gratilla* over other species of potential host sea urchins using visual cues in the absence of chemical cues; (4) does *G. mineri* preferentially move towards *T. gratilla* over other potential host sea urchins using chemical cues in the absence of visual cues?

## 2. Materials and methods

### 2.1. Collection of animals and holding conditions

Three species of sea urchins, *T. gratilla*, *Heliocidaris tuberculata* (Lamarck, 1816) and *P. indiana* (Michelin, 1862) were assessed as potential hosts. *G. mineri* occurs on *T. gratilla* and *P. indiana* in other parts of the world whereas *H. tuberculata* is the most common morphologically similar species that is consistently abundant in temperate eastern Australia (Miskelly, 2002). Individuals were opportunistically collected using SCUBA from depths of 5 to 25 m from two sites that are representative of areas that *G. mineri* has been observed: Fish Rock (30°56'25"S, 153°06'05"E) at South West Rocks, and Camp Cove or Fairlight in Sydney (33°51'23"S, 151°14'35"E), Australia. The number of individuals collected varied between sea urchin species and were based on the relative abundance of that species. Both sites maintained a similar relative abundance of the three species of urchins and contained *G. mineri* at the time of collection (Williamson and Vaitilingon, personal observation).

Individuals were immediately bagged and transported back to the laboratory in Sydney where the number and location of *G. mineri* on

each individual was assessed (see below). After counting, urchins and their symbionts were individually held in the 30,000 L recirculating seawater facility at Macquarie University until used for experiments. Tanks within the facility were illuminated from above by Gro-Lux fluorescent bulbs ( $9.4 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ ), with day-length corresponding to the external environment. Seawater was maintained at a salinity of 34–36‰, at field temperatures (18–19 °C), and with an average flow of seawater into each tank at  $60 \text{ L.h}^{-1}$ . Animals were fed *ad libitum* a mixture of the kelp *Ecklonia radiata* and the brown alga *Sargassum linearifolium*, the common species of algae present in the collection habitats.

### 2.2. Abundance and location of *Gnathophylloides mineri* on sea urchins

Approximately 24 h after collection, the external features of each sea urchin were carefully examined for the number of resident *G. mineri* and their location. *G. mineri* was recorded as being either on the oral (bottom) or aboral (top) surface of a sea urchin, and whether they inhabited the ambulacral or inter-ambulacral regions on each surface. Data were analysed using chi-square tests comparing the observed abundance and location frequencies to an *a priori* expected frequency of 50:50. No shrimps were observed moving around or leaving their host at any time during this handling period. As with Maciá and Robinson's (2009) study, each *G. mineri* clung tenaciously to a spine of the host thus making it highly probable that most, if not all, of the shrimps remained on the same host from collection to examination. The dimensions of each sea urchin were recorded. Algae that the sea urchin was consuming at the time, along with the container that the individual inhabited, were also searched for *G. mineri*.

### 2.3. Use of vision in host detection by *Gnathophylloides mineri*

The role of vision in the detection of *T. gratilla* over other potential host sea urchins by *G. mineri* was determined by a series of visual tests in a 40 L tank as per VandenSpiegel et al. (1998). Here, the 40 L tank was filled with filtered seawater ( $0.22 \mu\text{m}$ ) and two identical clear plastic Ziplock™ bags were also filled with filtered seawater (400 ml) and placed at opposite ends of the tank, 14 cm apart. These bags held the various treatments used in the visual tests and allowed the test shrimp to see a particular treatment but not to be able to detect it through chemical means. Treatment combinations were: (1) *T. gratilla* versus filtered seawater, (2) *T. gratilla* versus *H. tuberculata*, and (3) *H. tuberculata* versus filtered seawater. Responses were tested against a control of filtered seawater versus filtered seawater.

At the start of each test one *G. mineri* was placed in the centre of the tank (7 cm from either treatment) and the time taken for the individual to move towards a treatment over 5 min was recorded. Tests were scored as "positive" if *G. mineri* touched a treatment bag within 5 min, and that treatment was recorded. Tests were scored as negative if the shrimp failed to touch a treatment bag within 5 min. Tests were repeated 50 times. Each *G. mineri* was used only once and water was changed between each trial. Plastic bags holding the treatments were checked at the conclusion of each test to ensure that there was no possibility of leaking chemical cues.

Results were analysed using chi-square tests comparing the observed preference frequencies to an *a priori* expected frequency of 50:50. Two different chi-square tests were employed. The first compared *G. mineri* that scored a positive result to those that scored a negative result, or failed to touch a treatment. If an individual scored a positive in the first test, a second chi-square test was done to distinguish which treatment was chosen. Response times for shrimp stimulated to move were compared across treatments using a one-factor ANOVA. Data were checked for normality prior to analyses.

#### 2.4. Use of chemical cues in host detection by *Gnathophyllodes mineri*

A laboratory experiment was done to test the role of olfaction, or recognition of a chemical cue, in host detection by *G. mineri* using a system similar to Davenport's "Y-maze" (Davenport, 1950; Fig. 1). This system allowed individual shrimp to distinguish between treatments using seawater flowing over the various treatments (and thus containing any chemical cue emitted from those treatments) without the shrimp being able to see any of the treatments. The Y-maze, constructed of clear Perspex (1 cm diameter), consisted of an 11 cm stem connected to two 11 cm arms of the Y at 60° apart. The arms terminated at a 45° elbow. Treatments were introduced into the Y via two separate 2 L aquaria (one treatment per aquarium, Fig. 1A and B), which were gravitationally fed from two separate sources of filtered water and connected with clear plastic tubing. A constant flow of water through the Y-maze (measured at the point where the shrimp was initially placed) was set at  $250 \text{ ml min}^{-1}$  and was regulated by a tap at the base of a common aquarium that the Y-maze drained into (Fig. 1). Treatments were left for 30 min to allow for a consistent amount of any chemical cues to be released into the water and a new urchin was used for each replicate and assay. Trials prior to the experiment using red food dye in either aquaria showed that flow was steady and laminar and that seawater flowing along the stem consisted of equal portions of water from both aquaria; one on either side of the stem. Thus shrimp placed at the end of the stem could distinguish between water on the left hand side originating from the left aquarium and right hand side water originating from the right aquarium. All tubing and apparatus connecting the aquaria and the Y-maze in the system was thoroughly flushed out with fresh filtered seawater between replicates to remove any odours from either the treatments or test individual from the previous trial.

Treatment combinations used were: (1) *T. gratilla* versus *T. gratilla*; (2) *T. gratilla* versus filtered seawater; (3) *T. gratilla* versus *H. tuberculata*;

(4) *T. gratilla* versus *P. indiana*; (5) *H. tuberculata* versus filtered seawater; (6) *P. indiana* versus filtered seawater; and (7) *P. indiana* versus *P. indiana*. Forty replicates were done for each treatment combination, with the exception of those containing *P. indiana* as this species was not always as readily available in the field as the others. Treatments were swapped from the left to the right arm and vice versa after half the replicates. Responses were tested against a control of filtered seawater versus filtered seawater to account for any preference for either the left or the right arm of the Y-maze (Putnam, 1962).

At the start of each trial, one *P. mineri* was carefully placed halfway between the common aquarium and the intersection of the Y-maze. The amount of time it took the shrimp to move from the starting point to the intersection of the Y-maze (Time A) was recorded, along with the time it took for the shrimp to move from the intersection of the Y-maze to halfway up either arm of the maze (Time B). Individuals that recorded a Time B were considered to have made an active choice towards that particular treatment. Those that did not record either Time A or B (i.e., did not move far enough into the Y-maze) within 20 min were recorded as having made no choice.

Results were analysed using two chi-square tests. The first test assessed individuals that made an active choice (recorded times for A and B) versus those that made no choice compared to an *a priori* expected frequency of 50:50. The second chi square test assessed which active choice the individuals made (i.e., which treatment) for those that had recorded times for both A and B. To account for any bias towards the left or right arm of the Y-maze, treatments in the second chi-square test were compared to the frequency obtained from the control (filtered seawater versus filtered seawater) rather than a frequency of 50:50. Response times for shrimp stimulated to move were compared across treatments using a one-factor ANOVA. Data were checked for normality prior to analyses.

### 3. Results

#### 3.1. Abundance and location of *Gnathophyllodes mineri* on sea urchins

A total of 216 *T. gratilla*, 68 *H. tuberculata* and 19 *P. indiana* were collected and surveyed for *G. mineri*. Of these 73% of the *T. gratilla* contained at least one *G. mineri*, whereas no shrimp were found on either of the other species. *T. gratilla* individuals that hosted *G. mineri* carried an average of  $1.86 (\pm 0.22 \text{ SE})$  shrimp. The average size of *T. gratilla* containing *G. mineri* was  $7.68 \pm 0.06 \text{ cm}$  (diameter) and  $4.48 \pm 0.042 \text{ cm}$  (height).

Measurements of the position of *G. mineri* on each *T. gratilla* revealed a clear trend. Significantly more *G. mineri* preferred the oral surface (80%) of the hosts to the aboral surface (20%) ( $\chi^2 = 9.0$ , 1 df,  $P = 0.003$ ). There was no significant difference between the number of shrimp on the oral ambulacral surface (36%) and the oral interambulacral surface (44%) of *T. gratilla* ( $\chi^2 = 0.2$ , 1 df,  $P = 0.655$ ). Although not significant, more shrimp inhabited the aboral ambulacral surface (16%) than the aboral interambulacral surface (4%) ( $\chi^2 = 1.8$ , 1 df,  $P = 0.178$ ). *G. mineri* was typically observed in an outward facing position along the spines of the host.

#### 3.2. Use of vision in host detection by *Gnathophyllodes mineri*

The first control series of filtered seawater versus filtered seawater showed that very few shrimp were stimulated to move and those that did move did not actively favour one side of the aquaria over another (Table 1a). These results eliminated the possibility of *G. mineri* favouring one treatment over the other for unknown experimental factors. In the next two series, the shrimp were actively attracted to their host urchin *T. gratilla*, in preference to either seawater or to another co-occurring species of sea urchin, *H. tuberculata* (Table 1b,c). When *T. gratilla* was not present and the shrimp had to choose between a non host species and seawater alone, there was no significant

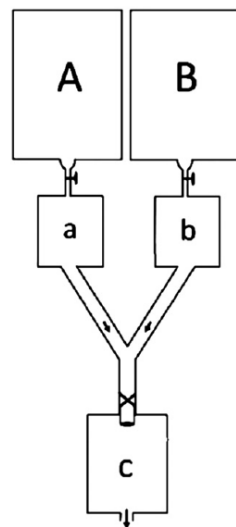


Fig. 1. Schematic diagram of the Y-maze used to test the behaviour of the symbiotic shrimp *Gnathophyllodes mineri* in response to chemical stimuli. 'X' marks the start position of the animal. 'A' and 'B' indicate the 2 L aquaria that held the treatments; 'a' and 'b' are the source aquaria that received seawater gravitationally fed from 'A' and 'B' respectively; and 'C' is the aquarium used to evacuate any remaining seawater at a constant flow.

**Table 1**

Visual host attraction of *Gnathophyllodes mineri* to the sea urchins *Tripneustes gratilla* and *Heliocidaris tuberculata* against each other and against control signals of filtered seawater (FSW).

	Time (s)		Choice		Results				Chi-square (p)	
	Minimum	Maximum	A	B	Assay	N	A	B	(1)	(2)
a	32	140	FSW	FSW	50	44	3	3	<0.001	1.0
b	2	104	<i>T. gratilla</i>	FSW	50	5	42	3	<0.001	<0.001
c	1	245	<i>T. gratilla</i>	<i>H. tuberculata</i>	50	5	37	8	<0.001	<0.001
d	3	110	<i>H. tuberculata</i>	FSW	50	17	30	3	0.124	<0.001

Time (s) shows the minimum and maximum reaction times. Choices A and B are the two proposed visual signals; Assay shows the number of replicates; N shows the number of individuals that showed no response; Results A and B show the number of shrimp that actively chose A or B respectively.

Chi-square (1) shows the probability significance of a shrimp making an active choice towards a signal; Chi-square (2) shows the probability significance of a shrimp choosing one signal over another. All results were compared to a theoretical ratio of 50:50.

difference between the number of shrimp that were stimulated to move compared to those that remained stationary. Individuals that actively moved, however, chose to move towards *H. tuberculata* in preference to seawater alone (Table 1d). There was no significant difference between response times for each treatment across all series for shrimp that were stimulated to move (one-factor ANOVA,  $F=1.451$ ,  $P=0.238$ ; Table 1a–d).

### 3.3. Use of chemical cues in host detection by *Gnathophyllodes mineri*

As with the visual experiment, the negative control series (seawater versus seawater) showed that relatively few shrimp were stimulated to make a choice and that, of those that did move to the intersection of the Y-maze, there was no significant difference in the arm of the tube that they chose (Table 2a). A positive control series (*T. gratilla* versus *T. gratilla*) showed that the majority of shrimp actively moved towards the host species, but that one arm of the Y-maze was not favoured over the other (Table 2b). These results show that *G. mineri* were able to perceive a cue and move towards it within the Y-maze and that there was no obvious asymmetry in the experimental design.

Shrimp were stimulated to move when *T. gratilla* was presented as an option against another signal, and actively moved towards their host species regardless of the type of other stimuli present (Table 2c–h). Only roughly half of the shrimp presented with either *H. tuberculata* versus seawater as signals were stimulated to move. Of those that did move, significantly more individuals actively moved towards *H. tuberculata* (Table i–j). Shrimps presented with a choice of *P. indiana* or seawater were not particularly stimulated to move or to actively choose one signal over another, thus acting in a similar manner to shrimp exposed to the seawater versus seawater controls (Table 2k–l).

In some of these cases, more individuals chose not to move at all than to make a choice. This was unexpected but a further series that was run testing *P. indiana* versus *P. indiana* resulted in a similar result where individuals were not stimulated to move or to make a choice either, thus supporting the results above (Table 2m). Interestingly, however, a similar number of choices were made by individuals when presented with a choice of *P. indiana* or *T. gratilla* (Table 2h).

The reaction times between treatments where shrimp were stimulated to move varied amongst individuals (see min/max times in Table 2). There was, however, no significant difference in reaction time between treatments for individuals that moved from the starting point of the Y-maze (Time A; one-factor ANOVA,  $F=1.423$ ,  $P=0.240$ ; Table 2a–m), or for individuals that moved from the intersection to halfway up an arm of the Y-maze (Time B; one-factor ANOVA,  $F=0.894$ ,  $P=0.447$ ; Table 2a–m).

## 4. Discussion

As in many other parts of the world, *G. mineri* forms a close association with *T. gratilla* in eastern Australia. *G. mineri* specifically inhabited *T. gratilla* despite the prevalence of other similar species of sea urchins that overlap in distribution with *T. gratilla*. Moreover, *G. mineri* has the ability to use both visual and chemical cues to locate its host from a distance, and rapidly swam against the water current towards its host in preference to other cues. When its host was absent, shrimp either remained stationary or moved at a slower rate towards another non-host sea urchin.

Locating a host is a fundamentally important stage in the life history of symbionts and, as such, the use of several sensory modes increases the likelihood of detecting an appropriate host. The ability

**Table 2**

Chemical host attraction of *Gnathophyllodes mineri* to the sea urchins *Tripneustes gratilla*, *Heliocidaris tuberculata* and *Pseudoboletia indiana* against each other and against control signals of filtered seawater (FSW).

	Min/Max time (s)		Choice		Results				Chi-square (p)	
	Time A	Time B	A	B	Assay	N	A	B	(1)	(2)
a	1/663	4/1200	FSW	FSW	20	11	5	4	0.655	0.739
b	1/11	3/346	<i>T. gratilla</i>	<i>T. gratilla</i>	20	1	9	10	<0.001	0.504
c	1/69	2/1126	<i>T. gratilla</i>	FSW	20	1	18	1	<0.001	<0.001
d	1/129	5/1200	FSW	<i>T. gratilla</i>	20	7	2	11	0.18	0.004
e	2/58	3/1200	<i>T. gratilla</i>	<i>H. tuberculata</i>	20	1	17	2	<0.001	<0.001
f	1/142	13/424	<i>H. tuberculata</i>	<i>T. gratilla</i>	20	0	4	16	<0.001	0.002
g	2/135	1/894	<i>T. gratilla</i>	<i>P. indiana</i>	17	5	11	1	0.09	0.001
h	1/154	3/432	<i>P. indiana</i>	<i>T. gratilla</i>	15	7	4	4	0.796	0.776
i	1/30	3/1200	<i>H. tuberculata</i>	FSW	20	11	2	7	0.655	0.048
j	1/65	5/1200	FSW	<i>H. tuberculata</i>	20	10	6	4	1	0.751
k	2/187	2/12	<i>P. indiana</i>	FSW	13	8	1	4	0.405	0.116
l	2/13	3/35	FSW	<i>P. indiana</i>	7	3	3	1	0.705	0.421
m	2/381	3/157	<i>P. indiana</i>	<i>P. indiana</i>	10	7	2	1	0.206	0.685

Time (s) shows the minimum and maximum reaction times for individuals that moved from the starting point to the intersection in the Y-maze (Time A), and those that moved from the intersection to halfway up an arm of the Y-maze, thus making an active choice (Time B). Choices A and B are the two proposed visual signals; Assay shows the number of replicates; N shows the number of individuals that showed no response; Results A and B show the number of shrimp that actively chose A or B respectively.

Chi-square (1) shows the probability significance of a shrimp making an active choice towards a signal; Chi-square (2) shows the probability significance of a shrimp choosing one signal over another for individuals that had recorded times for both Times A and B. All results were compared to a theoretical ratio of 50:50.



to use more than one sensory cue to detect potential hosts or predators is a relatively common strategy for marine organisms (Dalesman and Inchley, 2008; DeBose et al., 2008; Hay, 2009), and symbiotic decapod crustaceans routinely use a combination of visual and chemical cues for host location (Huang et al., 2005; Sikkell et al., 2011). The ectosymbiotic shrimp *Synalpheus demani* uses visual cues for small-scale host detection but uses chemical signals from the host crinoid to increase activity and orientate towards the host from a distance (Huang et al., 2005). The parasitic isopod *Gnathia marleyi* is more successful in locating its host fish in the field when it is able to employ a combination of visual and chemical signals rather than just one signal (Sikkell et al., 2011). There should be strong selection for modes of ability to detect and locate appropriate hosts since failure to find a host or inappropriate host choice increases the risk of predation and lowers reproductive success.

While our experiments showed that *G. mineri* responded rapidly and with accuracy towards its host using two remote signals, they did not determine the relative importance of visual versus chemical cues in the detection of *T. gratilla*. Visual and chemical cues in host detection often occur in terms of hierarchy and sequence: symbionts use chemical means to locate the general area of an appropriate host, then visual signals in the final stages of host detection (DeBose and Devitt, 2008; Segura et al., 2007). Chemical cues are generally considered reliable mediators of habitat choice for different life history stages in marine specialist invertebrates as they provide a unique signature from the host (Williamson et al., 2000; Williamson and Steinberg, 2012). If the host itself is either variably abundant or cryptic and thus likely to produce more subtle or unreliable chemical signals, the relative importance of visual or chemical cues may change.

In temperate east Australia, *T. gratilla* occurs in patchy populations along its southern limit of distribution. At sites where *T. gratilla* occurs more reliably, such as our study site at South West Rocks, the species fluctuates from being highly abundant to relatively scarce from year to year (Williamson, personal observation). It is therefore reasonable to expect that chemical signals from *T. gratilla* should vary in strength, depending on the population density of *T. gratilla* at that time. Thus the host chemical cue should be somewhat unreliable at times for *G. mineri*. In light of this, it is perplexing that no shrimp were found on other similar species of sea urchins that overlapped in distribution with *T. gratilla*. *H. tuberculata* is consistently abundant in our study sites (Williamson, personal observation; Wright et al., 2005), albeit in lower densities than *T. gratilla* at times. It generally occurs in hollows in the rocky subtidal, potentially allowing substantial protection for any epibionts; a criterion deemed critically important in host choice for *G. mineri* in other areas (Maciá and Robinson, 2009). Moreover, the primary spines on *H. tuberculata* are, on average, roughly three times as long as those of *T. gratilla* (Miskelly, 2002), potentially offering a further increase in protection from predation (Hartney and Grorud, 2002; Tegner and Dayton, 1981).

Several hypotheses can be posed to explain the absence of *G. mineri* on *H. tuberculata*. As host choice is decided prior to physical contact with potential hosts and assuming that *G. mineri* in eastern Australia do move between individual urchins occasionally as suggested by Patton et al. (1985), all hypotheses indicate some form of adaptive associative learning by the shrimp to distinguish the appropriateness of sea urchins as hosts from a distance. First, a difference in the nutritional content or the constitution of pigment cells within the epithelia of the two species of sea urchins may drive this pattern of host use. Patton et al. (1985) suggests that the pigments of *G. mineri* most likely originate from sequestered host pigments. If this were the case, one would expect that the *T. gratilla* epithelium would be significantly more nutritious and/or contain more camouflaging pigments than other potential hosts for *G. mineri* but has yet to be tested.

An alternative hypothesis is that *T. gratilla* may offer greater protection against predators for *G. mineri* through the morphology of the host's spines. Spines of *H. tuberculata* are oval in cross section

and substantially thicker than the spines of *T. gratilla* (Miskelly, 2002), thus potentially making them more difficult for these shrimp to inhabit. Certainly host-specific symbionts in other systems are constrained by the morphology of their host's point of attachment. For example, species of *Geomydoecus*, commonly known as gopher lice, occur in close association with particular species of geomyid rodents (Morand et al., 2000). The lice are extremely specialised and have difficulty surviving on hosts that have larger hair diameters than those of their native host. This is hypothesised to be a result of an increased risk of dislodgement by the lice (Reed et al., 2000).

*G. mineri* showed less interest in *P. indiana* as a host species than *T. gratilla*, despite associating with *P. indiana* in Hawaii (Titgen, 1989) and having similar spines and habitat to *T. gratilla*. As with the Hawaiian species, *P. indiana* in eastern Australia has a white test and spines, and shrimp occurring on this species are not cryptically camouflaged in terms of colour. Maciá and Robinson (2009) suggest that such mismatches with host colour show the relative importance of chemical cues over visual cues for this shrimp. Although no visual signals with *P. indiana* were tested in this study and this hypothesis cannot therefore be discounted, results in our visual experiment suggest that visual signals are important to the shrimp. *P. indiana* is patchily distributed in low densities along the temperate eastern Australia (Rowe and Gates, 1995), but appears more abundant in Hawaii (Titgen, 1989). The difference in the abundance may relate to the historical ability of *G. mineri* to assess the attractiveness of this host species through associative learning in Australia. By remaining stationary in the presence of signals originating from *P. indiana*, the shrimp may merely have been waiting for a familiar signal. The decreased mobility of shrimp in the absence of visual or chemical cues from *T. gratilla* suggest that remaining stationary in the absence of a reliable familiar cue may be a camouflage strategy in itself. These ideas, however, currently remain untested.

There are obvious benefits in this association for *G. mineri*, including nourishment from the epithelium of its host, the provision of a structure that protects it from predators (spines); and the cryptic coloration of *G. mineri* through sequestering ingested pigment cells into their own surface layers. Conversely, the advantages, if any, for *T. gratilla* are much less apparent. Although no damage to *T. ventriosus* by *G. mineri* has been previously reported due to the ability of the shrimp to move around the test and allow the epithelium to regenerate (Patton et al., 1985), the impact of epithelial grazing may depend on the density of its symbionts. Opportunistic predation by the melitid amphipod *Elasmopus levis* on the epithelium of the sea urchin *Lytechinus variegatus* can substantially affect the health of the host and even lead to death (Gibbs et al., 2011). Despite the various benefits for *G. mineri*, individuals residing on *T. gratilla* are exposed to various risks. For example, the urchin's pedicellariae, which are used for protection from predators and fouling organisms, may be fatal to the shrimp if attacked (Campbell and Rainbow, 1977; Mebs, 1984; Nakagawa et al., 2003; but see Guenther et al., 2007).

*G. mineri* did not generally occur in large numbers on *T. gratilla*. Many of those that did occur on *T. gratilla* inhabited the oral surface of its host, or the ambulacral areas where the urchin's primary spines are located. These results are consistent with previous research on both species from other areas of the globe (Maciá and Robinson, 2009; Patton et al., 1985). Moreover, previous authors have noted that if *T. ventriosus* are inverted all *G. mineri* inhabiting the oral surface will migrate towards the aboral surface, which was now underneath (Patton et al., 1985).

A similar study (VandenSpiegel et al., 1998) observed that another shrimp species, *Synalpheus stimpsonii*, regularly occurred in pairs on the underside of its host sea urchin. The abundance and distribution of *G. mineri* on *T. gratilla* has important implications for the reproductive success of the shrimp. There are huge costs for failure to reproduce, hence, and low numbers of shrimp on hosts may suggest issues in locating a mate, particularly if this population has an unequal male to female

ratio as documented for other species of caridean shrimps (Gherardi and Calloni, 1993; Nakashima, 1987).

To date, many aspects of this symbiotic relationship are unknown, and further research should focus on questions such as movement of shrimps over varying time scales, the reproductive mode of the shrimp, and the relative protection that *T. gratilla* offers *G. mini* in comparison with other co-occurring species of sea urchins. In addition, assessing how male and female *G. mini* successfully find their host through chemosensory capabilities and on the nutritional value of the epithelium as some epibiotas select host species on the basis of their quality as a habitat rather than as a source of food (Williamson et al., 2004). Finally, isolation and identification of the particular water-borne chemical cue (s) originating from *T. gratilla* that attracts *G. mini*, along with assessing the uniqueness of the cue in the habitat, would give an indication of the specificity of the cue as an indicator of chemical host detection.

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