An integrative approach to understanding the population structure & dispersal patterns of two commercial octopus species (*Octopus maorum* & *Octopus pallidus*)

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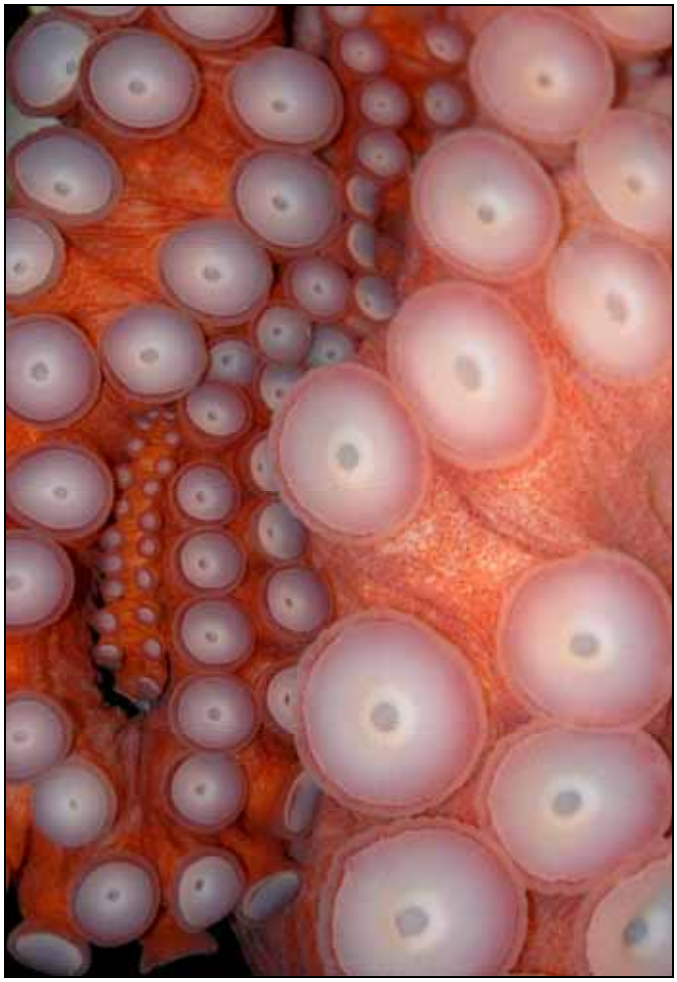

Frontispiece

Declarations

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Statement of Co-authorship

Chapters 2 – 5 of this thesis have been prepared as scientific manuscripts as identified on the title page for each chapter. In all cases experimental design, field and laboratory work, data analysis and interpretation, and manuscript preparation were the primary responsibility of the candidate. However, they were carried out in collaboration with supervisors and co-authors. Contributions of co-authors are outlined below:

Chapter 2

David Belton (CSIRO Exploration and Mining) provided technical and operating support for the nuclear microprobe and assisted with data analysis and presentation. Gretta Pecl and Jayson Semmens (both from the Tasmanian Aquaculture and Fisheries Institute, Marine Research Laboratories; TAFI MRL) contributed to data interpretation and manuscript preparation.

Chapter 3

Gretta Pecl contributed to method development, statistical analyses, data interpretation, and manuscript preparation. Jayson Semmens assisted with the collection and dissection of octopuses from sample sites, and contributed to data interpretation and manuscript preparation. Leonid Danyushevky (Centre for Ore Deposit Research, UTas) gave advice on sample preparation and laser ablation analysis.

Chapter 4

Similar contributions to Chapter 3.

Chapter 5

Jayson Semmens assisted with the collection and dissection of octopuses, and contributed to data interpretation and manuscript preparation. Adam Smolenski (Central Science Laboratory, UTas) provided technical support for the isolation of microsatellites (particularly protocol development) and screening of individuals. Paul Shaw (Royal Holloway University of London) contributed to data interpretation, statistical analyses and manuscript preparation.

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Abstract

The population structure and dispersal patterns of two commercially harvested octopus species, *Octopus maorum* and *Octopus pallidus*, were examined using a combination of natural elemental stylet signatures and genetic microsatellite markers. The early life history strategies of the two species are markedly different: *O. pallidus* produce large well-developed benthic hatchlings (holobenthic) and *O. maorum* produce small planktonic paralarvae (merobenthic). Such differences will influence the species' dispersal potential and population structure and thus resilience to fishing pressure. Both species were collected from several sample sites in Tasmania (Australia), and *O. maorum* was also collected from South Australia (SA) and New Zealand (NZ).

The spatial distribution of elements within octopus stylets (a small internal remnant 'shell'), was investigated using the nuclear microprobe. Proton induced x-ray emission (PIXE) was conducted using the Dynamic Analysis method and GeoPIXE software package, which produced high resolution, quantitative elemental maps of whole *O. pallidus* stylet cross-sections. The analyses indicated that Ca was a suitable internal standard for laser ablation inductively-coupled plasma-mass spectrometry (LA ICPMS), due to its homogeneous distribution and consistent concentration between individuals.

Elemental signatures representing the early life history region of the stylet were used to investigate connectivity and the common origins of adults. Using LA ICPMS stylets were analysed for 12 elements, several of which were excellent spatial discriminators. There was evidence of sub-structuring within the *O. maorum* population despite the species' high dispersal potential. Individuals from an aggregation in south-east Tasmania were particularly distinct and appeared to share a local common

origin. *Octopus pallidus* showed a relatively high level of population structure with all samples appearing distinct from each other, which is in accord with the species' limited dispersal potential. The stylet signatures of *O. pallidus* hatchlings were also analysed from three locations (collected 6 – 10 months prior to the adults), to determine if they could classify adults back to their natal site. Although hatchling signatures showed significant spatial variation, they were unable to be used as markers of natal origin.

Microsatellite markers and morphometrics were also employed to investigate the population structure of *O. maorum*, as they can provide information relevant to longer-term inter-generational structural patterns. Five polymorphic microsatellite markers were isolated from *O. maorum* DNA, and then used to investigate levels of gene flow and genetic structure. Overall, within-sample variability was very high (mean number alleles = 15, mean expected heterozygosity = 0.85). Multi-locus pairwise F_{ST} values revealed a significant level of structuring, which did not fit an isolation-by-distance model of population differentiation. Divergence was observed between most populations, except for SA and the southern Tasmanian populations which were genetically homogeneous, indicating a level of connectivity on a scale of 1,500 km. Morphometrics indicated divergence between Australian and NZ populations. The structural patterns identified can be explained largely in relation to the regional oceanographic features.

This study presents valuable insights into the population structure and dispersal patterns of both a merobenthic and holobenthic octopus species, and will provide essential information for the sustainable management of *O. maorum* and *O. pallidus*. Additionally, this study shows that the targeted elemental analysis of stylets will be a beneficial new tool for examining octopus populations, and that the utilisation of both genetics and elemental signatures is a robust and powerful method to investigate population linkages in marine species.

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General Introduction

POPULATION STRUCTURE – DEFINITIONS & IMPORTANCE

Throughout the scientific literature there is an extensive array of terms used to define a biological population. However, all definitions imply that there is a cohesive process that groups individuals together, and these cohesive processes can be broadly lumped into genetic (reproductive) and demographic (social and behavioural) categories (Waples & Gaggiotti 2006). A key difference between genetic and demographic populations is the level of spatial and temporal integrity by which they are defined. Compared to a demographic population, a genetic population has a high level of integrity, because a very low rate of exchange between individuals is sufficient to maintain genetic homogeneity (Carvalho & Hauser 1994). However, regardless of whether a population is defined as demographic or genetic they are rarely simple and commonly consist of a complex composite of subdivisions.

Intra-specific population differentiation can occur at varying levels, and is determined by the level of connectivity or exchange between individuals (see Fig. 1.1). It can be difficult to apply the concept of structural subdivisions within marine populations as many marine ecosystems lack obvious barriers to dispersal (Waples 1998); however, marine populations often consist of localised sub-populations that are relatively independent and have distinct biological and genetic properties (Gaffney 2000). Metapopulation concepts have been increasingly used to define the range of population structures which consist of partially closed networks of sub-populations, where connectivity occurs across a range of spatial scales (Kritzer & Sale 2004). Recognising such complex population systems is important for improving our understanding of spatial patterns in marine species and thus managing them more effectively.

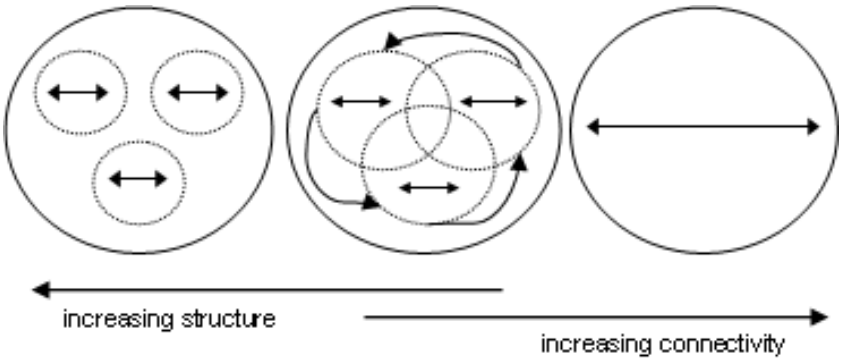

Figure 1.1 Three generalised levels of intra-specific population structure: discreet self-recruiting sub-populations; a complex of partially closed sub-populations (metapopulation); no subdivisions present, individuals disperse and inter-mix throughout the species' distributional range (figure adapted from Kritzer & Sale 2004).

Assessing and preserving structural complexity is crucial for maintaining healthy marine populations, and has been described as one of the 'ten commandments' for sustainable ecosystem-based fisheries management (Ryman et al. 1995; Francis et al. 2007). Population structure determines the spatial scale at which individuals operate within a population and to what extent they are connected (see Fig. 1.1). If a group of individuals form a relatively independent self-recruiting sub-population, for instance, it is logical that it should represent the management unit or scale for assessing population characteristics such as growth rate, age structure, mortality and productivity (Kassahn et al. 2003). Furthermore, understanding connectivity and dispersal is a critical component for designing effective management tools, such as no-take fisheries reserves, which can provide a buffer against overexploitation (Sale et al. 2005). Disregarding population complexity in an exploited population may lead to a decline in stock abundance and the number of localised sub-populations, and thus genetic diversity, which is crucial for maintaining a species' ability to evolve and adapt to environmental change (Carvalho & Hauser 1994; Stephenson 1999). This will not only have negative effects on fishery productivity, but will have large-scale repercussions for respective predator-prey populations and, ultimately, ecosystem stability (Bradbury et

al. 2008). Currently, traditional management practices typically assume, without prior knowledge, that populations are simple and singular entities (Stephenson 1999). It is vital, therefore, that the structure of commercially harvested marine populations are characterised.

POPULATION STRUCTURE & DISPERSAL – NOT A SIMPLE RELATIONSHIP

Dispersal potential (the movement of individuals away from the parental population) is a key factor in shaping the structure of populations (Nathan 2001). In marine species, it is generally assumed that a high capacity for dispersal in the early life history stage results in reduced intra-specific differentiation over smaller spatial scales (Palumbi 1995). For instance, species with planktonic larvae, relative to those with benthic larvae, can achieve thorough mixing of individuals from different source populations over a greater distance (Wilke & Davis 2000). Some studies have shown, through direct comparisons between species with planktonic and benthic larvae, that the level of population differentiation is congruent with larval dispersal potential (Wilke & Davis 2000; Lambert et al. 2003). However, more and more studies are revealing that population structure does not always reflect a species' dispersal capabilities. Population structuring has been observed in many species which have a high capacity for dispersal (see Palumbi 1995; Palumbi 2004 for examples). Such exceptions may be caused by a whole range of factors: ocean currents and bathymetry; behaviour, such as philopatry, habitat preferences and sex-biased dispersal; and historic events such as sea level change, extinction and recolonisation, can all create subdivisions within a population (Avise 2004). Although seemingly less common, a number of studies have revealed unexpected genetic homogeneity in species with apparently low dispersal potential (Palumbi 1995), with algal rafting as a potential vector for long-distance dispersal (see Highsmith 1985 and references therein). Exceptions which challenge the generalised paradigm (ie. increased dispersal equals decreased structure) highlight the inherent complexity and variation

associated with population structure and connectivity regardless of predicted dispersal potentials. This emphasises the need to conduct species- and fisheries-specific assessments of population structure rather than relying on predictions or extrapolations from different stocks or related taxa (Thorpe et al. 2000).

TOOLS FOR INVESTIGATING POPULATION STRUCTURE

There have been numerous methods used to study the movement patterns and population structure of marine species, including morphometric measurements of soft and hard body parts, differences in life history traits and population characteristics, parasite abundance and diversity, external and electronic tags, natural and artificial chemical signatures within biomineralised structures, and various genetic markers (Begg & Waldman 1999; Palumbi 2004; Semmens et al. 2007). To keep within the scope of the thesis, this Chapter will focus on natural chemical signatures, morphometrics and genetic markers.

Molecular markers are heritable genetic characters (ie. proteins or segments of DNA) which have multiple variants or polymorphisms (alleles); this variation can be used to examine the genetic relationships between individuals, populations and taxa (Sunnucks 2000). The use of polymorphic genetic markers in marine biology has proliferated over the last decade, and have been shown to be valuable tools in the fields of population biology and fisheries science (Gaffney 2000; Thorpe et al. 2000; Shaw 2002). Genetic markers can determine the level of exchange or reproductive isolation between conspecific individuals over an inter-generational time-scale, thus providing information on longer-term structural trends within a population (Purcell et al. 2006; Semmens et al. 2007). There are a variety of molecular markers used to study genetic variation at the intra-specific level, including allozymes, sequences of mitochondrial DNA regions, and microsatellites (see Sunnucks 2000 for

review). Microsatellite markers are short tandem repeat sequences (usually di-, tri- or tetra-nucleotides) of nuclear DNA, and, as a consequence of their rapid mutations rates, can be highly polymorphic (ie. have several variations in repeat number) (Avise 2004). This hypermutability makes them advantageous for determining finer scale structuring and resolving structure in species with low levels of genetic variability (Shaw et al. 1999; Chistiakov et al. 2005). Microsatellites, therefore, are ideally suited to marine species, as they commonly exhibit high gene flow and limited genetic structure due to fewer physical barriers to dispersal and high dispersal potentials of eggs and larvae (Ryman et al. 1995; Palumbi 2004).

Analysing phenotypic variation in morphological characteristics, which can include soft body parts or hard structures such as otoliths, is one of the simplest methods to examine structure at the intra-specific level (see Swain & Foote 1999 for examples). However, a main disadvantage of morphometric data is that phenotypic plasticity may have environmental, rather than genetic, origins, which may lead to the identification of sub-populations which in reality are highly connected. To disentangle the causal factors of variation, such studies, therefore, can be particularly useful once combined with genetics (eg. Begg & Waldman 1999; Milton & Chenery 2001; Kassahn et al. 2003).

Natural chemical signatures are based on the premise that the concentration and spatial distributions of elements within biomineralised structures are influenced by the chemical, biological and physical environment. Biomineralised structures with temporally-related incremental growth, such as teleost otoliths and mollusc shells, incorporate elements into their matrix chronologically, thus potentially providing information on an individual's movement history (Elsdon & Gillanders 2005). The environmental time-recording properties of these structures can be exploited using microprobe analysis, which allows temporally-specific microstructural regions of interest, such as the pre-hatch region, to be targeted and their elemental 'signature' analysed. Microprobe analysis is

more powerful than solution-based analysis, as the latter involves dissolving structures whole and thus interpreting a signature that reflects the entire life cycle of the individual. There are a variety of microprobe-based instruments; however, the most common used in marine biology is laser ablation inductively-coupled plasma mass spectrometry (LA ICPMS). Laser ablation involves the targeted ablation of a particular area using a pulsed laser beam of variable diameter, the released material is then transported to the ICPMS via gas flow for analysis of selected chemical constituents (see Mokgalaka & Gardea-Torresdey 2006 for more details).

INTEGRATING MULTIPLE TECHNIQUES

The most robust inferences on population structure are achieved through the integration of multiple techniques, as different techniques can identify structural characteristics at different spatial and temporal resolutions (Begg & Waldman 1999; Feyrer et al. 2007). Although the number of integrated studies are currently limited, the simultaneous utilisation of both genetic markers and elemental signatures has provided both corroborative and additional information on population structure in teleosts (see Miller et al. 2005; Ashford et al. 2006; Feyrer et al. 2007). Genetic markers can be valuable for investigating levels of dispersal and connectivity relevant to long-term inter-generational population changes, however, they may have relatively little ability in identifying more complex metapopulation structures (Thresher 1999; Waples & Gaggiotti 2006). This is because only a very low exchange rate (as little as 1 % of individuals per generation) is required to maintain genetic homogeneity (Carvalho & Hauser 1994) – a major limitation of genetic markers. In comparison, natural elemental signatures are directly related to the movement of the individuals analysed, and thus reflect the present movement patterns of a population. Therefore, they may reveal distinct sub-populations that are otherwise genetically homogenous due to periodic low-level mixing or

mixing during the early stages of the life cycle (Campana & Thorrold 2001; Arkhipkin et al. 2004). By integrating techniques, such as genetics and elemental analysis, both demographic and genetic population characteristics can be defined, allowing for a more holistic approach to the spatial management of marine species.

CEPHALOPODS – WHY STUDY THEM?

Cephalopods are an ecologically important and highly successful group, as shown by their global geographical distribution, trophic importance and large contribution to global biomass and productivity throughout all marine habitats (Boyle & Boletzky 1996; Lipinski 1998). They are voracious, versatile and opportunistic predators consuming a variety of crustaceans, fish and molluscs (Rodhouse & Nigmatullin 1996), and are an important prey group to large teleosts, sharks, marine birds, seals and cetaceans (Boyle & Rodhouse 2005). Cephalopods have unique life history traits, including rapid and non-asymptotic growth, short life spans, and high individual variability (Clarke 1996; Moltschaniwskj 2004). However, this makes them sensitive to ecological perturbations, caused by fishing activity or environmental change, which can trigger dramatic and rapid inter-annual fluctuations in cephalopod abundance (Boyle & Boletzky 1996). Due to their high feeding rates and unusually high trophic efficiency (that is, the ratio of food consumed to somatic weight gained) even minor changes in cephalopod biomass could potentially have profound flow-on effects to predator-prey populations (Clarke 1996; Boyle & Rodhouse 2005).

Worldwide, the majority of finfish and shellfish fisheries are either commercially extinct, declining, or at best remaining stable (Caddy & Rodhouse 1998; Thorpe et al. 2000). On the other hand, cephalopod fisheries have undergone a dramatic global increase in catch, and continue to remain, in many regions, a highly exploitable resource. Between 1990 and 1999 cephalopod catch levels increased by 42 % from 2.4 to 3.4 million

tonnes per year; in contrast, total fishery catch increased by less than 9 % (Boyle & Rodhouse 2005). Although a large proportion of harvested cephalopod stocks are squid, octopus catch levels have increased significantly over the past 50 years, with over 350,000 tonnes landed globally in 2005 (FAO 2008) (see Fig. 1.2). Their distinctive life histories, inherent vulnerability to population fluctuation and significant large-scale linkages between other trophic levels highlights the importance of gaining further insights into their population biology and developing cephalopod-specific management strategies for current and emerging commercial species.

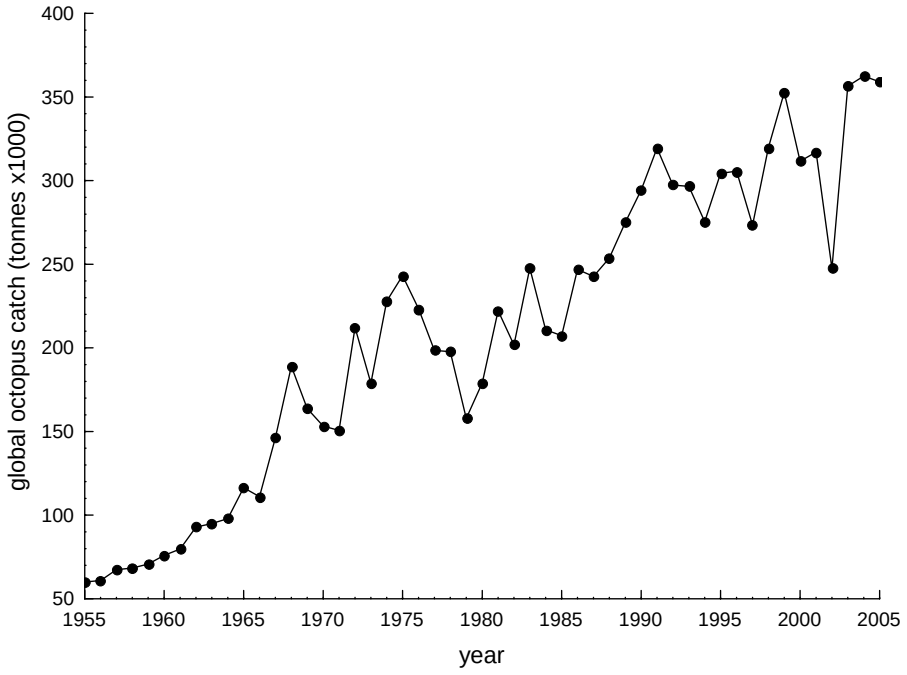

Figure 1.2 Global octopus catch from 1950 – 2005 (FAO 2008).

POPULATION STRUCTURE IN CEPHALOPODS – EXTENT & METHODS

Various methods have been adopted to examine the movement patterns of cephalopods, including tag recapture methods, electronic tags, morphometrics and genetic markers, with emerging research into parasites, and artificial (ie. fluorescent compounds) and natural (ie. trace elements) chemical signatures (see Semmens et al. 2007 for review). However,

overall, there is little known about the population structure and dispersal patterns of most cephalopod species. Genetic markers have been used to investigate the population structure of several species (mostly squid); however, a common problem is that cephalopods tend to have very low levels of genetic variability (Thorpe et al. 2000). Consequently, allozyme and mitochondrial markers have had limited success (Shaw et al. 1999) compared to microsatellites, which have shown greater power in resolving genetic structuring within cephalopods (eg. Perez-Losada et al. 2002; Buresch et al. 2006). Although more commonly used to investigate inter-specific relationships, multi-variate morphometrics have been successfully used to resolve intra-specific differentiation (Vega et al. 2002; Kassahn et al. 2003). Natural chemical signatures is a relatively new field in cephalopod research; however, utilising statoliths, solution-based methods and LA ICPMS have been used to examine the population structure of squid (Arkhipkin et al. 2004; Zumholz et al. 2007).

Only a handful of studies investigating population structure in octopus populations have been conducted, and they are predominantly restricted to *Octopus vulgaris*. These are largely genetic based (Murphy et al. 2002; Oosthuizen et al. 2004; Cabranes et al. 2008), however, population characteristics (Oosthuizen & Smale 2003) and morphometrics (Hermosilla 2004) have also been utilised. A key factor limiting further genetic studies on a wider range of species is that microsatellite markers, which are species-specific, have only been developed for *O. vulgaris* (see Greatorex et al. 2000). Elemental analysis has yet to be applied to octopuses; primarily due to the lack of a suitable increment-based biomineralised structure. Octopus statoliths, unlike other cephalopod statoliths, show no internal differentiation (Jackson 1994). However, octopus stylets, an internal 'shell' unique to Octopoda, have been shown to be a promising increment-based structure for the application of targeted elemental analysis (Doubleday et al. 2006).

STUDY SPECIES – *OCTOPUS MAORUM* & *OCTOPUS PALLIDUS*

Octopus maorum (Hutton, 1880) is a very large species growing up to 15 kg, which is common throughout southern Australian and New Zealand temperate and sub-Antarctic coastal waters (Stranks 1996) (Fig. 1.3). It inhabits depths up to 549 m and prefers rocky reefs and beds of seagrass or seaweed (Stranks 1998). A particular mystery is the occurrence of an exceptionally large year-round aggregation of *O. maorum* in Eaglehawk Bay, southeast Tasmania, Australia – the only known location of this kind of phenomenon worldwide (Grubert et al. 1999). The reason for the aggregation and the source of the animals remain unknown, and despite the vast majority of individuals being fully mature, there is no evidence of spawning (Grubert & Wadley 2000). The Eaglehawk Bay aggregation supplies small but intensive recreational and commercial fisheries. Approximately 4,000 individuals were captured from within the small very narrow bay by commercial fishers in the 2001/02 summer (Department of Primary Industries and Water, unpubl. data). Significant quantities of *O. maorum* are also sold as bycatch by rock lobster fishers in South Australia, Tasmania and New Zealand.

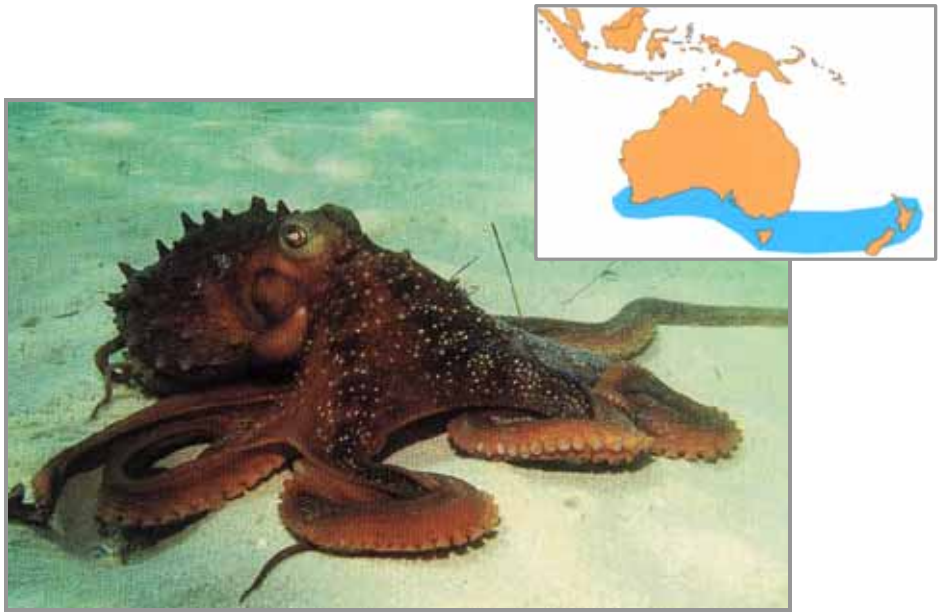

Figure 1.3 *Octopus maorum* (image from: Norman 2000) and its distribution (map from: Norman & Reid 2000).

Octopus pallidus (Hoyle, 1885) is a small species growing up to 1 kg which is commonly found throughout temperate inshore south-east Australian waters (Stranks 1996) (Fig. 1.4). It occurs on sandy substrates up to 275 m, commonly among sponges, seagrass, and ascidians (Stranks 1988). *Octopus pallidus* is currently harvested through a permit-based unbaited-pot fishery in Bass Strait, Tasmania. The relatively intensive fishery has undergone growth in the last several years, with 20,000 pots deployed simultaneously and an annual catch of approximately 80 tonne (Ziegler et al. 2007).

Figure 1.4 *Octopus pallidus* (image from: Norman 2000) and its distribution (map from: Norman & Reid 2000).

The early life histories of *O. pallidus* and *O. maorum* are strikingly different (see Table 1.1), which could have important implications for population structure and larval dispersal patterns. *Octopus pallidus* are holobenthic, producing a small number of adult-like hatchlings that are large, well-developed and benthic. In contrast, *O. maorum* are merobenthic, producing a large number of hatchlings or 'paralarvae' that are initially small and planktonic before settlement to the seafloor. These dichotomous strategies could have a profound impact on the way stocks will respond to

fishing pressure and stock depletion. For example, *O. pallidus* may be more vulnerable to localised depletion due to its potentially limited dispersal, whereas *O. maorum* may be more vulnerable to overfishing due to its potentially high degree of inter-annual variability (as a result of planktonic dispersal and high paralarval mortality).

Table 1.1 Comparison in life history traits between *Octopus pallidus* and *Octopus maorum* (see Stranks 1996; Grubert & Wadley 2000; Leporati et al. 2008).

Life history traits	*Octopus pallidus*	*Octopus maorum*
egg length	11 – 13 mm	4 – 6 mm
no. eggs per spawning event	~ 400	~ 100,000
hatchling size	20 mm	7 mm
hatchling 'life style'	benthic	planktonic

The sustainable level of production for the *O. pallidus* and *O. maorum* fisheries is not known and no defined management plans are in place, which is primarily due to the lack of basic biological information. This situation reflects a very similar trend for other octopus species worldwide, with many constituting large and economically important fisheries (eg. Arreguín-Sánchez et al. 2000; Rocha & Vega 2003; Rigby & Sakurai 2005; Cabranes et al. 2008).

THESIS AIMS

This project aims to increase our understanding of population structure and dispersal of both merobenthic and holobenthic octopuses, through the use of microsatellite DNA markers, basic morphometrics and natural stylet elemental signatures. In relation to the methods adopted there are three key objectives: to isolate and characterise several polymorphic microsatellite markers from octopus DNA, develop the methodology for the analysis of stylet elemental signatures and assess the potential utility of this novel tool for studying octopus populations. On a

broader conceptual level this project also seeks to compare the efficacy of the techniques employed and investigate the use of holistic integrative approaches to resolving population linkages in marine species. This thesis is primarily focused on understanding structure and dispersal within the context of commercially harvested populations and sustainable fisheries management.

THESIS STRUCTURE

This thesis is arranged into four data Chapters (2 – 5) and a general discussion Chapter (6). The key aims and themes addressed in each data Chapter is outlined below:

The main aim of the first data Chapter was to determine the suitability of stylets for LA ICPMS. This was a crucial component for the commencement of Chapters 3 and 4. More specifically, this Chapter identified an element which can be used as an internal standard – an essential requirement for LA ICPMS. Proton induced x-ray emission (PIXE) was conducted using the Dynamic Analysis method, which produced quantitative elemental maps of stylet cross sections. To broaden our understanding of stylet structure and stoichiometry, general compositional analyses were also included in this Chapter. This research is published in: *Nuclear Instruments and Methods in Physics Research B*. 2008. 266: 67 – 72 (see see Published Papers).

In the second data Chapter, targeted elemental analysis (LA ICPMS) was used to analyse the early life history region of the stylet. The potential of this novel technique to examine dispersal and population structure was explored in a merobenthic octopus population (*O. maorum*). This Chapter provides the first insights into the population structure and dispersal of an octopus species through the successful application of single and multi-elemental stylet signatures. This research is published in: *Marine Ecology Progress Series*. 2008. 360: 125 – 133 (see Published Papers).

The third Chapter builds upon the methods developed in the second Chapter, utilising stylet elemental signatures from the pre-hatch region of the stylet to investigate the population structure and dispersal of a holobenthic octopus population (*O. pallidus*). Additionally, elemental signatures from hatchling stylets were analysed to determine if they can be matched against the adult signatures and thus identify their natal origins. This Chapter is the first population-level study on a holobenthic octopus species. This research is published in: *Marine Ecology Progress Series*. 2008. 371: 1-10 (see Published Papers).

The last data Chapter employs genetic markers and morphometrics to determine the longer-term structural trends within the *O. maorum* population. Highly polymorphic microsatellite markers were isolated from *O. maorum* DNA and then used to investigate levels of gene flow and genetic structure within the population. A complex structural pattern was identified, which is explained largely in relation to the regional oceanographic features. This research is published in: *Marine Biology*. 2009. 156: 1183-1192 (see Published Papers).

2

Quantitative elemental imaging of octopus stylets using PIXE & the nuclear microprobe

This research is published as:

Doubleday Z, Belton D, Pecl G, Semmens J. (2008). Quantitative elemental imaging of octopus stylets using PIXE and the nuclear microprobe. *Nuclear Instruments and Methods in Physics research B.* 266: 67-72

ABSTRACT

Targeted microprobe-based analysis of chemical elements incorporated within the hard biomineralised structures of marine organisms has provided unique insights into the population biology of many species. The spatial distribution of elements within octopus stylets was investigated, using the nuclear microprobe, to assess their potential for determining dispersal and population structure in octopus populations. Proton induced x-ray emission (PIXE) was conducted using the Dynamic Analysis method and GeoPIXE software package, which produced high resolution, quantitative elemental maps of whole stylet cross-sections. Ten elements were detected within the stylets which were heterogeneously distributed throughout the microstructure. Although Ca decreased towards the section edge, this trend was consistent between individuals and Ca remained homogeneous in the inner region of the stylet, thus appearing as a suitable internal standard for future microprobe analyses. Additional analyses used to investigate the general composition of the stylet structure suggested that they are amorphous and largely organic, however, there was some evidence of phosphatic mineralisation. In conclusion, this study indicates that stylets are suitable for targeted elemental analysis, although this is currently limited to the inner hatch region of the microstructure.

INTRODUCTION

The quantitative and spatial distribution of chemical elements within the hard biomineralised structures of marine organisms provide a 'story' of an individual's life history and movement patterns, as they commonly reflect changes in behaviour, physiology and the surrounding environment. However, such chronological information can only be accurately measured if the structure contains regular temporally- or age-related growth increments. Due to their crumbly conglomerate structure and lack of growth increments (Jackson 1994; Lombarte et al. 2006) octopus statoliths, unlike other cephalopod statoliths or teleost otoliths, are of little use for ageing and therefore time-specific elemental studies. On a global scale, there are many unresolved questions regarding the population biology and dispersal patterns of octopuses, and this is largely due to the lack of available methods to study octopus populations. The development of such methods will be crucial for increasing our understanding of octopuses and enable the sustainable management of increasingly exploited commercial species.

Stylets (also known as vestigial shells) are a little known structure unique to Octopoda and are thought to represent a remnant shell (Bizikov 2004). Stylets consist of a pair of fine, cartilage-like rods embedded within the dorsal mantle musculature. Their composition has been suggested to be based on a calcium phosphate compound, such as hydroxyapatite (Napoleão et al. 2005) or chitin (Bizikov 2004). The stylet microstructure of *Octopus pallidus* has been found to have distinct concentric regions, a visible pre-hatch nucleus, and age-related growth in the form of daily growth increments (Doubleday et al. 2006). Due to this microstructure stylets, like squid statoliths (Semmens et al. 2007; Zumholz et al. 2007), are likely to incorporate elements from the environment on a chronological basis, and therefore, may be a useful tool to address ecological questions on the dispersal patterns of both juvenile and adult octopuses.

Laser ablation inductively-coupled plasma-mass spectrometry (LA ICPMS) is one of the most widely employed microprobe techniques for examining biomineralised structures on a temporal level, as it requires relatively little sample preparation and is capable of measuring elements to the trace level (Campana et al. 1997). However, LA ICPMS requires compositional information and standard materials to accurately measure elemental concentrations. For example, internal standardisation, a calibration method used for the analysis of many biological materials, requires the presence of one element (usually Ca) of known concentration which is homogenously distributed throughout the microstructural region of interest (Mokgalaka & Gardea-Torresdey 2006).

The nuclear microprobe (NMP) is a powerful microanalytical tool for investigating the distribution of minor and trace elements in biological materials (Przybylowicz et al. 1999). Proton induced x-ray emission (PIXE) is a reliable and effective NMP-based technique, which quantitatively maps the spatial distributions of elements within a structure (Lipinski et al. 1997). PIXE analysis is also standardless, and therefore does not require compositional information or the use of a standard (Ryan et al. 2002). Although the use of the NMP for analysing the biomineralised structures of marine species is not common, studies have included the analysis of squid statoliths (Lipinski et al. 1997), fish otoliths (Zenitani et al. 2003), octopus statoliths (Ikeda et al. 1999), and octopus stylets (Napoleão et al. 2005).

This is the first time octopus stylets have been analysed with the CSIRO-GEMOC NMP (CSIRO Exploration and Mining) using PIXE and the Dynamic Analysis method of analysis, which allows for simultaneous multi-elemental analysis and quantitative imaging at a high spatial resolution (down to 1.8 μm) and sensitivity (detection limits have been recorded at 0.2 ppm) (Ryan et al. 2002). Standard comparisons, using accepted reference and secondary standards, have shown that the standardless PIXE method has an accuracy level of 5 – 10 % for major and trace elements (Ryan 1995). The Dynamic Analysis method produces 'true'

quantitative images of the whole section which are resolved of elemental overlaps, background-subtracted, free of artefacts and generated in real-time (Ryan 2000). This capability will enable the spatial distribution of elements of whole stylet sections to be quantitatively mapped. In comparison, the study by Napoleão et al. (2005) examined the elemental distributions by targeting the proton beam at a selected number of single 2 – 3 μm points across the section of the stylet. Using the Dynamic Analysis method this study assesses the potential of stylets as environmental time-recorders and the suitability of Ca as an internal standard for LA analysis. Furthermore, to broaden our understanding of the stylet structure and stoichiometry, general compositional analyses (including x-ray diffraction and infrared spectrometry) were also be conducted.

MATERIALS & METHODS

General composition analyses

General composition analyses were performed on stylets collected from adult *O. pallidus* sourced from the commercial fishery in Tasmania in October 2005. Powder x-ray diffraction was conducted to determine mineral composition (Mineral Resources Tasmania, Australia). The air dried stylet samples were ground to approximately 10 – 75 μm and pressed into a 25 mm sample holder. The samples were run on an automated Philips X-ray System and analysed with Diffraction Technology software. To determine total inorganic content 'loss-on-ignition' (combustion of organic material) was also conducted (Mineral Resources Tasmania, Australia). Secondly, infrared spectroscopic analysis was conducted on the stylet with most of the outer sheath removed (see Doubleday et al. 2006 for more details on the microstructure). The sheath was removed to help identify, more clearly, the potential discriminating peaks characteristic of the calcified portion of the stylet. A crushed sample was mixed with anhydrous KBr and pressed into a 7 mm diameter pellet. The analysis was performed at 4 cm^{-1} resolution using a Fourier Transform Bruker IFS66

infra-red spectrometer (Central Science Laboratory, University of Tasmania).

Nuclear microprobe analysis

Five stylets were sourced from adult *O. pallidus* also collected from the commercial fishery. The mature adults were of similar size ranging from 550 – 760 g. Two 'juvenile' stylets were also sourced from five month old aquaria-reared *O. pallidus* in January 2006. These juveniles were reared in a natural seawater flow-through system with a simulated natural temperature regime at the Tasmanian Aquaculture and Fisheries Institute, Hobart, Australia. All stylets were removed from fresh mantle muscle and air dried for 48 hours. To remove excess tissue from the juvenile stylets they were soaked in a mixture of 30 % H_2O_2 buffered with NaOH for 24 hours, and then rinsed thoroughly in ultra-pure water (Milli-Q) prior to drying. Transverse stylet sections, 3 – 4 mm in length, were cut from the post-rostral zone of the stylet, proximal to the bend, using Teflon-coated razor blades (see Doubleday et al. 2006). The stylet sections were mounted vertically in a 25 mm disc of epoxy resin, which was oven dried for two hours at 60˚ C. The epoxy disc was ground using 1500 grit carborundum paper lubricated with ultra-pure water until all sections were exposed and then polished using 0.3 µm alumina powder on a suede polishing disc. Once polishing was complete the mounts were ultra-sonicated for five minutes in ultra-pure water and then rinsed further to remove surface contaminants before being allowed to air dry. To prevent charge build up each disc was carbon-coated using a standard sputter deposition technique.

Individual specimens to be scanned were viewed under a light microscope and then the respective x/y coordinates were digitised and subsequently translated to microprobe stage coordinates to permit the precise location of the beam for analysis. During analysis, the beam was rastered electrostatically in the y-direction while the stage was typically stepped in 1.6 µm increments in the x-direction (scan speed differed on

some samples and was varied to optimise beam current integration). X-ray data was captured using a Canberra germanium detector.

The analyses were performed with three MeV protons using the CSIRO-GEMOC NMP and utilising PIXE. Beam spot size was nominally 1 – 2 μm using beam currents of 3.5 nA. A 47 μm Al filter was used to reduce the intensity of the Ca K x-ray lines while allowing for the simultaneous measurements of Ca and trace elements. Two sections were scanned using a 200 μm Al filter, this improved detection of heavier elements but suppressed Ca more than anticipated and were therefore not included in the final analysis. Scan size was determined according to the size of the individual specimens. The maximum scanned region was 1 x 1.5mm (412 x 591 pixels). True quantitative elemental maps were developed with a spatial resolution of 2.5 μm using a rapid matrix transform method called Dynamic Analysis (incorporated within the GeoPIXE II software) (Ryan 2001), which directly relates x-ray yields obtained from the PIXE spectra to elemental concentrations (Przybylowicz et al. 1999). A multi-element red-green-blue (RGB) image was also produced (in this study green was replaced with black for easier visualisation on a white background). Multi-elemental images, although quantitative, are primarily used to examine spatial distributions due to the difficulties associated with producing a concentration scale (additional colours are produced where elements overlap). Images were generated and analysed using the GeoPIXE II software package.

To determine whether Ca concentration varied significantly between individuals elemental values were taken every 5 – 7 μm along a transect line, which ran from edge to edge across the centre of each stylet cross-section, resulting in 40 – 90 data points (depending on cross-section width) per individual. A one-way analysis of variance (ANOVA) was then conducted on three individuals to determine if patterns of Ca distribution varied significantly. One juvenile was not included in the analysis due to a detector artefact (variation in x-ray absorption caused by the stylet section

being misaligned within the epoxy), resulting in unreliable values in a portion of the image. To further examine the relationship between Ca and Br distributions, elemental values obtained from the transect line of one adult was plotted on a scatter graph.

RESULTS & DISCUSSION

No minerals were identified in the stylets by x-ray diffraction, and as such the stylets were therefore classified as amorphous. Loss-on-ignition showed a total inorganic content of 35 %, although a portion of this may be due to the halite (salt) found in the structure from the x-ray diffraction. The infrared spectra showed amide I and II bands suggestive of α-chitin, which forms complexes with proteins in invertebrates (Galat et al. 1979; Rinaudo 2006) (Fig. 2.1). The spectra also indicated the presence of calcium phosphate. Napoleão et al. (2005) found that although Ca and P both decreased from the core region to the edge of the stylets of *O. vulgaris*, the P/Ca ratio along all sections remained consistent between individuals, suggesting that the major mineral constituent is a calcium phosphate compound, such as hydroxyapatite. Although our data suggests the presence of some calcium phosphates, it also indicates that stylets are largely organic in nature and that any mineralised component consists of poorly crystallised or uncrystallised material.

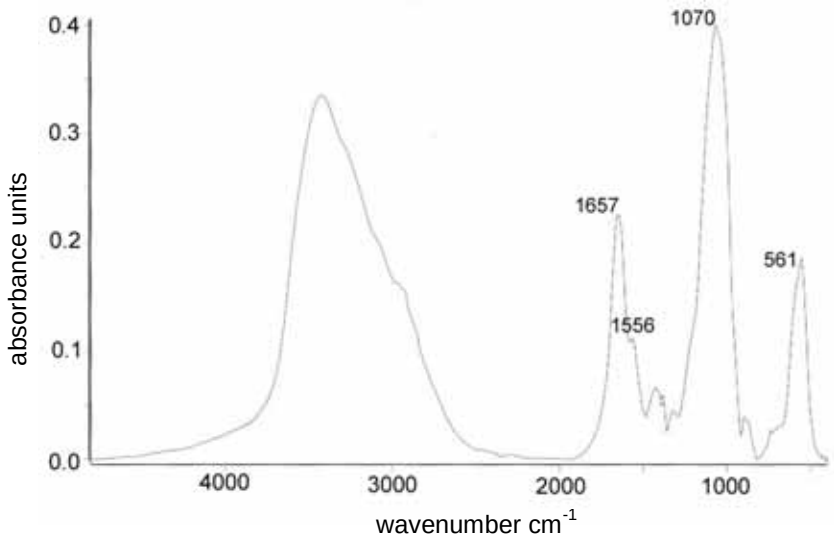

Figure 2.1 Infrared spectra of a stylet with the outer sheath removed. Amide bands I and II at 1657 and 1556 cm^{-1} suggest the presence of chitin, and the peaks at 1070 and 561 cm^{-1} suggest the presence of calcium phosphate.

A suite of differentially incorporated elements were detected within the stylet microstructure (Table 2.1). Calcium, Sr, Zn, Fe, Br, Mn, and Cu were detected in all sections and Co, Rb, As, Ni, and Ba were detected in much lower levels and in less than half of the sections. Some elemental concentrations varied between adult and pre-hatch regions of the microstructure (eg. Ca, Sr, Mn, and Ni), and others varied more significantly between individuals (eg. Zn, Cu, Co, and Fe). Such heterogenous distributions suggest, as stylets exhibit temporally-related concentric growth, that elemental concentrations vary on a temporal scale in relation to changes in the surrounding environment and an individual's physiology. Stylets from the juvenile octopuses had significantly more Zn than the adult stylets (Fig. 2.2). This may be due to the different water chemistry to which the juveniles were exposed and/or different physiological requirements due to faster growth rates and greater metabolic needs. In cuttlefish the assimilation efficiency of Zn (important for enzymatic function) was 22 % greater in juveniles than adults (Villanueva & Bustamante 2006). Such elemental enrichment has also been observed within the otolith core regions of a variety of fish species (Ruttenberg et al. 2005). Discrete 'particles' of Zn

and Fe were observed within the microstructure; however, they are assumed true components of the structure rather than contamination as they appeared to be contained within concentric regions (see Fig. 2.3a). There is evidence of possible contamination on the edges of some sections. In one sample, an area of very high Fe concentration was observed on the edge of the section, possibly being a fragment of a blade that was used to cut the stylet. This highlights the importance of careful section preparation if outer regions are to be targeted for trace elemental analysis.

Table 2.1 Average concentration (ppm), statistical error (based on total number of x-ray counts detected at each pixel) and detection limits (99 % confidence; DL) of elements identified within a randomly selected 50 µm circle area of four individuals analysed. a) = area selected within core 'pre-hatch' region, b) = area selected within outer 'adult' region. Dash (-) indicates elements which were not detected.

element	adult 1			adult 2			Juvenile 1			juvenile 2		
	ppm	error	DL	ppm	error	DL	ppm	Error	DL	Ppm	error	DL
Ca a	128,321	1,653	725	122,539	2,497	1,807	129,616	1,148	716	146,237	1,102	724
b	114,915	1,432	614	107,398	2,294	1,700						
Sr a	6465	163	109	5957	111	50	7,222	99	42	7,480	118	37
b	5721	141	92	4949	106	48						
Zn a	157	18	26	114	9	14	65	7	14	88	8	13
b	140	16	22	121	10	13						
Fe a	72	19	26	31	14	23	-	-	-	-	-	-
b	57	16	22	38	13	20						
Co a	58	13	19	26	9	15	-	-	-	-	-	-
b	39	11	17	26	8	12						
Mn a	140	31	48	92	27	38	73	18	31	68	21	31
b	129	27	37	71	21	33						
Ni a	38	15	25	31	8	16	-	-	-	-	-	-
b	-	-	-	-	-	-						
Cu a	46	13	26	25	7	14	-	-	-	-	-	-
b	42	11	22	29	7	13						
As a	-	-	-	-	-	-	38	6	12	29	6	12
Rb a	-	-	-	-	-	-	81	19	36	100	20	27

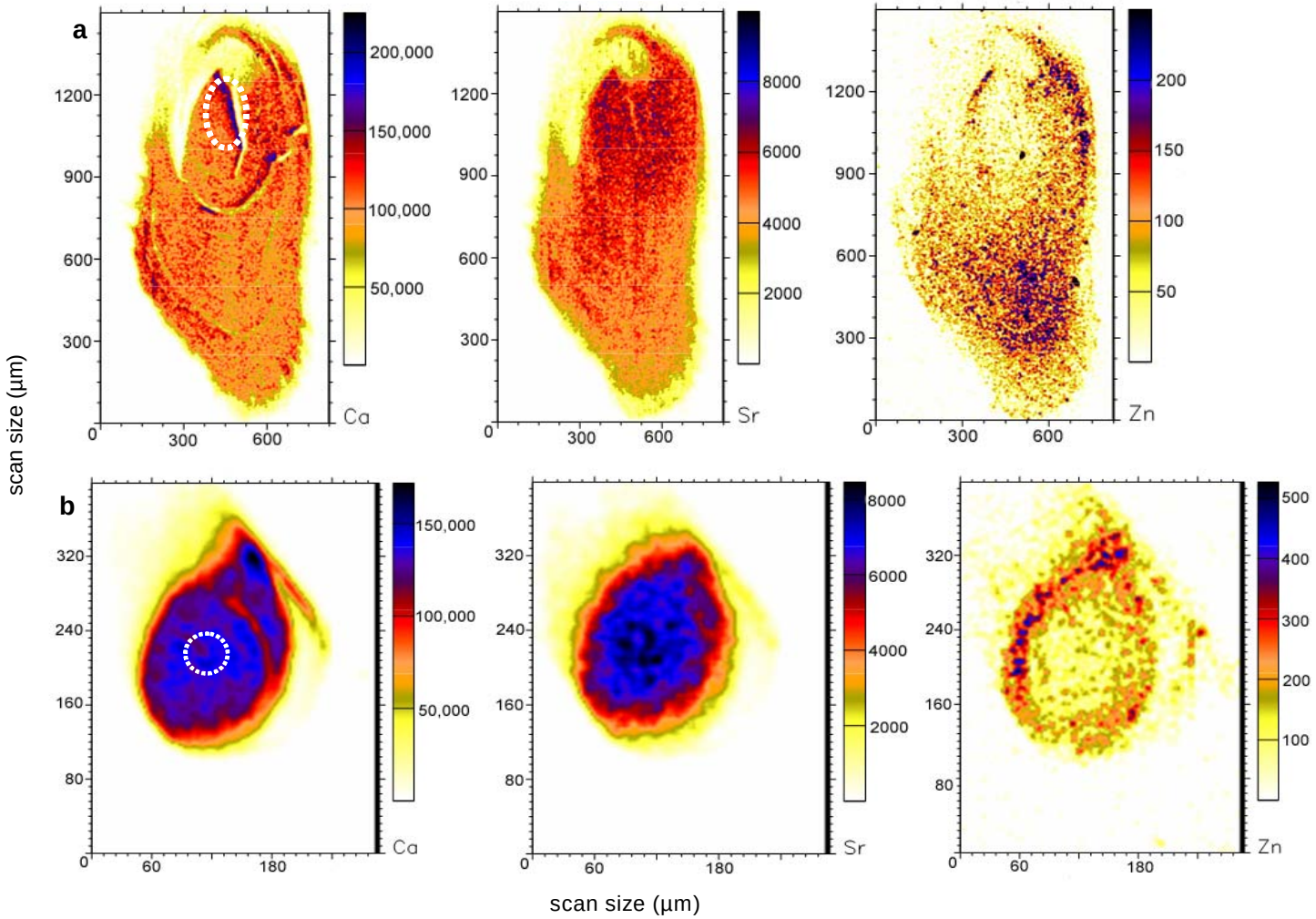

Figure 2.2 Elemental images of two stylet cross-sections showing distributions of Ca, Sr, and Zn, a) = 'adult' stylet, b) = 'juvenile' stylet. Dashed circles indicate approximate location of the pre-hatch region. Scale bars represent concentration (ppm).

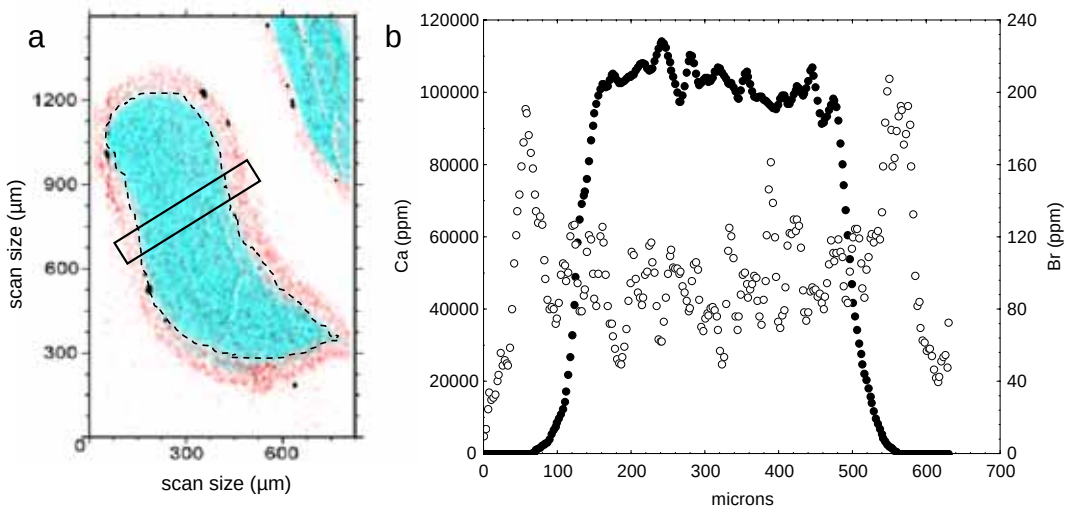

Figure 2.3 a) Multi-element image of a stylet cross-section (Ca = blue, Br = red, Fe = black). Dashed line indicates approximate position of the outer sheath. b) Concentration profiles of Ca (●) and Br (○) along a transect region outlined in Figure 2a.

There was a close relationship between Ca and Sr in all sections, with both elements decreasing from the pre-hatch region to the edge of the section (Fig. 2.2). A multi-element image revealed negligible Ca yield in the stylet's gelatinous outer sheath, which also appears to be a region of relatively high Br concentration (Fig. 2.3). Although the Br is probably the result of saltwater evaporation, the lack of Ca suggests that this region is largely organic. Zinc showed an inverse relationship to Ca and Sr, showing a higher concentration in the outer regions of the section (Fig. 2.2). The trends observed for Ca, Sr and Zn were also found in the adult stylets of *O. vulgaris* (Napoleão et al. 2005). Such patterns may give insight into life cycle and diet changes of the individual, and movement between different environments, on both a long- and short-term scale. Such similar trends between species with differing life histories (*O. vulgaris* are merobenthic whereas, *O. pallidus* are holobenthic) suggest they are the result of physiological processes. However, the physical and biological environment also significantly influences relative concentrations of elements in cephalopods. Although the number of studies are limited, diet and temperature has been found to significantly influence Ba concentrations in cuttlefish statoliths (Zumholz et al. 2006; Zumholz et al. 2007), and

temperature has shown to negatively correlate with Sr concentration in squid statoliths (Arkhipkin et al. 2004). Variation of such parameters on a geographic level, therefore, is likely to provide a useful indicator of a population's movement and dispersal history.

Although stylets are not composed of $CaCO_3$, like the majority of biomineralised structures used for laser ablation studies, Ca still appears a suitable element for an internal standard. Table 2.1 indicates that Ca concentration within the core region of the microstructure (which is about 13 %) only varied by 3 %, regardless of stage of maturity and collection location. Although Ca decreased towards the edge of section and disappeared altogether in the sheath, this trend was relatively stable and consistent between individuals. The transect data showed that Ca was not significantly different between individuals (ANOVA: $F_{2, 186} = 0.92$, $p = 0.40$). In contrast to the 40 % Ca standard used for $CaCO_3$-based material, a 13 % Ca standard for analysis of the inner region of the stylet should be adopted. Greater calcification and a potentially higher inorganic content in the inner region of the stylet, identified by the trends of Ca and P concentrations in *O. vulgaris* (Napoleão et al. 2005) and Ca in *O. pallidus* (Fig. 2.2), suggests that it is the most suitable area for microprobe analysis. Furthermore, as Ca is not homogeneously distributed through the microstructure, a different Ca concentration may be required if the mid-region of the stylet is to be targeted. The lack of calcification in the outer sheath indicates that edge analysis is currently not suitable for stylets.

These results support the potential of stylets as a useful tool for studying the dispersal and population structure of octopus populations through targeted elemental analysis, which has been applied so successfully to many teleost species. Firstly, there was a suite of detectable elements incorporated heterogeneously within the microstructure (which are most likely linked to the individuals' environmental and physiological history), and secondly, Ca appears to be a suitable internal standard for LA analysis. The combined information from all analyses and from past studies

(Napoleão et al. 2005) suggests that stylets are probably chitinous, with some inorganic component associated with phosphatic mineralisation. However, further analyses are required to define and quantify major organic and inorganic constituents more accurately. Additionally, this study further highlights the unique potential and efficacy of PIXE and Dynamic Analysis as a tool for examining the hard structures of marine species, providing insight into the physical and chemical structure, physiological processes involved in formation, and the biology and environmental history of the individuals analysed.

3

Assessing the efficacy of stylet elemental signatures for determining population structure in octopuses

This research is published as:

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ABSTRACT

A novel method was used to investigate the population structure and dispersal patterns of *Octopus maorum*, an octopus species with a planktonic paralarval stage, which forms a distinct and large aggregation in south-east Tasmania. Single and multi-elemental signatures within the early life history region of the stylet were used to determine levels of connectivity and the common origins of individuals collected from five locations across Tasmania, South Australia and New Zealand. Using laser ablation inductively-coupled plasma-mass spectrometry (LA ICPMS) the stylets were analysed for 12 elements, eight of which were found to be excellent spatial discriminators. There was evidence of sub-structuring within the *O. maorum* population; individuals from the aggregation in south-east Tasmanian were particularly distinct from other groups and appeared to share a local common origin. The two northern sample sites shared a similar elemental signature, which could be due to either similar water chemistries or long-distance dispersal. This study presents the first insights into the population structure of *O. maorum*, and provides vital information for the sustainable management of this species. Furthermore, this technique will have direct application to other more heavily fished octopus species around the world.

INTRODUCTION

It is generally assumed that species with planktonic larvae have a comparatively reduced population structure or are highly connected, as it is possible to achieve a inter-mixing of individuals from different source populations over large distances (Avise 2004; Becker et al. 2007). However, many species with planktonic larvae exhibit limited dispersal, resulting in highly structured populations or metapopulation complexes (see Avise 2004; Palumbi 2004 for examples). These exceptions emphasise the need to conduct species- or stock-specific assessments of population structure rather than relying on predictions which are based on the generalised paradigm (Thorpe et al. 2000).

In spite of the ecological importance and increasing commercial significance of octopuses on a global scale, little is known about the population structure or dispersal patterns of most octopus species or indeed cephalopods in general. Only a handful of studies investigating these parameters in octopus populations have been conducted (eg. Maltagliati et al. 2002; Oosthuizen et al. 2004), and are predominantly restricted to one species. Virtually nothing is known about the population structure and dispersal patterns of *O. maorum*, a merobenthic species with a high dispersal potential. This is also true for other commercial merobenthic species, including *Enteroctopus dofleini* (Rigby & Sakurai 2005), *Octopus mimus* (Rocha & Vega 2003).

The quantitative and spatial distributions of chemical elements within the hard biomineralised structures of marine organisms are influenced by their availability in the seawater, and commonly reflect changes in the chemical, physical and biological environment. Biomineralised structures with a temporally-related incremental structure may incorporate elements chronologically, providing a natural 'tag' that reflects an individual's movement history (Elsdon & Gillanders 2005). Targeted microprobe techniques (eg. LA ICPMS) have been employed

extensively to analyse increment-based structures such as fish otoliths (Campana 1999), and also, more recently, gastropod shells (Zacherl 2005), bivalve shells (Becker et al. 2005) and squid statoliths (Zumholz et al. 2007), providing information on the population structure, recruitment processes and dispersal or migratory patterns of many species. To determine the natal origins of adults and dispersal trajectories of juveniles, it is ideal to directly compare the elemental chemistry of juvenile hard structures to the pre-hatch region of the adult structure (eg. Hamer et al. 2005), however, this method is not suitable for all species. *Octopus maorum* hatchlings, for example, are only 5 mm long making it virtually impossible to successfully prepare and analyse hatchling stylets using microprobe techniques. However, when juveniles are difficult to obtain or process adult elemental signatures alone can provide information on common origins and the level of connectivity between geographically disparate groups within a population (eg. Ashford et al. 2006; Swan et al. 2006).

Although studies based on the targeted elemental analysis of cephalopod hard structures are few, it has been shown to be a useful technique in examining the population structure and movement patterns of squid (Zumholz et al. 2007). The paucity of elemental studies on octopuses may be partly due to the lack of a suitable biomineralised structure. Although octopus stylets are not composed of $CaCO_3$, like other structures used for targeted analysis, the central portion of this increment-based structure is suitable for laser ablation analysis (see Chapter 2).

This is the first time stylet elemental signatures have been used to examine the structure and dispersal patterns of an octopus population (*O. maorum*). In particular, the following key questions were investigated: (1) are elemental signatures from the early life history region of the stylet useful spatial discriminators of octopuses, (2) is there a level of connectivity between geographically isolated individuals, (3) do individuals from the same location share a common origin or alternatively come from a range of locations?

MATERIALS & METHODS

Stylet collection and preparation

Octopuses were collected from five sites: north-east Tasmania (NE), south-west Tasmania (SW), the Eaglehawk Bay aggregation (EHB) in south-east Tasmania, South Australia (SA) and New Zealand (NZ) (Fig. 3.1). Octopuses were collected as bycatch from rock lobster fishers (at depths between 10 – 40 m) from all sites except EHB, where they were collected from the commercial octopus fishery (at depths between 1 – 3 m). All specimens were frozen prior to dissection. Whole wet weight (kg) or mantle weight (g) (as often the arms were removed), sex, and stage of maturity were recorded. Males were classed as mature or immature, depending on the presence or absence of visible spermatophores in Needham's sac. Females were assigned a maturity stage from 1 – 3 (immature, maturing or mature) depending on ovary size and level of egg development (Smith et al. 2006). Octopuses ranged in size from 0.7 – 10.2 kg, all males and 50 % of the females were mature.

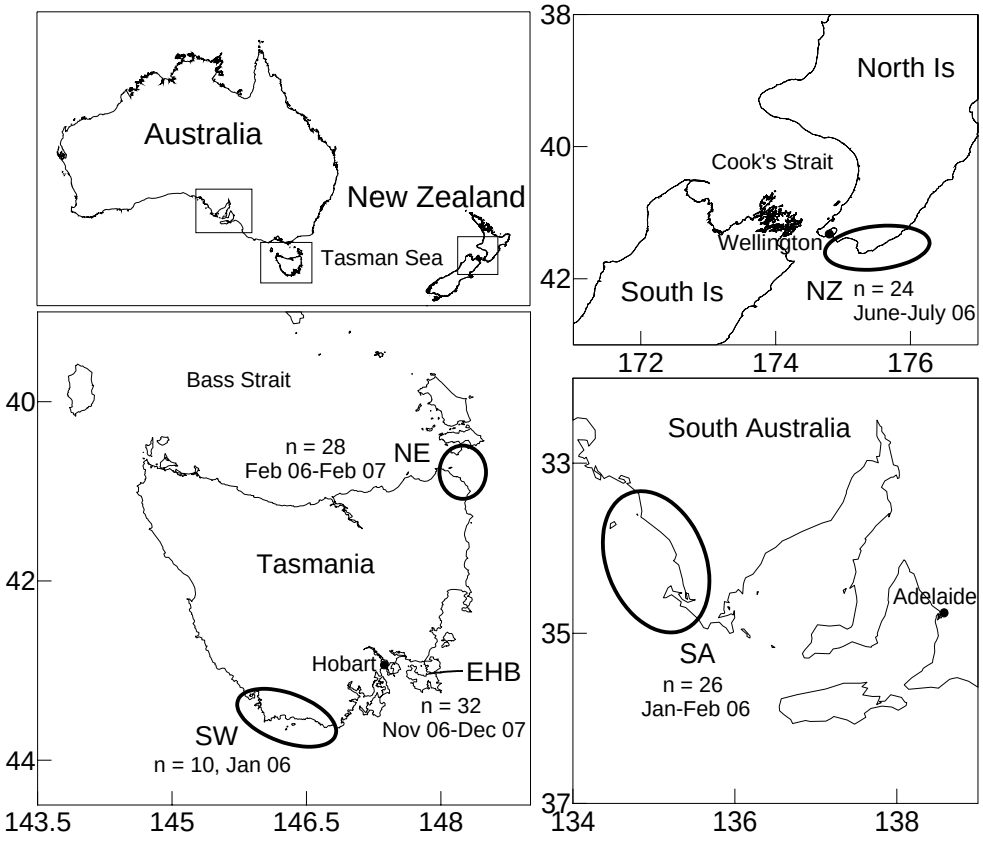

Figure 3.1 The sample locations, sample sizes (n), and collection dates of *Octopus maorum*. The sites are: South Australia (SA), north-east Tasmania (NE), New Zealand (NZ), south-west Tasmania (SW) and the Eaglehawk Bay aggregation (EHB).

Stylets were removed from thawed mantle tissue (see Bizikov 2004 for stylet location) and allowed to air dry for two days prior to being stored dry. Transverse sections, approximately 3 mm thick, were cut from the post-rostral zone proximal to the bend (see Fig. 3.2). The stylet sections were placed vertically onto double-sided tape and then surrounded by a thin layer of epoxy, which was left to partially set for 10 min before pouring on the remaining epoxy, and drying them in an oven for three hours at 60˚C. Once set, the mounts were ground using 1200 grit carborundum paper lubricated with ultra-pure water until all cross-sections were exposed, and then polished using 0.3 µm alumina powder on a pellon cloth polishing disc. The mounts were rinsed thoroughly in ultra-pure water and allowed to air dry.

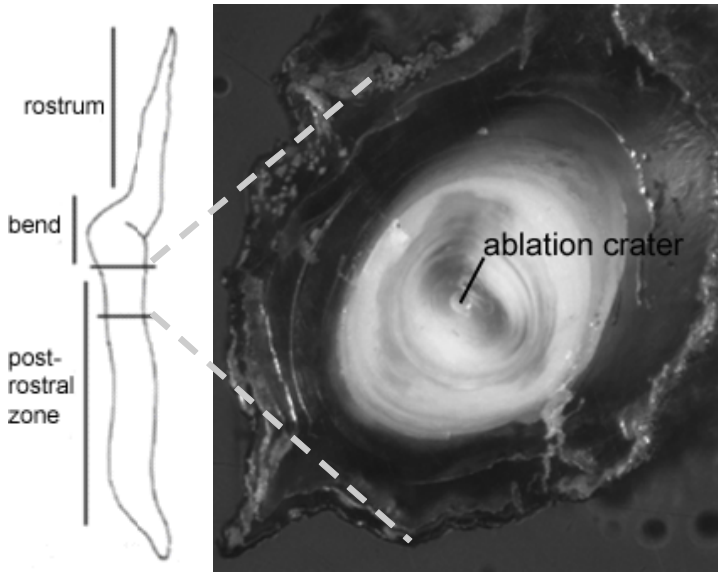

Figure 3.2 Location of elemental analysis within the stylet structure shown by the ablation crater (the result of a 35 µm spot ablation). The image is a stylet cross-section (63 x magnification) mounted in epoxy, which has been removed from the post-rostral zone (indicated by solid horizontal lines in the diagram).

Analytical methods

Elemental analyses were performed using LA ICPMS at the Centre for Ore Deposit Research (CODES), University of Tasmania. The New Wave UP-193 Nd:YAG Q-switched laser ablation system was connected to an Agilent HP7500cs Quadrapole ICPMS, and controlled by the MEOLaser 193 software package. For each sample a single spot ablation was performed, which targeted a distinct early life history region within the central region of the stylet cross section (see Fig. 3.2 for location). The concentric age-related growth of the stylets implies that this inner region of the stylet represents the earliest stages of the life cycle, however, the approximate size of the pre-hatch region has not been validated in the stylet microstructure of *O. maorum*. Furthermore, if a reasonable spot size and detection limits (DL) are to be maintained the pre-hatch region alone would be too small to target, as the width of one day old stylets, and thus the pre-hatch region within the adult stylet, would be < 10 µm. A 35 µm ablation spot size was used, which covered approximately 3 % of the whole stylet cross-section. Based on *O. maorum* age data (White 2007), it is estimated that

a 35 μm spot size would approximately incorporate the first seven days of an individual's life.

Laser ablation was performed with a laser pulse rate of 10 Hz and average laser energy of 4 – 5 J/cm^2. To remove surface contamination, each sample was pre-ablated at 1 Hz for 5 seconds prior to analysis. Background levels were measured for 30 seconds prior to each ablation, the average of which was subtracted from the subsequent sample analysis to correct for background levels. Due to the lack of matrix-matched standards, the international glass reference National Institute of Standards and Technology (NIST) 612 (Pearce et al. 1997) was used as the external standard. Although relying solely on NIST standards results in reduced analytical accuracy, in terms of absolute values, good analytical precision should allow valid comparisons between relative differences in elemental concentrations (eg. Zumholz et al. 2007). To account for instrument drift throughout the analyses the NIST standard was ablated twice every 90 minutes. The following suite of 12 elements was recorded for each analysis: ^{7}Li, ^{24}Mg, ^{55}Mn, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{208}Pb, ^{66}Zn, ^{75}As, ^{85}Rb, ^{88}Sr and ^{137}Ba. To correct for variations in ablation yield ^{43}Ca, set at a constant 13 %, was used as the internal standard. Calcium was chosen as the internal standard due to its consistent concentration and homogeneous distribution within the inner region of the stylet (see Chapter 2).

Data reduction was performed off-line using spreadsheet programmes. The DLs for each element were calculated from the concentration of analyte that yields a signal equivalent to three times the standard error of the background signal. The mean DLs for a 35 μm spot size were as follows (in ppm): Li – 0.033, Mg – 0.273, Mn – 0.093, Co – 0.013, Cd – 0.100, Ni – 0.059, Cu – 0.071, Pb – 0.009, Zn – 0.133, As – 0.203, Rb – 0.010, Sr – 0.068 and Ba – 0.026. All elements except Co and Pb were consistently above DLs. Although Co and Pb were below DLs in 10 and 15% of the samples respectively, 93.5 % of those samples were restricted two sites (SA and NE). Due to such concentration differences between sites Co

and Pb were retained in the analyses. One method to deal with values which are generated when an element falls below DLs is to substitute them with a constant, such as one-half of the DL (recommended by Farnham et al. 2002). Statistical analyses were performed using both the original values and substituted values based on one half of the DL (0.0045 for Pb and 0.0065 for Co), however, the results were the same.

Statistical analyses

To determine differences in elemental concentrations among sites for individual elements one-way analysis of variance (ANOVA) was performed. Elemental data were examined for normality and homogeneity of the variances using graphical methods and Levene's test. To meet model assumptions Li, Cu, Zn, Mn, Mg, Pb, Co and Ni were $\log_{10}(y + 2.5)$ and As was $y^{-0.5}$ transformed. All *a posteriori* comparisons among sites were conducted using Tukey's HSD test ($p < 0.05$). To test for differences between sexes and between sex and site, a separate two-way factorial ANOVA was performed for each element using animals from all sites (n = 120).

Elements which showed significant differences among sites in the univariate analyses were used for the multivariate analysis (n = 10). To determine the most important elements for the discrimination of sites, forward step-wise discriminant function analysis (DFA) was performed. This also served to reduce the number of dependent variables. All elements, except Ni and Rb, significantly contributed to the discrimination of sites. A second DFA was performed with these two elements removed. Differences in elemental concentrations between sites for multi-elemental signatures (n = 8) were determined using one-way multivariate analysis of variance (MANOVA). Pillai's trace was used as the test statistic. Canonical discriminant plots were used to visualise multivariate differences in elemental signatures among sites. To assess how accurately individuals could be assigned to site using multi-elemental signatures, cross-validation

classifications were performed using jack-knife 'leave one out' procedures. To compare the relative individual variability among sites, scatterplots of element concentration and site were constructed using untransformed data (the four most informative elements are presented in the Results and the remaining elements are shown in Appendix I). Cross-validation was performed using SPSS (v.13, SSPS Inc.) and all other analyses were performed using STATISTICA (v. 8, StatSoft).

RESULTS

All single elemental signatures detected within the stylets were significantly different among sites, except for Cu and Zn, with Pb, Mn, Mg, As, Co and Ba showing highly significant differences ($p < 0.001$) (Table 3.1). Lithium, Rb and Ni discriminated among sites the least, and Mn and Pb discriminated among sites the most (Table 3.2). Each site significantly differed from each other site by 3 – 4 elements, except for SA/NE and NZ/SW which both only differed by two elements. There were few similar among site trends between elements, except for Sr and Rb, which showed a striking similarity in relative concentrations. Prominent among site differences for individual elements included high levels of As in EHB, high levels of Ba in SA and NZ, low levels of Mg in SA, and low levels of Co and Pb in SA and NE. Individual variability in element concentration was relatively similar for Sr, Mg and Ba between sites. Manganese, Co, Pb, and As showed a larger range in concentration, which was particularly evident in the EHB site for Pb and As (Fig. 3.3).

Table 3.1 One-way ANOVAs comparing elemental concentrations among sites in the early life history region of the stylets of *Octopus maorum*. Element concentrations were transformed as described in Methods.

Source	df	MS	F	p
Li				
site	4	0.05	2.55	0.042
error	115	0.02		
Ba				
site	4	198.2	6.28	0.000
error	115	31.6		
Sr				
site	4	1.70	3.75	0.006
error	115	4.56		
Cu				
site	4	0.10	2.38	0.056
error	115	0.05		
Zn				
site	4	0.12	2.13	0.081
error	115	0.04		
Rb				
site	4	1.85	2.81	0.027
error	115	0.65		
Mg				
site	4	0.06	34.0	0.000
error	115	0.00		
Mn				
site	4	3.37	101.7	0.000
error	115	0.02		
As				
site	4	54.8	9.90	0.000
error	115	5.53		
Ni				
site	4	0.15	2.73	0.032
error	115	0.06		
Pb				
site	4	15.8	96.8	0.000
error	115	0.16		
Co				
site	4	3.88	33.4	0.000
error	115	0.11		

Table 3.2 Mean concentration of elements (ppm, ± SD) in pre-hatch region of adult stylets for each of the five sites. AP = mean analytical precision (ppm), T = results from Tukeys HSD, with significant differences ($p < 0.05$) indicated by letters (A, B, C). Sites are: South Australia (SA), New Zealand (NZ), north-east Tasmania (NE), south-west Tasmania (SW), and Eaglehawk Bay (EHB).

element	SA			NZ			NE			SW			EHB		
	mean ± SD	**AP**	**T**	**mean ± SD**	**AP**	**T**	**mean ± SD**	**AP**	**T**	**mean ± SD**	**AP**	**T**	**mean ± SD**	**AP**	**T**
Li	0.84 ± 0.20	0.08	AB	1.00 ± 0.34	0.08	AB	0.85 ± 0.26	0.07	A	0.90 ± 0.12	0.06	AB	1.11 ± 0.54	0.10	B
Mg	28,144 ± 4,038	1,884	A	36,274 ± 3,869	2,301	B	34,236 ± 1,989	1,984	B	34,544 ± 1,928	2,134	B	36,406 ± 3,437	2,342	B
Mn	14 ± 9.2	1.0	AB	60 ± 35.5	3.8	B	14 ± 7.6	0.85	A	20 ± 4.8	1.31	C	76 ± 38.0	4.89	B
Co	0.06 ± 0.09	0.02	A	0.23 ± 0.20	0.03	B	0.02 ± 0.02	0.01	A	0.10 ± 0.05	0.02	B	0.12 ± 0.12	0.02	B
Ni	0.62 ± 0.38	0.10	AB	0.55 ± 0.38	0.09	AB	0.52 ± 0.31	0.09	AB	0.67 ± 0.20	0.08	A	0.44 ± 0.25	0.08	B
As	56 ± 30	3.3	A	50 ± 23	2.9	A	50 ± 17	2.6	A	65 ± 19	3.6	A	117 ± 71	6.9	A
Rb	3.2 ± 0.7	0.22	AB	3.6 ± 0.9	0.25	A	3.3 ± 0.7	0.21	AB	2.8 ± 0.7	0.20	AB	3.1 ± 0.6	0.22	B
Sr	3,766 ± 282	250	AB	3,876 ± 213	244	A	3,840 ± 181	220	AB	3,653 ± 148	223	B	3,702 ± 189	231	B
Ba	27 ± 6.1	1.8	A	27 ± 4.6	1.7	A	20 ± 4.3	1.3	B	21 ± 5.2	1.3	AB	24 ± 6.7	1.61	AB
Pb	0.03 ± 0.03	0.01	A	0.10 ± 0.08	0.02	B	0.03 ± 0.01	0.01	A	0.12 ± 0.10	0.01	B	1.08 ± 0.60	0.07	C

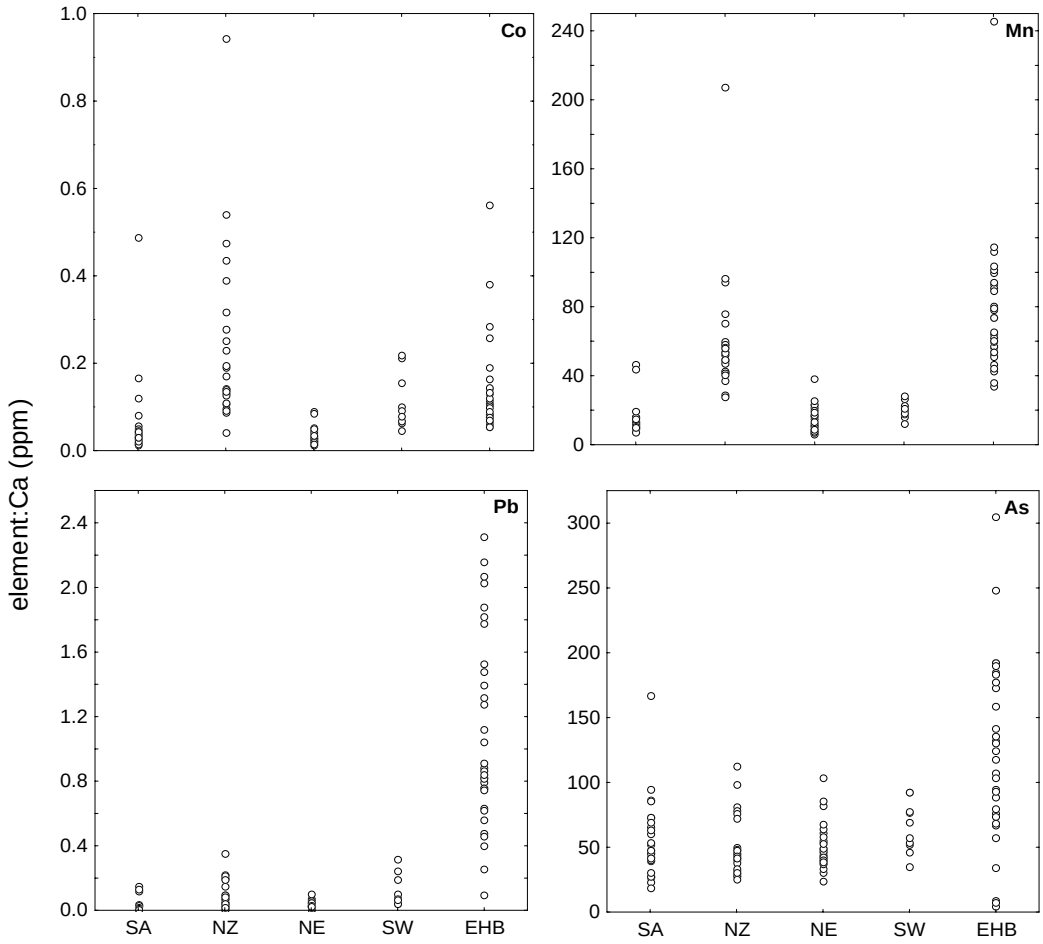

Figure 3.3 Individual variability in As, Co, Mn, and Pb concentrations for each site. Each circle represents an individual octopus. Sites are: South Australia (SA), New Zealand (NZ), north-east Tasmania (NE), south-west Tasmania (SW), and Eaglehawk Bay (EHB).

Multi-elemental signatures were highly significant among sites (MANOVA: Pillai's trace = 1.92, $F_{28, 448} = 14.9$, $p < 0.001$). All elements, except Ni and Rb, significantly contributed to the discrimination among sites (step-wise DFA: $F > 2.5$, $p < 0.05$). Discriminant functions 1 and 2 explained 78 and 11 % of the variation respectively. Overall, Pb contributed the most variation among sites, followed by Mn and Sr, while Li contributed the least (Table 3.3). EHB had the most distinct signature, which has been separated from the other sites largely by differences in Pb, Mn and As concentrations (Fig. 3.4). Although NZ shares some overlap with SW and SE, it showed some separation, particularly from Co, Mn and Mg, described by discriminant function 2. SA and NE shared the most

similar multi-elemental signature, which has been separated from the remaining sites largely by Sr. The overall cross-validated classification rate was 91.7 %, based on the eight elements entered in the final DFA (Table 3.4). Most misclassifications were associated with SA and NZ, which had 19.2 and 12.5% of their individuals misclassified respectively. Four out of five misclassified individuals from SA were classified as NE individuals. The most distinct sites were SW and EHB, with 100 % of individuals being correctly classified.

Table 3.3 Standardised canonical coefficients for discriminant functions (DF) 1 and 2 for each element entered in the final DFA. Coefficients represent the relative contribution of each element to each DF.

element	DF1	DF2
Mn	-0.69	-0.43
Pb	-0.74	0.64
Mg	-0.40	-0.40
Sr	0.61	-0.16
Co	-0.02	-0.62
Ba	0.35	-0.06
As	-0.23	0.63
Li	0.07	0.37

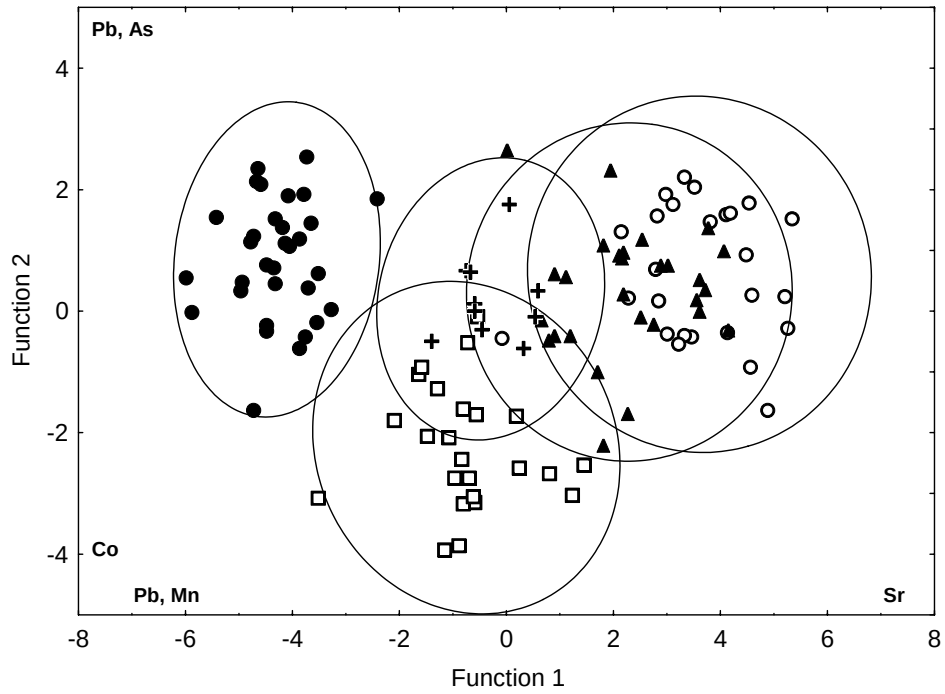

Figure 3.4 Canonical discriminant plots comparing multi-elemental signatures among sites. Elements that contributed most variation to the data (canonical coefficients) are indicated at both ends of each axis. Ellipses represent 95 % confidence intervals for each group. Sites are: South Australia (○), Eaglehawk Bay (●), New Zealand (□), north-east Tasmania (▲), and south-west Tasmania (+).

Table 3.4 Predicted group memberships from cross-validation classification procedures for each site, based on DFA scores from multi-elemental data. Results indicate the percentage of individuals classified to each site with correct classifications in **bold**. Sites are: South Australia (SA), New Zealand (NZ), north-east Tasmania (NE), south-west Tasmania (SW), and Eaglehawk Bay (EHB).

site	SA	NZ	NE	SW	EHB
SA	**80.8**	3.8	15.4	0	0
NZ	0	**87.5**	4.2	8.3	0
NE	0	3.6	**92.9**	3.6	0
SW	0	0	0	**100**	0
EHB	0	0	0	0	**100**

There was no significant effect of sex on elemental concentration for all elements, except for Ni and Co (two-way ANOVA: Co, df = 1, F = 9.19, p = 0.003; Ni, df = 1, F = 5.19, p = 0.025), with females having a significantly

lower concentration than males. However, site-sex interaction effects were not significant for Ni or Co (two-way ANOVA: Co, df = 4, F = 0.42, p = 0.78; Ni, df = 4 , F = 1.08, p = 0.35).

DISCUSSION

Single and multi-elemental signatures within the early life history region of stylets were informative spatial discriminators for *O. maorum*. The discrimination of element signatures between sites suggests that elemental concentrations in the early life history region may reflect the environment in which the individuals inhabited at that time in their life history. The elemental signatures indicate that there is a level of structure within the population, which adds to the growing evidence that populations with planktonic larval stages can be relatively structured. Although the duration of the planktonic paralarval stage in *O. maorum* is unknown it is likely that other factors are influencing its dispersal. Population structure observed in other cephalopods with planktonic paralarvae, including *O. vulgaris* (Murphy et al. 2002; Teske et al. 2007), *Loligo gahi* (Arkhipkin et al. 2004), and *Todaropsis eblanae* (Dillane et al. 2005), has been suggested to be caused by oceanographic conditions and historic climatic events.

The multivariate analyses suggest that individuals from the Eaglehawk Bay aggregation have a common natal origin, and are separated predominantly from the northern populations. It is assumed that individuals aggregating in Eaglehawk Bay as adults have hatched elsewhere, due to the scarcity of hard substrate for the attachment of egg broods within the bay, the dominance of particularly large mature animals in the fishery catch for 17 years, and no known observations of juveniles or egg broods. Although it is speculative to propose the natal origin of the Eaglehawk Bay individuals, particularly as relationship between water composition and stylet composition is unknown, the elevated levels of Pb

and As suggests that they may have hatched in coastal waters comparatively close to the industrialised Derwent estuary. Pollution has resulted in significantly elevated levels of Pb and As in the Derwent estuary, compared to surrounding waters (Jones et al. 2003), making the south-east region comparatively heterogenous in ambient elemental concentrations. If the aggregating animals are hatching within this region, it would explain the very broad range of As and Pb (and potentially Co and Mn) levels between the individuals from Eaglehawk Bay. Furthermore, all other sites are relatively exposed coastal waters and isolated from industrial pollution, suggesting that individuals from the aggregation are not hatching in such environments. In light of this, the *O. maorum* fishery at Eaglehawk Bay should be managed as a separate stock as replenishment from neighbouring sources may be limited.

South Australia and north-east Tasmania share a relatively similar elemental signature, with only two elements being significantly different between the two sites, resulting in the misclassification of some individuals. Such similarities could be due to long-distance dispersal (some individuals may share a common origin or undergo post-hatch mixing), or the two sites having similar water chemistries. Oceanic currents within the region support the possibility of such dispersal patterns: the Leeuwin current flows eastward along the south coast of Australia, which then flows southward (turning into the Zeehan current) along the west coast of Tasmania (Crawford et al. 2000), while pushing water eastward across Bass Strait (Tomczak 1981). There is no evidence to suggest that there is a regular exchange of individuals between Australia and New Zealand. The elemental signature from New Zealand is distinct from Australian signatures, with particularly high levels of Co. The possibility of trans-Tasman dispersal cannot be dismissed, however, as not all key regions within the distribution of *O. maorum* were sampled (eg. Victoria and the west coast of New Zealand). Furthermore, a small percentage of individuals from New Zealand were classified as Tasmanian and vice versa

for Tasmanian individuals, which is supported by the scatterplots of Mn and Co (key elements which separated New Zealand from the other sites) which show some individuals not fitting the general trend.

Time specific elemental studies are based on the assumption that the structure grows throughout the life cycle of the organism and is metabolically inert or undergoes no post-depositional modification (Campana 1999). Although it has been shown that stylets undergo temporally-related incremental growth (Doubleday et al. 2006); it is not known whether stylet material is reworked over time (in other words, the elemental signature may not reflect the environment to which the individual was exposed). Elemental incorporation into the stylet structure may also be under differing degrees of physiological control. Of the elements which were detected within the stylets, eight are classified as essential for cephalopod function (Mn, Mg, Sr, Co, Ni, Zn, Cu and Rb) (Villanueva & Bustamante 2006) and are likely to be under some level of internal regulation. However, four of these essential elements (Mn, Mg, Sr and Co) were excellent spatial discriminators, which suggests that environmental availability may also have some effect on stylet composition. Although, Co and Ni were significantly higher in males suggesting that concentration is controlled somewhat by physiological processes, the effect of geographical location out weighed the effect of sex. The lack of differentiation in Cu and Zn concentrations among sites indicates that these elements are either predominantly controlled by physiological process or are just similar between sites. However, two studies suggest that Cu and Zn are largely under metabolic control in *O. vulgaris*, both in the muscle tissues of adults (Raimundo et al. 2004) and in eggs (Villanueva & Bustamante 2006). Even though the mechanistic controls of elemental incorporation in stylets is not understood, and physiology could have some effect on elemental incorporation, consistent geographic patterns in elemental signatures can be exploited as natural tags regardless of whether the signature was governed directly by water chemistry or a complex

interaction of environment, physiology, and behaviour (Gillanders 2005; Swan et al. 2006).

There have been relatively few studies on the direct causal and regulatory mechanisms that govern the incorporation of elements into bio-mineralised structures. Barium (Zacherl et al. 2003) and Sr (Elsdon & Gillanders 2004) concentration in gastropod statoliths and fish otoliths respectively have been directly associated with seawater concentration, and temperature has shown to negatively correlate with Sr concentration in squid statoliths (Arkhipkin et al. 2004) and octopus statoliths (Ikeda et al. 1999). Food is likely to be an important source of element uptake for cephalopods, due to their rapid growth rate, high metabolism and food intake requirements (Bustamante et al. 2004), with diet found to significantly influence Ba concentrations in cuttlefish statoliths (Zumholz et al. 2006). Stylets, unlike statoliths and otoliths which are surrounded by endolymphatic fluid (the composition of which is suggested to be under significant physiological regulation) (Campana 1999), are in direct contact with muscle tissue and element uptake is likely to be effected by a different, and perhaps more direct, physiological pathway. It has been shown that adult and juvenile cuttlefish (*Sepia officinalis*) rapidly incorporate Co and Ag into their muscular tissue from seawater (Bustamante et al. 2004). To facilitate the interpretation of stylet elemental signatures, it will be valuable in the future to conduct controlled experiments examining the relationships between stylet composition and environmental variability.

This study shows that targeted elemental analysis is a particularly useful technique for understanding population linkages in merobenthic octopuses, providing valuable information without the necessity of juvenile individuals. Traditional tag-recapture methods have proven incredibly difficult for marine species with planktonic larvae, due to the small size and high mortality rates of offspring and the large number of individuals which need to be tagged to recover sufficient sample sizes (Thorrold et al. 2001; Gillanders 2002). Furthermore, adult octopuses are particularly difficult to

tag due to their tendency to remove external tags. Molecular markers are a powerful tool for investigating population structure in octopuses, but the recent utilisation of both elemental and molecular techniques within a single study has shown that elemental signatures do not just reproduce, but also complement genetic data (see Miller et al. 2005; Ashford et al. 2006). Genetic methods provide estimates of dispersal over long inter-generational time-scales, with, theoretically, an exchange of only a few individuals per generation required to maintain genetic homogeneity among populations (Semmens et al. 2007). On the other hand, elemental signatures can indicate distinct sub-populations that are otherwise genetically homogeneous, due to periodic low-level mixing or mixing during the early stages of the life cycle (Campana & Thorrold 2001; Arkhipkin et al. 2004), which would be particularly common for species with planktonic larvae.

In summary, this study has provided evidence of localised population structure within the *O. maorum* population through the analysis of elemental signatures within the early life history region of stylets. As it is difficult to discern whether similar signatures between sites are due to dispersal or similar water chemistries, levels of connectivity are hard to define. Larger sample sizes, a larger number of sample locations and some understanding of the relationships between the environmental availability of elements and elemental concentrations within the stylet, would improve such uncertainties. It would also be useful to conduct a similar study using a different technique, such as molecular markers, as an integrative approach to understanding such aspects of marine populations has been shown to provide both corroborative and complementary results (e.g. Kassahn et al. 2003; Miller et al. 2005). The results presented here will help determine the appropriate sustainable scale at which the *O. maorum* fishery ought to be managed. Additionally, we have shown that elemental analysis of the octopus stylet will be a powerful tool to resolving issues of population structure and dispersal in more heavily fished octopus species.

4

Stylet elemental signatures indicate population structure in a holobenthic octopus species

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ABSTRACT

Targeted elemental analysis was used to investigate the population structure and dispersal patterns of *Octopus pallidus*. Multi-elemental signatures within the pre-hatch region of the stylet were used to determine levels of connectivity and the common origins of individuals collected from five locations in Tasmania. To determine if hatchling elemental signatures could be used as tags of natal origin, hatchling stylets were also analysed from three of the locations. Twelve elements were analysed, using laser ablation inductively-coupled plasma-mass spectrometry (LA ICPMS), seven of which were excellent spatial discriminators. There was evidence of high level structuring within the adult *O. pallidus* population with distinct groupings between all sites (the two closest being 85 km apart), suggesting that all adults had hatched in or near their respective collection sites. The hatchling signatures showed significant spatial variation, with a high percentage of individuals successfully classified back to their collection locations. However, they could not be used to classify adults back to their natal site, due to significant differences in element concentrations compared to the adults, the likely result of differences in Ca concentration. This study presents the first insights into the population structure of a holobenthic octopus species, providing vital information for the sustainable management of *O. pallidus*, and will have direct application to other more heavily fished species.

INTRODUCTION

Population structure and connectivity determine the spatial scale at which localised populations operate; they may be predominantly self-recruiting, depend on some exchange from neighbouring populations, or freely interbreed across the species' distributional range (Fowler et al. 2005). Such parameters of marine populations, therefore, are key properties for determining the resilience of populations to harvesting and developing effective conservation and fisheries management strategies (Thorrold et al. 2001; Gillanders 2002). However, reconstructing the dispersal patterns of marine species throughout their life history cycle, particularly the early stages, has proved a continuing challenge (Jones et al. 2005; Bradbury et al. 2008). Traditional tag-recapture methods have often proved inadequate due to the small size, large number and high mortality rates of offspring (Gillanders 2005; Warner et al. 2005). Genetic markers can be valuable for investigating levels of connectivity relevant to long-term inter-generational population changes (eg. Shaw et al. 1999; Kassahn et al. 2003; Buresch et al. 2006), however, only a very low exchange rate is required to maintain genetic homogeneity (Carvalho & Hauser 1994).

A more direct approach of measuring dispersal employs the analysis of natural elemental signatures within the hard biomineralised structures of marine organisms, which can be influenced by the physical and biological availability of various elements in the seawater. Elemental signatures have the advantage that all individuals within a population are 'tagged' regardless of size (Semmens et al. 2007), and as they reflect the present movement patterns of a population they may reveal distinct sub-populations that are otherwise genetically homogenous due to low-level mixing (Campana & Thorrold 2001; Arkhipkin et al. 2004). The development of targeted microprobe techniques, such LA ICPMS, has enabled the selective elemental analysis of specific temporally-related regions within structures such as otoliths (Campana 1999), gastropod shells (Zacherl 2005), bivalve shells (Becker et al. 2005) and squid statoliths

(Zumholz et al. 2007). Furthermore, elemental signatures taken from the pre-hatch region of an adult structure have been matched together with signatures from equivalent juvenile structures, allowing the natal origins of adults and dispersal trajectories of juveniles to be determined (eg. Forrester & Swearer 2002; Hamer et al. 2005).

In spite of the ecological and increasing commercial significance of cephalopods, the population structure and dispersal patterns of most species are poorly understood. For octopuses, such studies are predominantly restricted to the merobenthic species *Octopus vulgaris*, and are largely genetic based (eg. Maltagliati et al. 2002; Oosthuizen et al. 2004). Targeted elemental analysis of the early life history region of adult stylets has been successfully applied to examine the structure and dispersal patterns of the merobenthic species *Octopus maorum* (see Chapter 3). However, to date, there are no published studies based on the targeted analysis of both juvenile and adult cephalopod hard structures. Although holobenthic octopus constitute both emerging and developed fisheries, such as the *Octopus pallidus* fishery in south-east Australia (Ziegler et al. 2007), the *Octopus maya* fishery in Mexico (Arreguín-Sánchez et al. 2000) and *Eledone cirrhosa* fishery in the Mediterranean (Orsi Relini et al. 2006), there has been no known population-level research conducted on this group, which are likely to have differing dispersal potentials and population structures to that of their merobenthic counterparts.

This study utilises targeted elemental analysis of both hatchling and adult stylets to examine the population structure of *O. pallidus*. More specifically we determine if: (1) elemental signatures from the pre-hatch region of adult stylets can be used to examine structure and dispersal patterns, (2) hatchling elemental signatures exhibit sufficient spatial variation to act as tags of natal origin, (3) hatchling signatures are temporally stable over short time periods, and (4) adult signatures can be matched back to the hatchling signatures and thus determine the natal origins of the adults.

MATERIALS & METHODS

Octopus collection

Octopus hatchlings were collected from three sites: Circular Head (CH) on the north-west coast of Tasmania, and Mercury Passage (MP) and Northwest Bay (NWB) on the south-east coast of Tasmania, Australia (Table 4.1, Fig. 4.1). Unbaited octopus pots attached to demersal longlines and set at depths between 12 – 30 m were used to provide nesting habitat for mature females. Pots were deployed for 3 – 5 months and were checked regularly for egg broods. If eggs were present a PVC mesh bag was tied securely around the pot to prevent predation and aid in hatchling collection and subsequently checked every 2 – 4 weeks. This procedure does not affect the mother as she does not feed or leave her 'den' during egg development. Each batch of samples were collected as 'near hatching' eggs or recently hatched hatchlings from one brood. To assess the short-term temporal variation in hatchling elemental signatures, two additional batches of hatchlings were collected from NWB (Table 4.1), with collections spaced 4 – 6 weeks apart. All animals were frozen upon collection.

Table 4.1 Details of *Octopus pallidus* samples.

sample site	collection date (hatchlings)	no. of hatchlings	collection date (adults)	no. of adults
west Flinders Is (WFI)	-	0	June 2006	17
east Flinders Is (EFI)	-	0	April 2007	19
Northwest Bay (NWB)				
collection 1	Nov 06	40	May – Sept 2007	8
collection 2	Dec 06	33		
collection 3	Feb 07	36		
Mercury Passage (MP)	June 2006	72	Feb – April 2007	19
Circular Head (CH)	May 2006	32	November 2006	28

Using pots and longlines adult octopuses were collected 6 – 10 months after the hatchlings were collected from the same sites described above. According to an age study of *O. pallidus*, mature adults have an average age of eight months (Leporati et al. 2008), therefore, it is likely that

these adults hatched at a similar time to when the hatchlings were collected. To minimise potentially confounding temporal variation in elemental signatures it is recommended that adults are assigned to natal site based on juvenile elemental signatures that are from a similar age cohort as the adults (Gillanders 2002; Gillanders 2005). Adults without an 'equivalent' hatchling sample were additionally collected from west Flinders Island (WFI) and east Flinders Island (EFI) (Table 4.1, Fig. 4.1). For each adult animal, whole weight (g), sex and maturity stage was recorded. Males were classed as mature or immature, depending on the presence or absence of visible spermatophores in Needham's sac. Females were assigned a maturity stage from 1 – 5. Stages 1 – 4 (immature – mature) were identified according to ovary size and level of egg development, and stage 5 was assigned to post-spawning females (Leporati et al. 2008). Octopuses ranged in size from 280 – 1,012 g, all males were mature and most females were either stage 3 or 4.

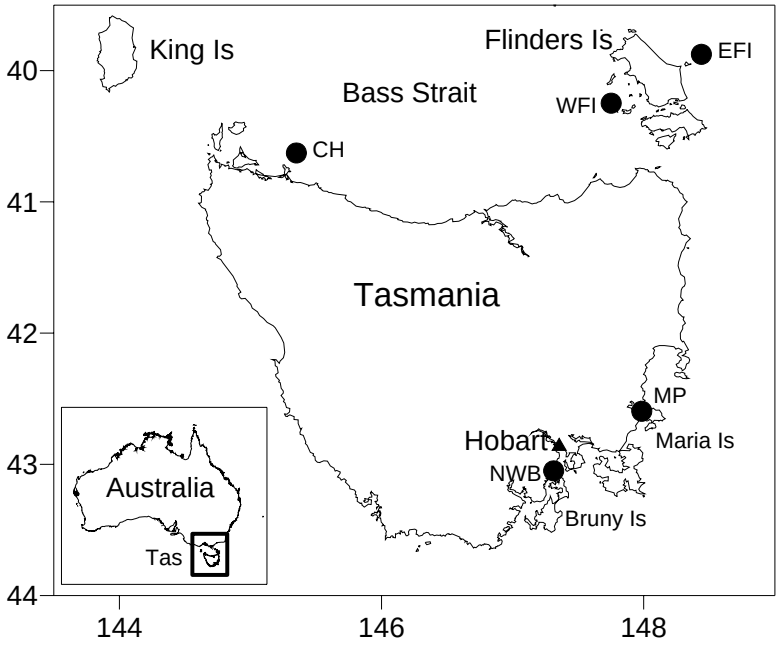


Figure 4.1 Location of *Octopus pallidus* sample sites. The sites (●) are: Circular Head (CH), west Flinders Island (WFI), east Flinders Island (EFI), Mercury Passage (MP), and Northwest Bay (NWB)

Stylet preparation

Hatchling stylets were carefully removed from partially thawed animals under a dissecting microscope. Stylets were placed on glass Petri dishes and oven dried overnight at 60° C. Once dried, the main bulk of tissue was scraped away from the stylets, which were then placed flat onto double-sided tape stuck onto glass plates. Epoxy resin was poured into 25 mm moulds placed on the tape, with each containing about 20 stylets, and then oven dried for three hours at 60° C. The mounts were not sanded or polished as this immediately removed the stylets from the epoxy, and many samples remained exposed on the surface of the mount. Individual photos were taken of each mounted stylet using a light microscope to aid the detection of the stylet material once in the ablation chamber. Adult stylets were removed from mantle tissue (see Bizikov, 2004 for stylet location) and processed according to the methods outlined in Chapter 3. All animals were fresh except for 50 % of the EFI individuals and all of the WFI individuals, which were frozen prior to dissection.

Analytical methods

Elemental analyses were performed using laser ablation inductively-coupled plasma-mass spectrometry (LA ICPMS) at the Centre for Ore Deposit Research (CODES), University of Tasmania. For more details on the laser ablation system, analytical specifications, detection limits (DLs) and external and internal standards used refer to Chapter 3. For the adults, the pre-hatch region visible within the stylet cross section was targeted (see Doubleday et al. 2006); ablation spot size was 35 µm (which is the approximate size of the pre-hatch region) with a laser pulse rate of 10 Hz. To remove surface contamination, each sample was pre-ablated at 1 Hz for 5 seconds prior to analysis. For the hatchlings, the laser was targeted closest to the 'bend' region of the stylet (see Doubleday et al. 2006), where possible, to achieve maximum ablation yield. The presence of Ca in the analysis was used to distinguish the surrounding tissue from the stylet, and determine

the position of the integration interval (see Fig. 4.2). Samples with integration intervals of ≤ 2 seconds were discarded. Hatchling ablation spot size was 20 µm with a laser pulse rate of 5 – 10 Hz. Pre-ablation was not performed on the hatchling stylets; however, surface contamination spikes were not included in the integration interval, as the laser was ablating through epoxy and tissue in the first few seconds (see Fig. 4.2). The following suite of 12 elements was recorded for each analysis for both adults and hatchlings: ^{7}Li, ^{24}Mg, ^{55}Mn, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{208}Pb, ^{66}Zn, ^{75}As, ^{85}Rb, ^{88}Sr and ^{137}Ba. Cobalt and Ni were below DLs in a large proportion of the hatchling samples and were therefore excluded from further analysis. Cobalt, Ni, As and Pb were below DLs in a small number of adult samples. One method of treating values generated by an element falling below DLs is to substitute them with a constant, such as one half of the DL (recommended by Farnham et al. 2002). Statistical analyses on the adult stylets were performed using both the original values and substituted values based on one half of the DL (Pb = 0.008, Co = 0.006, Ni = 0.032, and As = 0.081), however, the results were the same (the latter are presented).

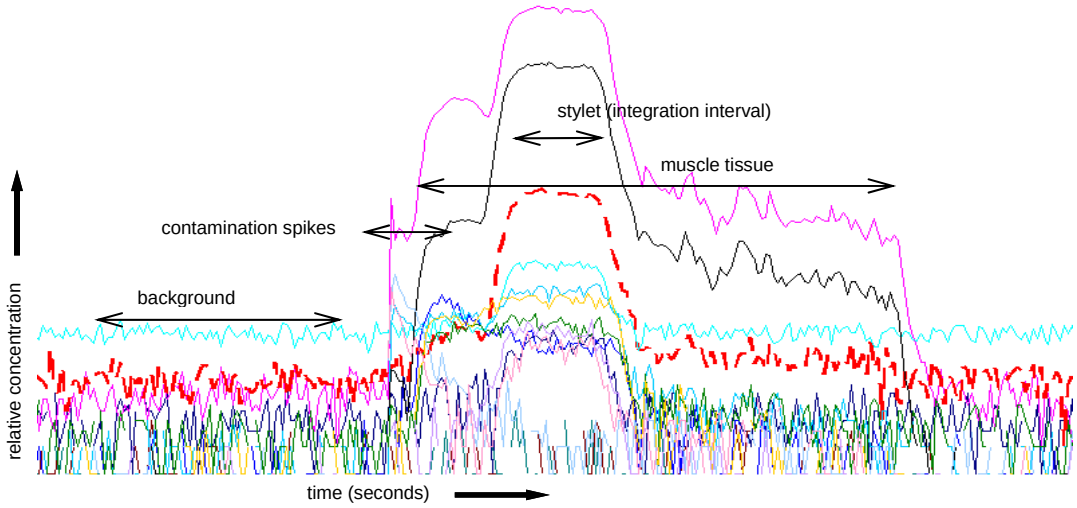


Figure 4.2 Example of a raw data output from the laser ablation analysis of a hatchling stylet, each colour represents a different element (red dashed line = Ca). An increase in Ca concentration, as shown, indicates the ablation of stylet material, and allows for correct selection of the integration interval.

Statistical analyses

To determine differences in adult elemental signatures among sites one-way analysis of variance (ANOVA) and multivariate analyses were performed. To meet ANOVA assumptions all elements were $\log_{10}$ transformed. All *a posteriori* comparisons among sites were conducted using Tukey's HSD test. All elements, except Ni, showed significant univariate differences and were included in the multivariate analyses (n = 11). Forward step-wise discriminant function analysis (DFA) was performed, which showed that Zn, Sr, Ba and Cu did not contribute to the discrimination of sites. To reduce the number of dependent variables, a second DFA was performed with these elements removed. Differences in multi-elemental signatures (n = 7) among sites were determined using one-way multivariate analysis of variance (MANOVA) and visualised using canonical discriminant plots. To assess how accurately adults could be assigned to their site of origin using multi-elemental signatures, cross-validation classifications were performed using jack-knife procedures. To test for differences between sexes and sex and site a separate two-way ANOVA analysis was performed for each element using all individuals (n = 91). Regression was used to examine the relationship between age and element concentration using individuals from CH (n = 28). Age data for the CH individuals was derived from a separate study (Leporati et al. 2008). To determine if hatchling elemental signatures were useful tags of natal origin, a series of univariate and multivariate analyses were performed using the same procedure described above for the adults. Strontium, Mn and Zn were excluded from the second DFA leaving a total of seven elements.

To assess the extent of short-term temporal variation within hatchling elemental signatures, a DFA and MANOVA were performed on the three hatchling collections from NWB using all detected elements (n = 10). A second canonical analysis and cross-validation was also performed on the hatchling spatial data which included all the NWB collections pooled together. To determine whether the adults, collected 6 – 8 months later,

matched the age-cohort of the hatchlings (as a mismatch of cohorts may introduce temporal bias), age data for the CH individuals was analysed. Cross-validation was performed using SPSS (v.13, SSPS Inc.) and all other analyses were performed using STATISTICA (v. 8, StatSoft).

RESULTS

Adult signatures

All single elemental signatures, except Ni, were significantly different among sites (one-way ANOVAs: $p < 0.05$; Table 4.2). All 11 elements were significantly different between EFI and MP; in contrast, EFI and WFI were only separated by differences in Mn, Sr, and Cu (Table 4.3). Major among site differences for individual elements included high levels of As at EFI and WFI and Co at CH, exceptionally high levels of Pb in NWB, and low levels of Mn at WFI and NWB. The multi-elemental signature was also highly significant among sites (MANOVA: Pillai's trace = 2.87, $F_{28, 332} = 30.6$, $p < 0.001$). All elements, except Zn, Sr, Ba and Cu, significantly contributed to the discrimination of sites (DFA: $p < 0.05$), with discriminant functions (DF) 1 and 2 describing 50 % and 27 % of the variation respectively. Magnesium contributed the most variation among sites, followed by As and Pb, while Li and Co contributed the least (Table 4.4). The canonical plot showed a clear separation between CH, MP and NWB, mainly due to differences in Mg, Rb and Mn, with MP appearing to have the most variable signature (Fig. 4.3). EFI and WFI were separated from the other sites largely by differences in As and Mg, and had relatively similar signatures. Based on the seven elements entered in the final DFA, the overall cross-validation rate was very high (98.9 %), with only one WFI individual misclassified to EFI (Table 4.5). There was a minimal effect of the two different pre-dissection treatments (frozen versus fresh animals) on the spatial analyses, with 100 % of the EFI individuals classified correctly back to site (even though 50 % were frozen), and with only two elements differing significantly between WFI (100 % frozen) and all the other sites.

There was no relationship between age and element concentration (r^2 were < 0.1) and no significant effect of sex on elemental concentration was found for any element (two-way ANOVAs: $p > 0.05$).

Table 4.2 One-way ANOVAs comparing single-elemental adult signatures among sites. Element concentrations were transformed as described in Methods

Source	df	MS	F	p
Li				
site	4	0.41	56.24	0.000
error	86	0.01		
Ba				
site	4	0.02	4.44	0.003
error	86	0.01		
Sr				
site	4	162941	9.98	0.000
error	86	16321		
Cu				
site	4	0.42	5.41	0.000
error	86	0.07		
Zn				
site	4	0.04	3.12	0.017
error	86	0.02		
Rb				
site	4	0.85	56.24	0.000
error	86	0.01		
Mg				
site	4	0.03	88.00	0.000
error	86	0.00		
Mn				
site	4	1.60	47.08	0.000
error	86	0.04		
As				
site	4	8.19	87.35	0.000
error	86	0.08		
Ni				
site	4	0.16	1.81	0.132
error	86	0.09		
Pb				
site	4	6.44	62.49	0.000
error	86	0.09		
Co				
site	4	0.84	10.30	0.000
error	86	0.07		

Table 4.3 Mean concentration of elements (ppm ± SD) in pre-hatch region of adult stylets for each of the five sites. AP = mean analytical precision (ppm), T = results from Tukeys HSD, with significant differences ($p < 0.05$) indicated by letters (A, B, C). The sites are: Circular Head (CH), west Flinders Island (WFI), east Flinders Island (EFI), Mercury Passage (MP), and Northwest Bay (NWB).

element	CH			EFI			WFI			NWB			MP		
	mean ± SD	**AP**	**T**	**mean ± SD**	**AP**	**T**	**mean ± SD**	**AP**	**T**	**mean ± SD**	**AP**	**T**	**mean ± SD**	**AP**	**T**
Li	0.86 ± 0.56	0.11	A	0.41 ± 0.23	0.03	B	0.49 ± 0.28	0.04	B	0.79 ± 0.12	0.8	A	0.82 ± 0.55	0.09	A
Mg	38,427 ± 4,440	4,385	A	31,968 ± 1,797	1,865	B	32,415 ± 2,265	2,009	B	33,465 ± 1,494	2,865	B	40,787 ± 5,828	3,985	C
Mn	19 ± 17	2.2	A	23 ± 27	1.3	A	5.3 ± 5	0.3	B	18 ± 9	1.5	A	6 ± 4	0.5	B
Co	0.03 ± 0.03	0.01	A	0.01 ± 0.03	0.01	B	0.02 ± 0.01	0.01	BC	0.01 ± 0.03	0.01	ABC	0.012 ± 0.01	0.01	C
As	1.0 ± 1.9	0.3	A	16 ± 13.5	0.8	B	26 ± 28.2	1.3	B	2.3 ± 2.2	0.1	C	3.2 ± 3.7	0.2	C
Rb	1.0 ± 0.5	0.1	A	2.5 ± 0.9	0.2	B	2.5 ± 1.6	0.2	B	0.6 ± 0.2	0.1	A	1.8 ± 1.0	0.2	C
Sr	3,549 ± 295	401	A	3,689 ± 195	211	B	3,610 ± 180	220	A	3,552 ± 161	299	ABC	3,436 ± 321	330	C
Ba	15 ± 6.3	1.8	A	14 ± 4.0	0.8	A	14 ± 6.1	0.8	AB	15 ± 3.7	1.2	AB	12 ± 5.9	1.3	B
Pb	0.03 ± 0.05	0.02	A	0.03 ± 0.09	0.01	A	0.02 ± 0.05	0.01	A	1.62 ± 1.00	0.09	B	0.05 ± 0.07	0.02	C
Cu	1.5 ± 3.2	0.2	AB	0.8 ± 2.0	0.1	A	1.4 ± 1.2	0.1	B	1.5 ± 1.1	0.2	B	2.1 ± 3.4	0.2	B
Zn	11 ± 12.3	1.3	AB	13 ± 8.3	0.8	A	12 ± 6.2	0.8	AB	11 ± 6.4	0.8	AB	9 ± 4.0	0.9	B

Table 4.4 Standardised canonical coefficients from adult data for discriminant functions (DF) 1 and 2 for each element entered in the final DFA. Coefficients represent the relative contribution of each element to each DF.

element	DF1	DF2
Mg	-0.76	0.51
Pb	-0.32	-0.23
Mn	-0.11	-0.75
As	0.81	-0.10
Rb	-0.03	0.63
Li	0.13	0.11
Co	-0.03	-0.28

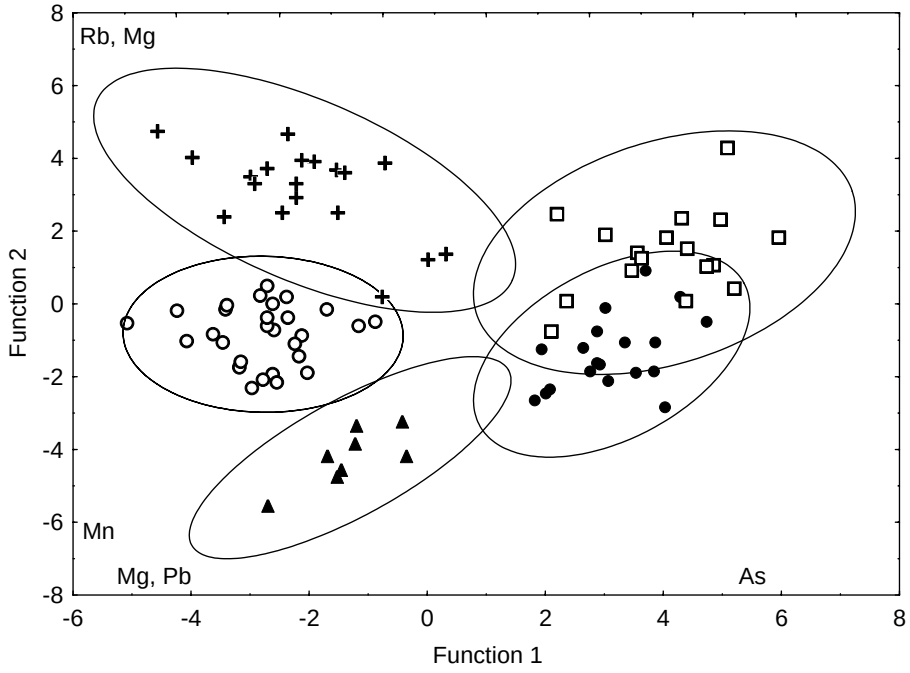


Figure 4.3 Canonical discriminant plots comparing multi-elemental signatures of adults among sites. Elements that contributed most variation to the data (canonical coefficients) are indicated at both ends of each axis. Ellipses represent 95 % confidence intervals around each group. Sites are: Circular Head (○), east Flinders Island (●), west Flinders Island (□), Northwest Bay (▲), and Mercury Passage (+).

Table 4.5 Predicted group memberships from cross-validation classification procedures for each site, based on DFA scores from adult data. Results are given as a percentage of the total number classified to each site with correct classifications highlighted in **bold**. The sites are: Circular Head (CH), west Flinders Island (WFI), east Flinders Island (EFI), Mercury Passage (MP), and Northwest Bay (NWB).

site	CH	WFI	EFI	NWB	MP
CH	**100**	0	0	0	0
WFI	0	**94.7**	5.3	0	0
EFI	0	0	**100**	0	0
NWB	0	0	0	**100**	0
MP	0	0	0	0	**100**

Hatchling signatures

All single stylet elemental signatures, except Sr, were significantly different among sites (one-way ANOVAs: $p < 0.05$; Table 4.6). All elements, except Ba, differed between CH/NWB, in contrast, only four and five elements differed between CH/MP and MP/NWB respectively (Table 4.7). Hatchling concentrations were several magnitudes higher in comparison to the adult elemental concentrations, with only two elements (Mg and Mn) sharing a similar relative concentration pattern to any adult signatures from CH, NWB and MP (see Tables 4.3 and 4.7). The hatchling multi-element signature was also highly significant among sites (MANOVA: Pillai's trace = 1.56, $F_{14, 78} = 20.45$, $p < 0.001$). All elements, except Zn and Mn contributed significantly to the discrimination among sites (DFA: $p < 0.05$), with DF 1 and 2 describing 78 % and 22 % of the variation respectively. The canonical coefficients indicated that, overall, Rb, Cu and Mg were major contributors to the variation among sites (Table 4.8). The canonical plot showed a distinct separation between the three sites, with CH and MP separated from NWB largely by differences in Rb and Mg/Cu respectively (Fig. 4.4). However, MP and CH, which both had small sample sizes, had considerable within-site variation. There was some similarity between CH and NWB elemental signatures, which is reflected in misclassification of

three CH individuals to NWB (Table 4.9). However, overall classification success was high (84 %).

Table 4.6 One-way ANOVAs comparing single-elemental hatchling signatures among sites. Element concentrations were transformed as described in Methods

Source	df	MS	F	p
Li				
site	2	0.41	21.04	0.000
error	44	0.02		
Ba				
site	2	1.04	21.39	0.000
error	44	0.05		
Sr				
site	2	0.00	0.8	0.436
error	44	0.00		
Cu				
site	2	1.42	12.66	0.000
error	44	0.10		
Zn				
site	2	0.60	11.56	0.000
error	44	0.04		
Rb				
site	2	0.94	17.26	0.000
error	44	0.05		
Mg				
site	2	0.25	16.54	0.000
error	44	0.01		
Mn				
site	2	0.07	5.72	0.005
error	44	0.01		
As				
site	2	0.96	12.73	0.000
error	44	0.08		
Pb				
site	2	2.17	29.61	0.000
error	44	0.06		

Table 4.7 Mean concentration of elements (ppm ± SD) in hatchling for each of the three sites. AP = mean analytical precision (ppm), T = results from Tukeys HSD, with significant differences ($p < 0.05$) indicated by letters (A, B). The sites are: Northwest Bay (NWB), Mercury Passage (MP), and Circular Head (CH), n = number of successful ablations, II = mean integration interval (seconds).

element	**CH** (n = 11; II = 6.3)			**MP** (n = 9; II = 3.8)			**NWB** (n = 27; II = 7.2)		
	mean ± SD	**AP**	**T**	**mean ± SD**	**AP**	**T**	**mean ± SD**	**AP**	**T**
Li	19 ± 18	7.7	B	13 ± 7.5	3.5	B	7 ± 3.4	2.0	A
Mg	133,023 ± 110,380	19,544	B	133,348 ± 71,886	25,082	B	78,869 ± 31,878	8,621	A
Mn	86 ± 40	25	A	63 ± 22	16	AB	63 ± 35	9	B
Rb	71 ± 73	12	A	36 ± 30	7	B	23 ± 25	3.2	B
Cu	442 ± 653	84	B	401 ± 371	81	B	153 ± 238	19	A
As	310 ± 270	77	A	132 ± 145	33	B	107 ± 32	32	B
Pb	169 ± 286	28	A	43 ± 46	10	B	27 ± 32	4	B
Ba	46 ± 52	16	A	186 ± 144	43	B	64 ± 79	11	A
Zn	729 ± 1,063	255	A	831 ± 445	153	A	1,621 ± 776	77	B

Table 4.8 Standardised canonical coefficients from hatchling spatial data for discriminant functions (DF) 1 and 2 for each element entered in the final step-wise DFA. Coefficients represent the relative contribution of each element to each DF.

element	DF1	DF2
Pb	0.74	0.58
Ba	1.04	0.27
Rb	-1.81	0.74
Mg	1.35	0.18
Cu	1.21	0.85
As	-0.72	-1.84
Li	-0.47	0.53

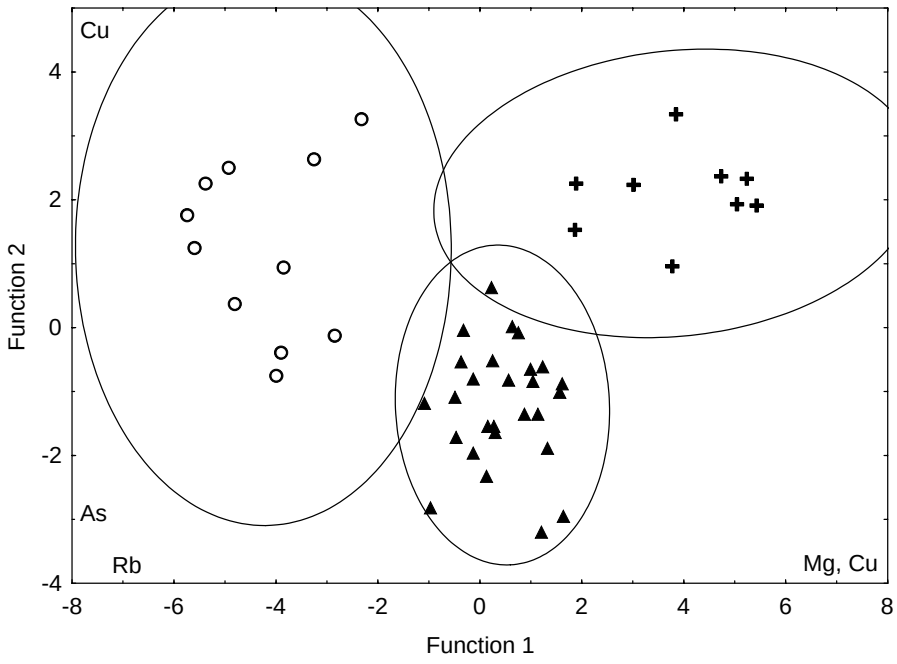


Figure 4.4 Canonical discriminant plots comparing multi-elemental signatures of hatchlings among sites. Elements that contributed most variation to the data (canonical coefficients) are indicated at both ends of each axis. Ellipses represent 95 % confidence intervals around each group. Sites are: Northwest Bay (▲), Circular Head (○), and Mercury Passage (+).

Table 4.9 Predicted group memberships from cross-validation classification procedures for each site, based on DFA scores from hatchling spatial data. Results are given as a percentage of the total number classified to each site with correct classifications highlighted in **bold**. The sites are: Northwest Bay (NWB), Mercury Passage (MP), and Circular Head (CH).

site	NWB	MP	CH
NWB	**96.4**	0	3.6
MP	16.7	**83.3**	0
CH	20.0	6.7	**73.3**

Temporal variation of hatchling signatures

Temporal variation in multi-elemental hatchling signatures from NWB were highly significant (MANOVA: Pillai's trace = 1.28, $F_{14, 104} = 13.16$, $p < 0.001$). However, Sr, Mn, Rb, Pb and As, did not significantly contribute to temporal differences, with differences largely attributed to Mg and Zn (Fig. 4.5). Discriminant functions 1 and 2 described 84 % and 16 % of the variation respectively. The third collection from NWB had the most distinct signature, with the first and second collections sharing some overlap. The second canonical plot of the hatchling spatial data showed that the degree of separation between the three sites was little changed with the addition of the NWB individuals from the second and third collections. Furthermore, using the pooled NWB samples, the classification success of the hatchlings was only marginally lower, with only one extra individual from CH misclassified to NWB.

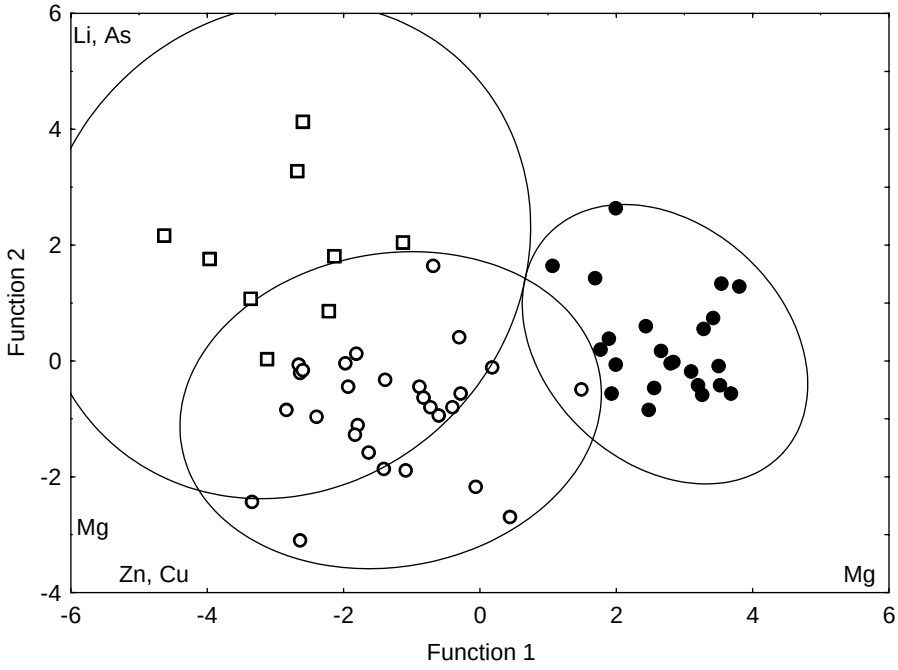


Figure 4.5 Canonical discriminant plots comparing multi-elemental signatures of hatchlings collected at Northwest Bay (NWB) at three different times from the same location. Elements that contributed most variation to the data (canonical coefficients) are indicated at both ends of each axis. Ellipses represent 95 % confidence intervals around each group. Sites are: NWB 1 (○), NWB 2 (□), and NWB 3 (●).

Ageing results showed that the adults from CH (n = 28) had a mean age of 262 (SE ± 13.1) days or 8.7 months (Fig. 4.6). This is close to the desired age-cohort of the adults, which were collected 234 days or 7.8 months after the hatchlings (ie. adults would have hatched at a similar time to the hatchling analysed for the hatchling signatures). Although the majority were ≤ 6 months old, a third of the animals were over 10 months old.

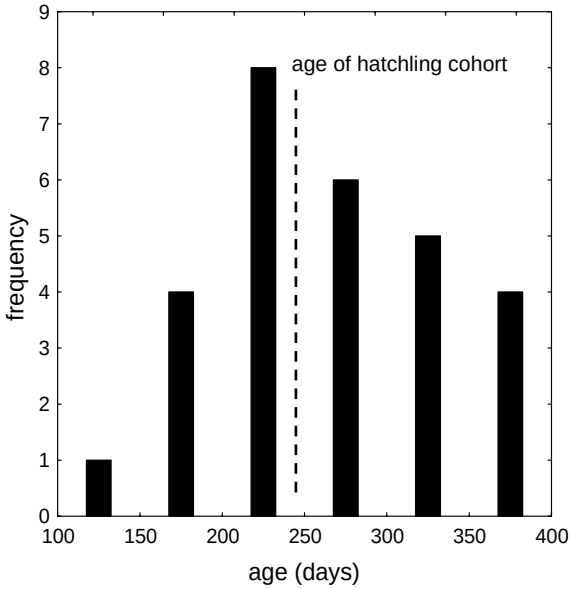


Figure 4.6 Age distribution of individuals (n = 28) collected from Circular Head (CH). Dotted line represents the theoretical age of the hatchling cohort at the time the adults were collected (7.8 months later).

DISCUSSION

The multi-elemental signatures of the five adult groups suggest that *O. pallidus* has a highly structured population with the vast majority of individuals from within each group appearing to share a common origin. Although there was some differentiation between the two closest sites, west and east Flinders Island, which are approximately 85 km apart, it is possible that the localised spatial range of each sub-population is substantially smaller. Little is known about the post-hatchling behaviour of cephalopods, however, it has been suggested that horizontal dispersal distance may be as small as one kilometre in small holobenthic cephalopods (Boletzky 2003). Based on the consistency of the results and the holobenthic life history strategy of *O. pallidus* it is likely that the natal origins of each sub-population are proximate to the respective collection sites. The very high level of Pb found in all Northwest Bay signatures is also suggestive of such dispersal patterns. Northwest Bay is likely to have higher Pb levels than the other sites due to its proximity to the Derwent estuary, a relatively polluted

water body with significantly elevated levels of Pb (Jones et al. 2003). The population structure of *O. pallidus*, therefore, reflects an expected congruence between early life history strategy and level of dispersal from the parental population. In contrast, a stylet elemental signature study of the merobenthic *O. maorum* population indicated some structure, but also a degree of connectivity (see Chapter 3). There are no published studies which have utilised elemental signatures to investigate holobenthic cephalopod populations. However, using genetic markers, high levels of structure have also been identified in two species of holobenthic cuttlefish, with populations showing subdivision on a scale of only 300 km (Perez-Losada et al. 2002; Kassahn et al. 2003).

The structure of the *O. pallidus* population has important implications for the commercial fishery, which includes the areas of Circular Head and west and east Flinders Island. Due to limited replenishment from neighbouring sources and limited dispersal from their natal habitats, these apparently localised sub-stocks may be vulnerable to rapid depletion from fishing and natural environmental change. Disregarding stock complexity in fisheries management may cause a reduction in productivity, stock abundance, genetic diversity, and a species' adaptive potential (Stephenson 1999; Hauser et al. 2002). Furthermore, models have predicted that biological parameters obtained from several aggregated sub-stocks and applied to a single population management approach are likely to be inaccurate and non-conservative (Frank & Brickman 2000). Additional repeat sampling on smaller spatial scales will be needed to define the maximum size of the *O. pallidus* sub-stocks within the region, and a further genetic study would elucidate if such population subdivisions occur on an inter-generational time scale. However, currently, to maintain population complexity as identified in this study, the stocks at Circular Head and west and east Flinders Island should be considered as discrete management units.

Hatchling signatures could not be used to classify adults back to their natal site due to significant differences between hatchling and adult signatures. Elemental concentration in the hatchling stylets, compared to the adult stylets, were orders of magnitude higher suggesting that the procedure which was used to determine the stylet material from the tissue and epoxy (see Fig 4.2) was unsuccessful or that the level of Ca (the internal standard) may be lower than 13 % in the hatchling stylets. In consideration of the latter, the raw data from the laser ablation analysis indicate that hatchling stylets contain Ca; however, it is plausible that this soft pliable and transparent structure (in comparison to the tough opaque adult structure) is not fully calcified at hatching. There are several studies which have successfully used juvenile signatures, obtained from $CaCO_3$-based structures, to classify adults back to their site of origin (eg. Hamer et al. 2005; Becker et al. 2007; Feyrer et al. 2007). However, stylets are not composed of $CaCO_3$ and are thought to be largely organic (see Chapter 2 for more details). It is important, therefore, that the process of stylet formation is examined further and that the concentration of Ca is quantified in hatchling and juvenile stylets. If stylets became 'completely' calcified within 1 – 2 weeks after hatching, day-old hatchlings collected from the wild could be raised in a closed aquaria system (containing water from the sample site) for 1 – 2 weeks prior to dissection. Although this may slightly alter the water chemistry, confounding effects may be only marginal as embryos would still develop *in situ* for 3 – 4 months. Obtaining larger stylets from older hatchlings would also greatly improve laser ablation success. Hatchlings signatures had lower discriminating power compared to the adults, due to higher detection limits (smaller spot size) and lower analytical precision (lower ablation yield). Furthermore, small sample sizes, particularly at Mercury Passage, were wholly due to many stylets being too small to discern from the surrounding tissue. If these changes could be applied targeted elemental analysis could potentially be an effective

technique for determining juvenile and adult connectivity, natal origins, and important source populations of octopuses.

Regardless of the problems associated with the hatchling analyses the adult data should still be valid. Calcium concentration has been found to be consistent, at 13 %, between individuals within the inner regions of the adult stylet (see Chapter 2); and, in this study, the near 100 % classification success of adults back to site also suggests that Ca concentration was stable. In this study, as with many others, accurate absolute values are of relatively little importance due to the lack of matrix-matched standards, however, if analytical precision is good relative comparisons in signatures can be made (see Becker et al. 2005; Zumholz et al. 2007). However, to provide an accurate measure of an individual's environmental history, a key assumption of targeted elemental studies is that the structure does not undergo post-depositional modification (Campana 1999). The lack of relationship between elemental concentrations and age does suggest that elements do not undergo modification (calcification) from at least 4 – 12 months of age. Until it is determined that there is no such relationship between the ages of 1 – 4 months, it is possible to argue that the signature from the pre-hatch region could represent the first four months of an individual's life. Although it seems unlikely that the individuals collected at each site hatched elsewhere, such dispersal patterns may be masked by a signature which represents several months rather than just the pre-hatch stage.

The site separation and classification success of hatchling individuals indicates that Ca concentration was relatively consistent between stylets, and thus relative comparisons between signatures can still be made. The assessment of short-term temporal stability of hatchling signatures indicates some temporal variation within a time scale of three and half months, however, the results indicated that spatial variation largely outweighed temporal variation. The age data from Circular Head found that although many individuals were the correct age (ie. matched the hatchling cohort)

some were over 10 months old. However, as temporal variation has shown to have a minimal effect on signatures within a three month time period, this suggests that the majority of adults were within the correct age range if they were to be classified back to their natal origins. However, *O. pallidus* has been aged up to 18 months old (Leporati et al. 2008), therefore, it would be important to either assess the level of temporal variation over at least an eight month time scale and its influence on spatial variation or select individuals which are the correct age.

This study indicates that *O. pallidus* has a highly structured population, which reflects the species' early life history strategy. The results from the adult analyses further support that targeted elemental analysis is an effective method for examining the population structure of octopuses. However, further studies investigating the relationships between stylet composition and factors such as water chemistry and temperature, will be important to facilitate the interpretation of the data. Temporal variation in elemental signatures should be considered and assessed in future studies to ensure that it is does not confound spatial analyses, particularly if hatchling signatures will be used to classify adult samples back to their natal origins. The results from the hatchling signatures indicate that they can be used to discriminate between sites, but, due to their possibly lower Ca levels and incredibly small size, are currently limited in their potential as tags of natal origin. However, improvements to the methods, such as retaining hatchlings for several weeks in aquaria prior to dissection, may rectify these initial problems. This is the first known study to use stylet element signatures to examine the population structure of a holobenthic octopus species, and as such will not only have important implications for the commercial *O. pallidus* fishery, but will have beneficial applications to other commercial species around the world.

5

Microsatellite DNA markers & morphometrics reveal a complex population structure in a merobenthic octopus species

This research is published as:

Doubleday ZA, Semmens JM, Smolenski AJ, & Shaw PW. 2009. Microsatellite DNA markers and morphometrics reveal a complex population structure in a merobenthic octopus species (*Octopus maorum*) in south-east Australia and New Zealand. *Marine Biology*. 156: 1183-1192.

ABSTRACT

Five polymorphic microsatellite loci were developed and then used to assess the population genetic structure of a commercially harvested merobenthic octopus species (*Octopus maorum*) in south-east Australian and New Zealand (NZ) waters. Beak and stylet morphometrics were also used in conjunction with the genetic data. Overall, genetic variation across all loci and the five sample populations was very high (mean number alleles = 15, mean expected heterozygosity = 0.85). Microsatellites revealed a significant level of structuring (overall F_{ST} = 0.024, $p < 0.001$), which did not fit an isolation-by-distance model of population differentiation. Divergence was observed between Australian and NZ populations, South Australia and north-east Tasmania and two relatively proximate Tasmanian sites. The South Australian and southern Tasmanian populations were genetically homogeneous, indicating a level of connectivity on a scale of 1,500 km. The morphometrics also indicated significant differences between Australian and NZ populations. The structural patterns identified can be explained largely in relation to the regional oceanographic features.

INTRODUCTION

The genetic structure of a population is shaped by the level of reproductive exchange between individuals, and the spatial and temporal scale at which this occurs. Populations may be freely inter-breeding (panmictic), consist of partially closed sub-populations which depend on some exchange from neighbouring sources, or form relatively independent sub-populations (Kritzer & Sale 2004; Waples & Gaggiotti 2006). Although it is generally assumed that the scale of population substructure is inversely related to dispersal potential, population structuring has been observed in many species which have a high capacity for dispersal (see Palumbi 1995; Palumbi 2004 for examples). Structural patterns, therefore, are often defined (particularly in species with planktonic larvae) by a complex interaction of factors, such as oceanographic features, behaviour, and historic environmental changes (Avise 2004). It is vital that any population division at the genetic level is correctly identified and incorporated into fisheries and conservation management plans, in order to prevent localised extinction and to conserve the genetic diversity within and between genetically distinct stocks (Carvalho & Hauser 1994). Genetic diversity is essential for maintaining a population's adaptive potential and thus its ability to deal with changing environmental conditions, such as global warming (Hauser et al. 2002).

Compared to teleosts, surprisingly few genetic studies have been conducted on commercially important marine invertebrates (Thorpe et al. 2000). In spite of the increasing commercial significance of cephalopods, very little has been studied on their genetic population structure (Buresch et al. 2006). Due to the generally very low levels of genetic variability observed in cephalopods relatively conservative markers, such as allozymes (Brierley et al. 1995; Triantafillos et al. 2004), mitochondrial DNA (Oosthuizen et al. 2004) and Random Amplified Polymorphic DNA (RAPD) (Hermosilla 2004), have had mixed success at resolving cephalopod populations. Highly polymorphic microsatellite markers have successfully

identified genetic structure in several cephalopod species (see Semmens et al. 2007 for review), and in some cases, have revealed structure which was previously undetected by more conservative markers (eg. Shaw et al. 1999; Perez-Losada et al. 2002). Although microsatellites are a favourable choice for genetic studies of cephalopods, to date species-specific microsatellites have been isolated from only one octopus species, *Octopus vulgaris* (Greatorex et al. 2000). Consequently, microsatellite-based population studies have been solely restricted to this species, identifying genetically differentiated sub-populations in all cases (see Casu et al. 2002; Murphy et al. 2002; Cabranes et al. 2008).

Analysing phenotypic variation in morphological characteristics is one of the simplest methods to examine structure at the intra-specific level (see Swain & Foote 1999 for examples). However, because phenotypic plasticity may have environmental rather than genetic origins it is useful to combine examinations of morphological variation with genetic studies to determine the underlying cause of variation (eg. Milton & Chenery 2001; Kassahn et al. 2003). Morphometrics have been used successfully to delineate cephalopod populations, and although cephalopods exhibit high levels of morphological plasticity and their soft body parts can be significantly altered or damaged by handling and storage, hard structures such as beaks have been shown to be particularly useful for population discrimination (Pierce et al. 1994; Vega et al. 2002; Hermosilla 2004).

Nothing is known about the genetic population structure of *O. maorum*, which is also true for other commercial merobenthic species such as *Enteroctopus dofleini* (Rigby & Sakurai 2005) and *Octopus mimus* (Rocha & Vega 2003). Although stylet elemental signatures have identified possible sub-structuring in the *O. maorum* population at the demographic level (see Chapter 3), information obtained from natural chemical signatures (derived from hard biomineralised structures within the organism) is directly related to the immediate structural and movement patterns of a population. In contrast, only genetic-based methods can determine the average levels of

gene flow among groups of individuals over an inter-generational time scale, thus providing information on levels of genetic diversity and longer-term structural trends within a population (Purcell et al. 2006; Semmens et al. 2007).

The overall aim of this study was to isolate species-specific polymorphic microsatellite markers from *O. maorum*, and then, using these markers, investigate levels of gene flow and genetic structure within the population in south-east Australian and New Zealand waters. Data on beak and stylet morphometrics taken from the same individuals were also analysed and compared to the genetic results.

MATERIALS & METHODS

Octopus collection

Octopuses were collected from five sites: north-east Tasmania (NE), south-west Tasmania (SW), the Eaglehawk Bay aggregation (EHB) in south-east Tasmania, South Australia (SA) and New Zealand (NZ) (Fig. 5.1). For more details on octopus collection and assessment of maturity see Chapter 3. A tissue sample was taken from either the arm or mantle muscle and preserved in 70 – 80 % ethanol. For the morphometric analyses stylets were air dried for 24 hours prior to dry storage in vials and beaks were preserved in 70 % ethanol.

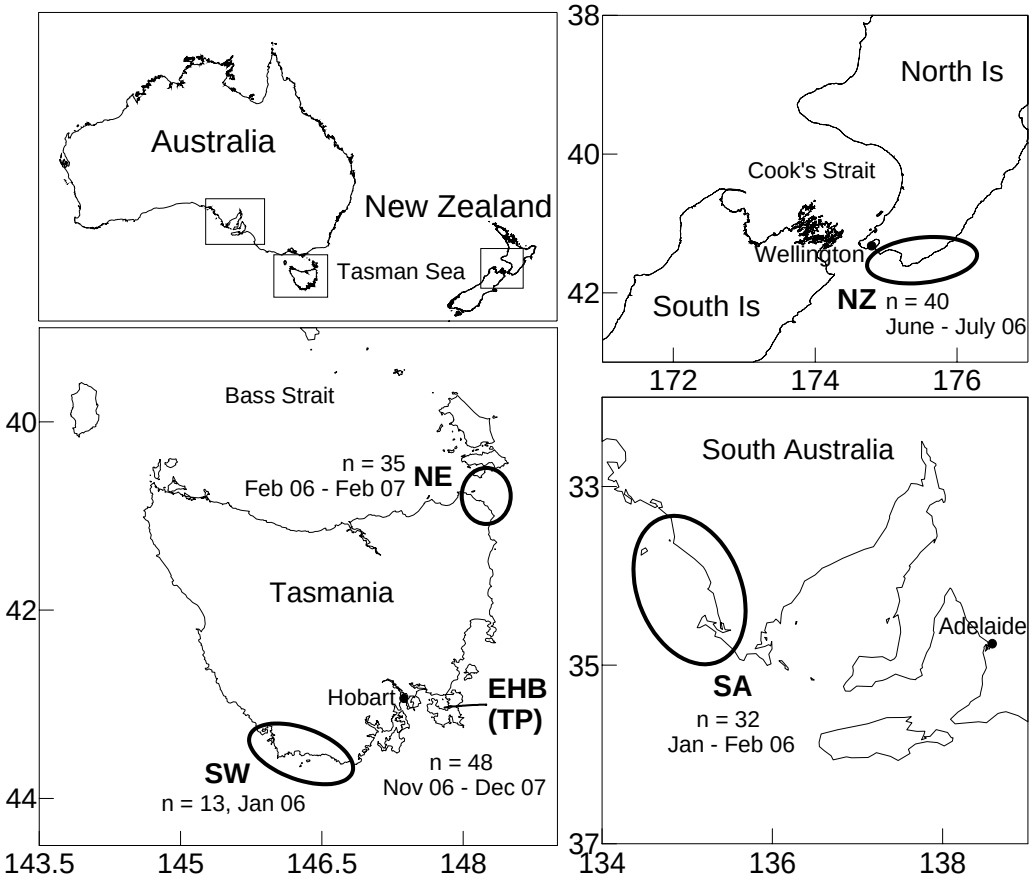


Figure 5. 1 The sample locations, sample sizes (n), and collection dates of *Octopus maorum*. Sites are: South Australia (SA), north-east Tasmania (NE), New Zealand (NZ), south-west Tasmania (SW), and the Eaglehawk Bay aggregation on the Tasman Peninsula (EHB – TP).

Microsatellite isolation, screening and data analysis

Total DNA was extracted from one individual using a standard CTAB phenol-chloroform protocol for the development of microsatellite loci. An enriched size-selected genomic library was constructed following the FIASCO (fast isolation by ALFP of sequences containing repeats) protocol (Zane et al. 2002) with some modifications. The DNA was digested with *Mse*I restriction endonuclease (New England BioLabs) and ligated to an *Mse*I-adaptor (Geneworks). The digested-ligated fragments were amplified (see Zane et al. 2002 for conditions) with *Mse*I adaptor-specific primers and hybridised with three biotinylated probes [$(ATT)_9$, $(CA)_{18}$ and $(AAAG)_6$] for 15 min at room temperature. DNA molecules hybridised with the probes were selectively captured by streptavidin-coated beads using the

Dynabeads kilobaseBINDER kit (Dynal Biotech) following the manufacturer's protocol. DNA was released from the biotinylated probe-bead complex with 10 mM Tris at 85° C for 3 min. Recovered DNA was re-amplified with the *Mse*I adaptor-specfic primers as described in Zane *et al*. (2002). The fresh PCR products were ligated to a pCR 4-TOPO plasmid vector for 30 min at room temperature and cloned using TOPO TA Cloning kit (Invitrogen) with One Shot TOP10 chemically competent cells following the manufacturer's protocol. About 175 clones were amplified with universal vector-specific M13 primers, visualised with gel electrophoresis, and sequenced using the Dye Terminator Cycle Sequencing with Quick Start Kit and a CEQ8000 Genetic Analysis System (Beckman Coulter) or by a commercial sequencing service (Macrogen Inc, Korea).

Twenty-two sequences contained a microsatellite repeat which was of sufficient quality for primer design. Primer pairs were designed using PRIMER 3 (Rozen & Skaletsky 2000), and amplification efficacy was tested using Amplify 3 (http://engels.genetics.wisc.edu/amplify). After being optimised over a range of annealing temperatures (T_A), ten primer pairs produced a single product of predicted size. PCR reactions were performed under the following conditions: initial denaturation of 3 min at 95° C, followed by 35 cycles of 30 s denaturation at 95° C, 35 s at the specific annealing temperature, and 50 s extension at 72° C, then a final extension step of 72° C for 5 min. Each reaction mixture (25 µl total volume) contained: 1.5 µl (20 – 50 ng) of template DNA, 2.5 µl of 10x buffer (670 mM tris pH 8.8, 166 mM $(NH_4)_2SO_4$, 4.5 % Triton-X, 2 mg/ml gelatin), 1 – 1.5 mM of $MgCl_2$, 0.4 µM of each primer, 125 µM dNTPs (Bioline), and 1 unit of *Taq* polymerase. To test for polymorphism each forward primer was end-labelled with different WellRED fluorescent dyes (Sigma Genosys), and fragments were sized using CEQ8000 Genetic Analysis Systems (Beckman Coulter). Five loci produced consistent amplifications and displayed levels of allelic polymorphism suitable for population level analyses (see Table 5.1

for details). Six microsatellite loci isolated from *O. vulgaris* (Greatorex et al. 2000) were also trialled, but they failed to amplify the *O. maorum* DNA.

Table 5.1 Characteristics of five microsatellite loci isolated from *Octopus maorum*: primer sequences, repeat motif, optimal annealing temperature (T_A, °C), $MgCl_2$ concentration (mM), and allele size range (bp) and number of alleles (N_A) from screening all individuals (n = 168).

locus	primer sequence 5'-3'	repeat motif	T_A	$MgCl_2$	size range	N_A
Om5	F: TCTTTGTGTTTGTCTGGGTGTC	$(AGT)_9$ $(GGT)_3$ . . .$(GGT)_4$	52	1.5	191 – 284	20
	R: TCCTGAGTAACACGATGATGA					
Om8	F: AAGAAAATTGTATTCAAACTGTCA	$(GTAT)_{19}$ $(GT)_{32}$	51	1.5	163 – 247	32
	R: AAGGTTAGGTCTTCAAAGGTG					
Om21	F: GCAGGTGAGTAGTAATGAGGCTA	$(GA)_{42}$	62	1.0	104 – 180	28
	R: CATCCCTTTGCACAGCCTAC					
Om3	F: ATGGTGAGAAGTGGGAATAGT	$(GA)_{30}$ $(GT)_{12}$	63	1.0	140 – 238	29
	R: CGTCACGTTCAACAGTAAACAC					
Om20	F: TGAGTAAGGTAATGGTGGTGAG	$(TTG)_8$ $(GGT)_6$	56	1.5	164 – 199	11
	R: CTCCAACAGTAGCAGTACCATC					

Total DNA was extracted from all sampled individuals using the HotSHOT method (Truett et al. 2000). Prior to DNA extraction muscle tissue was freed from skin and underlying mucous layers and thoroughly macerated. All individuals from all six sites were screened for variation at each of the five *O. maorum*-specific loci (see Table 5.1), using the PCR conditions described above. To improve specificity, locus Om3 was amplified using a commercial high-fidelity Sahara *Taq* (Bioline) following the manufacturer's protocol. High-fidelity *Taq* was also used on samples which failed to initially amplify due to poor quality DNA. PCR products were resolved on a CEQ8000 Genetic Analysis System and allele sizes were scored using associated Beckman Coulter Fragment Analysis software.

Allele frequencies and observed and expected heterozygosities were calculated for each locus at each population using Genalex (v. 6.1; Peakall & Smouse 2006). All loci pairs were tested for genotypic linkage disequilibrium to confirm independence, and deviations from Hardy-Weinberg (HW) expectations were estimated for each locus in each population using exact tests, with level of significance determined by the Markov chain method (Genepop, v. 4.0; Raymond & Rousset 1995). All loci were tested for possible genotyping errors, associated with null alleles and mis-scoring using Micro-Checker (v. 2.2.3; Van Oosterhout et al. 2004). Simple differentiation between populations in allele frequencies was tested using the exact G test, with multi-locus probabilities constructed using Fisher's method (Genepop). Global tests of genetic differentiation and pairwise tests of differentiation among sample populations were conducted using F_{ST} values estimated by theta (Weir & Cockerham 1984), with significant values determined using permutation procedures (FSTAT, v. 2.9.3; Goudet 1995). Significance levels were adjusted for multiple tests using the sequential Bonferroni correction (Rice 1989). To test for isolation by distance as a potential explanation for population differentiation, a Mantel matrix correlation test of geographical distance (km) against genetic divergence (F_{ST} values) was performed in Genepop.

Morphometric analyses

A total of 11 morphometric characteristics from the beaks and stylets were measured: stylet weight (SW), anterior stylet length (ASL), total stylet length (TSL) (see Fig. 5.2), beak height upper and lower (BHU and BHL), beak width upper and lower (BWU and BWL), beak length upper and lower (BLU and BLL), and hood length upper and lower (HLU and HLL) (see Fig. 5.3). Length measurements were made with digital callipers with a precision of 0.01 mm, and stylet weight was measured to 0.1 mg.

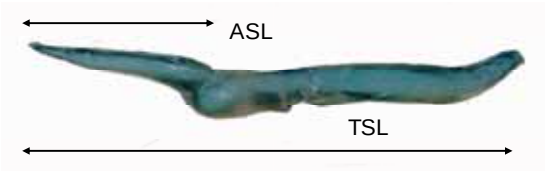


Figure 5.2 Stylet measurements: ASL = anterior stylet length, TSL = total stylet length.

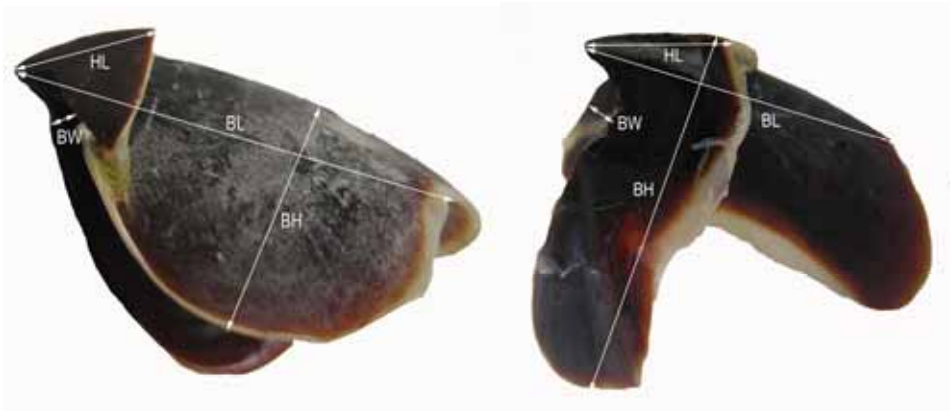


Figure 5.3 Beak measurements (upper beak – left, lower beak – right): beak height (BH), beak width (BW), beak length (BL), and hood length (HL).

Regression analyses indicated that BHU, BHL, BLU, BLL, TSL and SW had a moderate to strong relationship with body weight ($r^2 > 0.5$), and were therefore excluded from further analyses as body weight differed significantly between sites. Analysis of covariance (ANCOVA) could not be performed as all variables did not fit the assumption of homogeneity of the slopes ($p < 0.05$). One-way analysis of variance (ANOVA) were conducted

on each of the variables which had a negligible relationship to weight ($r^2 < 0.2$), which were BWU, BWL, HLU, HLU, and ASL. All *post hoc* comparisons were conducted using Tukey's HSD ($p < 0.05$). Discriminant function analysis was performed to determine the predicted group memberships of individuals based on the five characteristics listed above. Classification was based on cross-validated jack-knife procedures.

RESULTS

Within-population genetic variability

Overall, there were consistently high levels of within-population variability observed across populations and loci (see Table 5.2). The mean number of alleles for each population ranged from 10 – 18.2, and mean observed and expected heterozygosity ranged from 0.75 – 0.80 and 0.82 – 0.88 respectively. Levels of variability were consistent between populations, except for the SW, which had a markedly smaller sample size than the other sites (n = 13, compared to a grand mean of n = 31). The two tri-nucleotide loci, Om5 and Om20, were the least variable, having a mean of 11.2 and 6 alleles respectively, whereas Om8, a compound di-/tetra-nucleotide repeat, was the most variable locus having a mean of 23 alleles. No linkage disequilibrium was found between any pair of loci ($p > 0.05$). After sequential Bonferroni correction, six out of 25 single locus tests showed significant departures from HW assumptions, four of which were confined to the most polymorphic locus Om8. In contrast, Om20 had a slight excess of heterozygotes, which resulted in NE not conforming to HW expectations. Micro-Checker confirmed that overall both Om8 and Om21 had an excess of homozygotes due to the possible presence of null alleles, however, only one site significantly departed from HW expectations at the Om21 locus.

Table 5.2 Genetic variability and deviations from Hardy-Weinberg (HW) expectations within five *Octopus maorum* sample populations at five microsatellite loci. Sites are: South Australia (SA), north-east Tasmania (NE), New Zealand (NZ), south-west Tasmania (SW), and Eaglehawk Bay (EHB). N = number of individuals genotyped, N_A = number of alleles, H_O = observed heterozygosity, H_E = expected heterozygosity, p-value = probability of deviations from HW (values in **bold** remained significant after sequential Bonferroni correction).

locus	site					means
	EHB	SW	NE	SA	NZ	
Om8						
N	42	13	30	29	37	30
N_A	26	18	24	21	26	23
H_O	0.62	0.77	0.72	0.78	0.88	0.75
H_E	0.94	0.91	0.95	0.91	0.94	0.94
p value	**0.000**	**0.000**	**0.000**	**0.000**	0.030	
Om5						
N	3	13	31	29	37	31
N_A	16	8	6	15	11	11.2
H_O	0.85	0.77	0.84	0.78	0.69	0.78
H_E	0.86	0.82	0.67	0.84	0.72	0.79
p value	0.237	0.694	0.006	0.183	0.144	
Om3						
N	46	12	25	30	36	30
N_A	21	11	16	20	14	16.4
H_O	0.85	0.92	0.79	0.82	0.79	0.83
H_E	0.90	0.85	0.87	0.92	0.84	0.87
p value	0.632	0.378	0.078	0.092	0.331	
Om20						
N	43	12	35	31	40	32
N_A	6	4	7	6	7	6
H_O	0.71	0.82	0.76	0.73	0.77	0.77
H_E	0.74	0.68	0.71	0.75	0.74	0.72
p value	0.013	0.897	**0.000**	0.197	0.006	
Om21						
N	48	13	31	31	38	32
N_A	22	9	20	22	19	18.4
H_O	0.74	0.68	0.76	0.81	0.79	0.75
H_E	0.92	0.80	0.93	0.93	0.83	0.89
p value	**0.000**	0.220	0.021	0.012	0.005	
mean N	44	13	30	30	38	31
mean N_A	18.2	10.0	14.6	16.8	15.4	15
mean H_O	0.75	0.80	0.77	0.78	0.78	0.78
mean H_E	0.87	0.82	0.82	0.88	0.82	0.85

Among-population genetic variability

Overall, genetic differentiation among populations was highly significant (F_{ST} = 0.024, $p < 0.001$). Multi-locus pairwise F_{ST} values ranged from 0.001 – 0.035, with seven out of ten population pairs showing significant differentiation (see Table 5.3). F_{ST} values fell into two distinct groups: relatively high and significant values between NZ and all Australian sites (SA, NE, EHB, SW), and between NE and all other Australian sites (SA, SW, EHB, NZ) (F_{ST} = 0.025 – 0.035); and relatively low and non-significant values among the SA, SW and EHB sites (F_{ST} = 0.001 – 0.012). In comparison to F_{ST} pairwise values, after Bonferroni correction, exact tests revealed highly significant heterogeneity in allele frequencies for all multi-locus pairwise comparisons ($p < 0.001$), except for SW/EHB ($p = 0.011$). However, exact tests are much more sensitive (than F_{ST} tests) to differences in frequencies of rare alleles, particularly if loci are highly polymorphic and sample sizes are limited. The F_{ST} values, therefore, are likely to be a more realistic representation of genetic structure given the limited sample sizes used here. The Mantel test indicated no significant correlation between genetic and geographic distance among the five sample populations ($p = 0.18$). Due to a number of significant departures from HW expected genotype proportions at the Om8 locus, the FSTAT analyses were conducted again with this locus removed. Patterns of significance remained the same (as shown in Table 5.3), but overall F_{ST} values were slightly higher (overall F_{ST} = 0.03) with multi-locus pairwise values ranging from 0.001 – 0.049. Furthermore, F_{ST} values for each locus (jacknifed across populations) ranged from 0.003 – 0.058, with Om8 being the lowest, indicating that it contributed the least to observed population differentiation.

Table 5.3 Multi-locus pairwise estimates of F_{ST} among *Octopus maorum* sample populations. Sites are: South Australia (SA), north-east Tasmania (NE), New Zealand (NZ), south-west Tasmania (SW), and Eaglehawk Bay (EHB). Significant difference after Bonferroni correction is denoted as * ($p < 0.05$).

site	EHB	SW	NE	SA	NZ
EHB	-				
SW	0.007	-			
NE	0.026*	0.035*	-		
SA	0.001	0.012	0.033*	-	
NZ	0.032*	0.035*	0.025*	0.026*	-

Morphometrics

All characters not significantly correlated to body weight displayed significant differentiation between sites (one-way ANOVAs: $p > 0.001$). The *post hoc* tests clearly indicated significant differentiation in both beak and stylet morphometrics between Australian and NZ populations (Table 5.4). All Australian populations appeared homogeneous, except for NE which had one characteristic (BWU) similar to NZ. Cross-validation success was low for all Australian populations (22 – 29 %), however 65 % of the NZ individuals were successfully classified back to site (Table 5.5).

Table 5.4 Mean lengths (mm, ± SE) for each of the following morphometric characteristics (MC) for each site: anterior stylet length (ASL), beak width upper and lower (BWU and BWL), and hood length upper and lower (HLU and HLL). Sites are: South Australia (SA), north-east Tasmania (NE), New Zealand (NZ), south-west Tasmania (SW), and Eaglehawk Bay (EHB). Significant differences described by Tukey's HSD ($p < 0.05$) are indicated by letters (A, B).

MC	site				
	SA	**NE**	**EHB**	**NZ**	**SW**
BWU	6.2 ± 0.15 A	5.5 ± 0.15 B	6.2 ± 0.12 A	5.4 ± 0.15 B	-
BWL	6.0 ± 0.15 A	5.8 ± 0.15 A	6.1 ± 0.12 A	5.0 ± 0.15 B	-
HLU	10.6 ± 0.26 A	10.5 ± 0.25 A	10.8 ± 0.21 A	9.0 ± 0.25 B	-
HLL	7.3 ± 0.22 A	7.2 ± 0.20 A	7.3 ± 0.16 A	6.2 ± 0.19 B	-
ASL	5.6 ± 0.19 A	5.7 ± 0.19 A	6.0 ± 0.17 A	4.6 ± 0.18 B	5.7 ± 0.27 A

Table 5.5 Predicted group memberships from cross-validation classification procedures for each site, based on DFA scores from the multivariate data on stylet and beak morphology. Results indicate the percentage of individuals classified to each site with correct classifications in **bold**. Sites are: South Australia (SA), New Zealand (NZ), north-east Tasmania (NE), and Eaglehawk Bay (EHB).

site	SA	NE	EHB	NZ
SA	**23.8**	33.3	19.0	23.8
NE	4.5	**22.7**	45.5	27.3
EHB	39.3	21.4	**28.6**	10.7
NZ	5.0	5.0	25.0	**65.0**

DISCUSSION

The high levels of polymorphism observed across populations and loci in this study further indicates that microsatellites have the potential to be highly effective markers for resolving genetic population structure in octopuses and cephalopods in general. Significant levels of microsatellite variability have also been observed in *O. vulgaris* populations, with mean levels of expected heterozygosity reaching 0.875 (Cabranes et al. 2008) and 0.902 (Murphy et al. 2002). Microsatellites have also revealed high levels of variability (mean $H_E > 0.7$) in squid (Reichow & Smith 2001; Shaw et al. 2004; Dillane et al. 2005) and cuttlefish (Perez-Losada et al. 2002). In contrast to microsatellites, mitochondrial DNA sequences (COIII gene region) and allozyme markers have revealed little variability (mean H_E = 0.089) within *O. vulgaris* populations (Maltagliati et al. 2002; Oosthuizen et al. 2004).

In the present study, one locus in particular (Om8) exhibited an overall excess of homozygotes, which appears to be a common occurrence in octopuses (Murphy et al. 2002; Cabranes et al. 2008) and other cephalopod populations (see Wolfram et al. 2006 references therein). Although homozygote excess can be the result of non-amplifying ('null') alleles, non-random mating or the Wahlund effect (mixing of populations); in the present case the locus-specific nature of the deviations are suggestive

of null alleles as indicated by the Micro-checker analysis. Null alleles have been found to overestimate F_{ST} and genetic distance in the presence of significant population differentiation (Chapuis & Estoup 2007), however the results suggest that in this study the Om8 locus did not significantly inflate F_{ST} values.

Microsatellites revealed significant genetic structuring of the *O. maorum* population across southern Australia and New Zealand, but genetic homogeneity was also observed among a sub-set of the sample populations. Homogeneity was apparent between South Australia and southern Tasmania, sites separated by approximately 1,500 km of continental shelf waters. Over large spatial scales, the rate and direction of larval dispersal is largely influenced by the passive transport of ocean currents (Bradbury & Snelgrove 2001). Prevailing oceanic currents within the region support the possibility of long-distance dispersal between South Australia and Tasmania: the Leeuwin Current (LC) flows eastward along the south coast of Australia, which then flows southward (now called the Zeehan Current) along the Tasmanian west coast to the southern end of the island (Ridgway 2004) (see Fig. 5.4a). The Zeehan Current (ZC) is a remarkably consistent oceanographic feature, and in winter when it is strongest, flows northward around the island to the mid-east coast (see Fig 5.4b) (Crawford et al. 2000; Ridgway 2007). A model of the broad-scale transport and regional connectivity of southern rock lobster larvae in southern Australian and New Zealand waters (Bruce et al. 2007) suggests similar dispersal patterns to those suggested here for *O. maorum*. The rock lobster model predicted a relatively high level of connectivity between southern Tasmania and South Australia facilitated by the LC and ZC, and showed that larval recruitment to south-west and south-east Tasmanian populations was predominantly sourced from Tasmanian and South Australian waters. Rock lobsters have a very protracted larval stage of up to two years and thus probably a much higher capacity for dispersal than *O. maorum*. The duration of the planktonic stage in *O. maorum* is unknown, but a laboratory study has

shown that *O. vulgaris* larvae are planktonic for two months (Villanueva 1995). Data from satellite drifters indicated a travel time of approximately five months from South Australia to south-east Tasmania via the LC and ZC (Ridgway 2004), suggesting that octopus larvae may have the potential for at least occasional dispersal between the two areas. Genetic homogeneity on scales of 2,500 km (Reichow & Smith 2001) and 1,000 km (Shaw et al. 1999) has also been observed in neritic squid, however, such long-distance dispersal would probably be aided by extensive adult migrations. A study of stylet elemental signatures, obtained from the same individuals utilised in this study, suggested that the Eaglehawk Bay aggregation was a distinct sub-population from South Australia (see Chapter 3). This indicates that the degree of connectivity between the regions is probably low, as differences in elemental signatures reflect movement patterns which have occurred within the lifetime of the individuals sampled. As only low levels of exchange (as little as 1 % of individuals per generation) is required to maintain genetic homogeneity (Carvalho & Hauser 1994), and genetic differences reflect movement patterns averaged over past generations, it is likely that large scale genetic homogeneity of *O. maorum* populations from South Australia to southern Tasmania results from occasional long-distance dispersal by planktonic larvae.

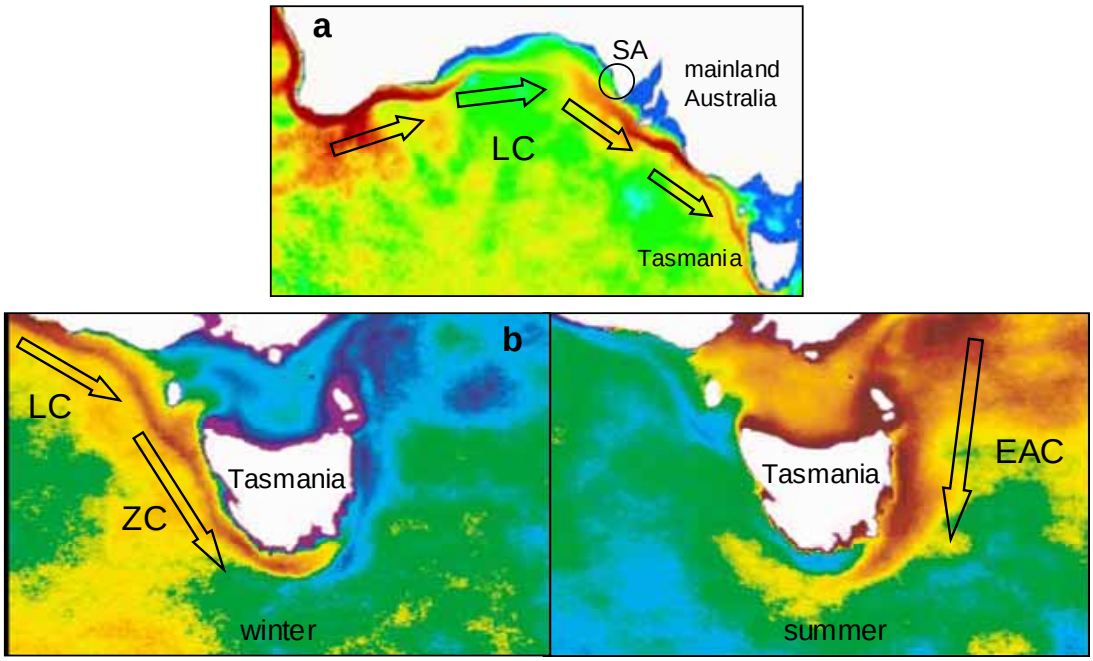


Figure 5.4 a) Sea surface temperature (SST) image showing extent of the Leeuwin Current (LC) flowing east along the south coast of Australia. The South Australian (SA) collection site has been added for reference. Image adapted from Ridgway (2004). b) SST images showing the Zeehan Current (ZC) in winter and East Australian Current (EAC) in summer around Tasmania. Images adapted from Ridgway (2007). red/yellow = warmer water, blue/green = cooler water.

In contrast, to the genetic homogeneity observed between South Australia and southern Tasmania, significant divergence was observed between South Australia and north-east Tasmania, which are also 1,500 km apart. This indicates that there is little transport of paralarvae across Bass Strait, which was also observed in the lobster model (Bruce et al. 2007). This further highlights the influence of the LC/ZC current system on the dispersal distance and direction of larval dispersal in the region. Surprisingly, the genetic results also indicated little connectivity between Eaglehawk Bay and north-east Tasmanian populations, which are only 450 km apart. Such restricted dispersal, which obviously does not conform to the isolation-by-distance model of differentiation, may be the result of local oceanographic conditions. Although the southerly flowing East Australian Current (EAC) reaches south-east Tasmania in summer, at the Tasman Peninsula it is sharply diverted from shallow shelf waters and follows a

highly variable trajectory according to the position of offshore eddies (Ridgway 2007) (see Fig. 5.4b). Eaglehawk Bay lies in a sheltered location deep within the western side of the Tasman Peninsula, and would be probably protected from the EAC current before it is diverted offshore (see Fig 5.1). Modelled dispersal trajectories of rock lobster larvae in north-east Tasmania were found to be largely influenced by EAC-associated eddies which draw water away from the shelf, with many dispersing offshore and being lost to the Tasman Sea (Bruce et al. 2007). Analysis of stylet elemental signatures from the same *O. maorum* individuals also indicated divergence between north-east Tasmania and Eaglehawk Bay locations (see Chapter 3). Although little is known about the extent of adult movement in *O. maorum*, the differentiation between north-east Tasmania and Eaglehawk Bay suggests that it is limited. Genetic differentiation on the scale of 100s of km has been observed previously in other cephalopods, for example cuttlefish populations, suggested that in the absence of larval dispersal (hatchlings are benthic), the adults do not effect widespread dispersal (Kassahn et al. 2003). Several studies have also indicated relatively fine-scale differentiation in *O. vulgaris* (Murphy et al. 2002; Cabranes et al. 2008) and squid populations (Shaw et al. 1999; Dillane et al. 2005; Buresch et al. 2006), which has been suggested to be caused by oceanographic features, bathymetry, behaviour and historic environmental changes.

Genetic divergence between Australian and New Zealand populations of *O. maorum* suggests that there is no ongoing population connectivity across the Tasman Sea. Trans-Tasman divergence has also been observed in two teleost species, even though both had a planktonic stage of nine months (Grewe et al. 1994; Tracey et al. 2007), indicating that this 2,000 km wide and 4,500 m deep tract of sea is a significant barrier to gene flow even to species with a high dispersal potential. Furthermore, satellite drifters released in Tasmanian waters took 18 months to reach New Zealand (Cresswell 2000), giving some idea of the dispersal potential which would be required to maintain connectivity. It is likely, therefore, that the

dispersal potential of *O. maorum* is just too limited to maintain panmixia on such a scale, particularly in the absence of a facilitating current. Concordance between the genetic and morphological data further confirms divergence between Australian and New Zealand populations. Furthermore, the morphological homogeneity found throughout the Australian populations suggests that morphological differences (ie. between Australia and New Zealand) may reflect longer-term divergences at a phylogenetic and temporal level compared to differences only expressed by microsatellite genotypes (ie. between north-east Tasmania and Eaglehawk Bay). Stylet elemental signatures also from the same individuals showed distinct differences (see Chapter 3), further supporting the division of sub-populations between the countries.

This study has identified a general broad-scale picture of the genetic structure of *O. maorum*. However, it would be useful to conduct a more detailed study which would include a greater number of sample locations, particularly from the extreme western end of the species' distribution in southern Western Australia, which also lies in the path of the easterly flowing LC (see Fig 5.2a). Larger sample sizes would also be preferential, particularly given the high levels of polymorphism and within-sample variability identified. Shaw et al. (1999), for instance, found that a samples size of 40 – 50 gave adequate statistical precision with 11 alleles (grand mean across samples and loci). In this study, loci Om8 and Om21 had a mean sample size of 31, but had a shared mean of 20.5 alleles, indicating that the discriminating power of these loci in particular could be improved with larger sample sizes. Furthermore, due to the presence of null alleles associated with the Om8 locus, in future studies it should either be replaced or the Om8 primers should be re-designed and re-tested.

Understanding how populations are partitioned at the genetic level is essential, not only for a biological and ecological understanding of a taxon, but for the development of sustainable management practices which aim to preserve intra-specific population complexity. This study provides an

insight into the genetic structure of a merobenthic octopus species through the application of both microsatellite markers and morphometrics, which will provide a base for the effective management of this commercial and recreational fishery. The complex structural patterns identified seem to be largely influenced by the regional oceanographic features rather than geographic distances between sites, with currents appearing to both facilitate (LC/ZC system) and restrict (EAC) connectivity between regions. This highlights the need to conduct species-specific assessments of population structure and not base them on pre-conceived assumptions based on predicted dispersal potentials.

6

General Discussion

UNDERSTANDING STRUCTURE & CONNECTIVITY – INTEGRATIVE & EMERGING METHODS

"As depletion of marine communities and resources continue, the success of conservation efforts will depend in part on the connectivity of these ecosystems and a clear understanding of them" (Bradbury et al. 2008b)

Our current understanding of spatial structure and connectivity in marine populations is sparse and insufficient for the effective conservation of marine ecosystems (Gillanders 2002; Bradbury et al. 2008). However, reconstructing the dispersal patterns of marine species throughout their life history cycle, particularly the early stages, remains one of the toughest challenges for marine biologists (Jones et al. 2005; Sale et al. 2005; Bradbury et al. 2008). Currently, there are a range of inter-disciplinary methods available to study the movement of marine organisms, both traditional and emerging, and all with inherent advantages and disadvantages.

The utilisation of several methods to investigate the population structure and dispersal patterns of a population can not only provide a basis for validating circumstantial results or results from new untried techniques, but can also provide synergistic data which covers a range of spatial and temporal resolutions. Regardless of the advantages of an integrative approach over a single method approach, surprisingly, it has been applied to only a handful of teleost populations (Milton & Chenery 2001; Miller et al. 2005; Cimmaruta et al. 2007; Feyrer et al. 2007; Bradbury et al. 2008). These latter studies and the research presented in this thesis indicate that the integration of markers which are inherited and markers which are retained for only the lifetime of the individual are a particularly useful combination. By comparing the results from the elemental data (Chapter 3) and the genetic and morphometric data (Chapter 5) several salient points can be made. The elemental signatures revealed the finest level of structure (ie. South Australia and Eaglehawk Bay were genetically homogenous but

elementally distinct), which is indicative of the techniques' differing levels of sensitivity toward delineating population subdivision (see also Ashford et al. 2006). Differences in elemental signatures reflect movement patterns which have occurred within the lifetime of the individuals sampled, whereas differences in genotypes or genetically-driven phenotypic variation reflect movement patterns averaged over past generations. The use of elemental signatures here allowed for the detection of sub-populations which are not genetically divergent but independent at a level probably more relevant for fisheries management purposes. Furthermore, the morphological homogeneity found throughout the Australian populations, which was not expressed by the microsatellite genotypes, suggests that the morphological differences between Australia and New Zealand may reflect a relatively long-term divergence. In turn, the microsatellite data elucidated some of the ambiguity inherently present in the elemental data (see also Feyrer et al. 2007). The genetic dissimilarity between north-east Tasmania and South Australia indicates that the relatively similar elemental signatures between these two sites were the result of similar water chemistries and not connectivity; two potential causes which are difficult to disentangle from elemental data alone. Congruent results undoubtedly confirm the reliability of the data and validates the techniques used (see Milton & Chenery 2001; Miller et al. 2005; Feyrer et al. 2007). In this study, for instance, congruent results confirm the more questionable genetic divergence between the relatively proximate sites in north-east Tasmania and Eaglehawk Bay, and further validate the efficacy of stylet elemental signatures as a new technique for resolving population structure in octopuses. These comparisons, and those presented in the relevant literature, highlight the synergistic benefits of using a multiple method approach, and that both concordant and discordant results help build a more complete, detailed and realistic picture of a species' population structure.

This thesis represents the first study to utilise stylet elemental signatures to investigate population linkages in octopuses. The preliminary assessment of stylet structure in Chapter 2 indicated that Ca was a suitable internal standard for laser ablation inductively-coupled plasma-mass spectrometry (LA ICPMS) due to its homogeneous distribution and consistent concentration between individuals. The following results presented in Chapters 3 and 4 indicated that elemental signatures were excellent spatial discriminators, providing unique and informative data on the population structure and dispersal patterns of both species. Targeted elemental analysis of stylets using LA ICPMS will have widespread application to other commercial octopus species, many of which may not currently have adequate management due to the lack of spatial data. However, it is important to recognise that differences in elemental signatures may reflect factors other than environmental variability, and it will be valuable to examine this in future studies. In elaboration of this technique, a profile analysis consisting of two or more spot analyses or a continuous quantification of elements along a transect could be taken across part of the stylet cross section; trends in elemental concentration could then be related to movement (Elsdon et al. 2008) and correlated with age or life history stage (eg. Fowler et al. 2005; Bradbury et al. 2008). However, a good understanding of the relationship between environmental variability and elemental variability is required to exploit the full potential of profile analysis (Elsdon et al. 2008). This can be achieved by conducting controlled laboratory experiments and testing the singular and interactive effects of different environmental parameters, such as temperature and ambient elemental concentrations (eg. Elsdon & Gillanders 2004).

Currently, one drawback of stylet elemental signatures are the difficulties associated with analysing hatchling signatures, thus limiting their ability to act as tags of natal origin and define the dispersal trajectories of early life history stages (see Chapter 4). Although larval movement is much more challenging to define than adult movement (Palumbi 2004), due

to large numbers of small larvae and high mortality rates, it is the key to understanding connectivity and structure in a definitive way. In marine populations, the early life history stage is typically the main conduit of dispersal, and thus plays a dominant role in defining the spatial structure of adult populations (Bradbury & Snelgrove 2001). Levin (2006) states that the two most pertinent questions surrounding larval dispersal are "where do they go?" and "where do they come from?". One of the most unambiguous approaches to answering such questions is to subsequently identify a natural tag, or apply an artificial one, to early life history stages at the natal site which can identify them in the adult population (Jones et al. 1999; Jones et al. 2005). Without validating the natal origins of adult recruits in a population, it is difficult, if not speculative to determine the spatial scale at which individuals have dispersed. Natural statolith signatures from hatchling squid have successfully identified the natal origins of adults (G. Pecl, unpubl. data); however, octopus statoliths are of little use for this application as they lack age-related growth (Lombarte et al. 2006).

Artificial markers overcome the key impediments associated with measuring larval dispersal, as they allow the mass-marking of individual larvae regardless of size. This can be achieved by immersing eggs or the early life history stage in a solution containing fluorescent dye (such as tetracycline and alizarin), which then becomes permanently incorporated into the embryonic biomineralised material (Jones et al. 2005). An emerging and potentially more efficient method for applying a marker artificially is transgenerational marking, which involves the maternal transmission of enriched stable isotopes (injected into the mother) to the embryonic material (Thorrold et al. 2006). This exciting new approach has potential application for cephalopods (see Semmens et al. 2007 for review), and particularly octopuses due to the analytical problems associated with natural signatures derived from hatchling stylets (see Chapter 4). However, as stylets are not composed of $CaCO_3$ (see Chapter 2), fluorescent dyes or enriched isotopes which have only been used to artificially mark fish otoliths, may not be

readily incorporated into the stylet matrix. Initial trials to mark stylets with tetracycline and alizarin have not been successful (White 2007). Much preliminary work would be required to apply these techniques to octopus, particularly aquaria-based experiments to determine a suitable compound which could be retained in the stylet throughout the lifetime of the individual.

The efficacy of highly polymorphic microsatellite markers for resolving population structure in taxa with low genetic variability and high dispersal potentials, traits common to many marine species including cephalopods, is further supported in Chapter 5. The five microsatellite loci isolated identified both a level of divergence and homogeneity among the sample populations of *O. maorum*, and were less conservative at defining sub-populations than the morphometrics measures. Although species-specific microsatellites can be laborious and require relatively specialist skills to isolate, if loci are purchased commercially such disadvantages can be overcome. Once structural patterns have been identified, microsatellites can also be used to monitor an exploited population over time to ensure that genetic diversity among sub-populations is maintained (Reichow & Smith 2001). One study has demonstrated that even populations of several million individuals are vulnerable to harvesting-induced declines in genetic diversity (see Hauser et al. 2002), such monitoring therefore should be an important part of any management strategy. Microsatellites have also been recently used to directly measure dispersal distance and direction in a species of reef fish, by conducting paternity analysis of adults and juveniles which had recruited to the population (Jones et al. 2005). Although microsatellites are one of the most effective markers for investigating intra-specific level relationships, an emerging new genetic marker, single nucleotide polymorphisms (SNPs) has some promising characteristics, including high genotyping efficiency, genome-wide coverage, and analytical simplicity (ie. unlike microsatellites, SNPs mutate in a way which can be effectively described by simple mutational models) (Morin et al.

2004). The power and versatility of genetic markers are continuing to be increased through recent developments in analytical techniques (eg. coalescent approaches, Bayesian-based clustering, and assignment testing), which are providing more accurate, detailed and direct estimations of migration, population subdivision, and the population origin of individuals (Pearse & Crandall 2004; Semmens et al. 2007).

POPULATION COMPLEXITY & FISHERIES MANAGEMENT

*"Continuing to rely on traditional stock assessments that either ignore or artificially delineate the true spatial structure of [*marine*] populations is clearly a recipe for disaster"* (Francis et al. 2007)

Worldwide, many marine fisheries are either on the brink of collapse or subject to declining catch levels (Caddy & Rodhouse 1998; Thorpe et al. 2000; Sale et al. 2005). The ineffective management of many fisheries, is in part due to the disregard or lack of understanding of the structural complexity within a harvested stock (Begg & Waldman 1999). Many studies investigating the population structure and dispersal patterns of commercial populations, including teleosts (Miller et al. 2005; Bradbury et al. 2008), bivalves (Becker et al. 2007), crustaceans (Puebla et al. 2008) and cephalopods (Dillane et al. 2005; Buresch et al. 2006), have revealed levels of structure that often render current management plans either inappropriately simplistic (the most common conclusion) or artificially complex. Such studies have not only defined the appropriate scale at which stocks ought to be managed, but have identified critical natal or 'source' habitats (Forrester & Swearer 2002; Gillanders 2005) and provided data for the effective design of marine protected areas (MPAs) (Thorrold et al. 2001). Research on population structure and dispersal, therefore, has been and will be critical for the continued protection of marine species against the potentially deleterious effects of fishing.

The traditional paradigm that marine populations are 'open' is becoming increasingly challenged as more and more studies show that populations with high dispersal potentials are surprisingly 'closed' (Levin 2006). The complex relationship between dispersal potential and structure, outlined in Chapter 1, is illustrated in this thesis through the study of both a holo- and merobenthic octopus species and through the application of multiple methods (see Table 1). Chapter 4 indicated the presence of discreet sub-populations within the Tasmanian *O. pallidus* population on a scale of < 100 km; a pattern which is probably due to the species' limited dispersal potential. Such high levels of population structure have also been observed in two species of cuttlefish, which is also indicative of their poor dispersal capability (ie. cuttlefish lack a planktonic stage) (Perez-Losada et al. 2002; Kassahn et al. 2003). Chapters 3 and 5 indicated that *O. maorum* has a less structured population than *O. pallidus*, which is in accord with their differing dispersal potentials. Low-level long-distance dispersal (enough to maintain genetic homogeneity) between South Australia and southern Tasmania is probably facilitated by the passive transport of the paralarvae by ocean currents. However, a more complex picture emerges with *O. maorum* which fails to fit the generalised paradigm of a completely open homogenous population, as both elemental signatures and genetic markers also indicated the presence of sub-populations. Both at a demographic and genetic level, for example, little connectivity was evident between Australian and New Zealand and north-east Tasmanian and Eaglehawk Bay populations. Although it is likely that the duration of the planktonic paralarval stage is just too short to enable effective dispersal across the Tasman Sea, the relatively close proximity between the latter two populations indicates that other factors, such as current systems, are restricting connectivity. Several studies have indicated population differentiation in merobenthic *Octopus vulgaris* using microsatellites (Murphy et al. 2002; Cabranes et al. 2008), and also in highly mobile squid species, using both statolith elemental signatures (Arkhipkin et al. 2004) and

microsatellites (Shaw et al. 1999; Dillane et al. 2005). Such surprisingly restricted dispersal in cephalopods has been suggested to be caused by a variety of factors, which commonly include oceanographic features (eg. currents, eddies, and upwelling) and historic climatic events (eg. changes in sea level and temperature).

Table 6.1 Summary of findings for *Octopus maorum* and *Octopus pallidus* detailing: key structural patterns and their potential causal factors, the methods by which they were identified, and the relative level of divergence and connectivity between sample populations as indicated by the different methods. Sites are: South Australia (SA), north-east Tasmania (NE), New Zealand (NZ), south-west Tasmania (SW), and Eaglehawk Bay (EHB). Techniques are: genetic markers (GM), morphometrics (M), and elemental signatures (ES).

species & life history	key structural patterns	potential causes	identifying techniques	relative level of divergence / connectivity
O. maorum merobenthic	i) connectivity between SA & EHB/SW (long distance)	passive transport of larvae via current systems	GM	not indicated by ES, suggests sample populations are not homogenous at the demographic level
	ii) divergence between Australia & NZ (long distance)	limited dispersal potential	GM, M & ES	long-term divergence, very little connectivity
	iii) divergence between NE & EHB (short distance)	restriction/diversion of larval transport by current systems	GM & ES	long-term divergence, little connectivity
	iv) divergence between EHB & all other populations	restriction/diversion of larval dispersal by current systems	ES	not indicated by GM in all cases, suggests low-level connectivity with other populations
O. pallidus holobenthic	all sample populations were divergent on scales of $\leq$ 100 km	limited dispersal potential	ES	short-term divergences, require GM / M study to determine if long-term

The results presented in Chapters 3 – 5 clearly indicate that the two octopus populations investigated are not simple singular units, and that structure is defined (particularly in species with planktonic larval stages) by a complex interaction of several factors (see Table 1). It is essential, therefore, that the management and conservation of cephalopods, or indeed other marine taxa, be based on species-specific assessments of population structure and not pre-conceived assumptions based on predicted dispersal potentials. Although the fisheries examined in this thesis are relatively small and developmental, it will be important to incorporate the structural patterns identified into fisheries management plans to help ensure the sustainability of *O. maorum* and *O. pallidus* stocks. For *O. pallidus*, the elemental data clearly indicated divergence between western and eastern Bass Strait stocks (Chapter 4), and although the population has only been investigated using a single method it would be prudent to adopt a precautionary approach (assume that the sub-populations are discreet) until additional studies are conducted (see Table 1) (Stephenson 1999). For *O. maorum*, the individuals from the intensively fished aggregation in Eaglehawk Bay were genetically homogenous with individuals from South Australia, indicating a level of connectivity between the two regions (Chapter 5). This is in discordance with the elemental data, which suggests that the Eaglehawk Bay individuals were predominantly sourced from a local common origin (Chapter 3). However, as genetic markers are much more conservative at defining sub-populations the latter results are probably more relevant for fisheries management applications.

FUTURE DIRECTIONS & CONCLUSIONS

The research presented in this thesis is one example of the successful employment of a novel and integrative approach to study the structural and dispersal patterns of a marine invertebrate. It is evident that natural stylet elemental signatures are a valuable new tool for studying octopus

populations which will have worldwide application to other commercial species. However, controlled experiments investigating the relationship between stylet composition and factors such as water chemistry and temperature, diet, and physiology, will be an important step to facilitate the interpretation of stylet elemental signatures. It will be also very valuable to adapt the technique so that larval dispersal patterns can be directly determined. Chapter 3, for example, suggests that individuals from the Eaglehawk Bay aggregation, the location of the fishery, are sourced from a local common origin. To effectively manage this fishery it will be important to validate this assumption and define the location and geographical extent of their natal origins. The next step, therefore, will be to define or create a unique stylet marker which can be used to 'tag' the early life history stage at the natal site. Artificial markers applied either through maternal transmission or direct immersion (see Jones et al. 2005; Thorrold et al. 2006) offer a promising alternative to natural signatures for examining the dispersal trajectories of early life history stages of both holo- and merobenthic octopuses. Initially, however, the chemical constituents of the stylet need to be defined more accurately (see Chapter 2) and analysed in more species, particularly as stylet morphology can vary considerably at the inter-specific level. Models which combine the biological characteristics of the species, such as mortality and duration of the planktonic stage, and the hydrodynamic characteristics of the local oceanographic features could also offer further insights into dispersal patterns of planktonic larvae (see Cowen et al. 2000; Bruce et al. 2007). Little is known about the development, behaviour and mortality of the early history stages of many cephalopods (Boletzky 2003). Therefore, further studies, such as determining the duration of the paralarval stage of *O. maorum* and 'swimming' behaviour of both paralarvae and benthic hatchlings (ie. do they actively seek preferential habitat or remain close to the parental population), will be an important step in obtaining a thorough understanding of the dispersal patterns of octopuses.

Although the most robust and holistic inferences on population structure are achieved through integration, there is a surprising paucity of such studies. It will be important therefore that more multi-disciplinary approaches are adopted and that the most informative combinations are defined. The synthesis of Chapters 3 and 5 outlined earlier in this Chapter indicates that inherited markers (genetics and morphometrics) and elemental signatures are a particularly informative combination due to their differing levels of sensitivity at resolving population structure. Variation in parasite fauna has also been successfully used as a biological marker, and, like elemental signatures, represents the movement patterns of an individual for part or all of its life, and are thus especially useful when combined with genetic studies (MacKenzie 2002). There has been several parasitological studies on squid with some promising results, however, much more research is required into this area before parasites can be utilised as natural markers (Semmens et al. 2007). The use of tracking technology to directly examine population structure and connectivity has been limited, although one study has successfully used acoustic telemetry to investigate fine-scale movement of adult squid within and between geographically discreet spawning grounds (Pecl et al. 2006). The integration of tracking with genetic and elemental or parasitological information could add a third more detailed layer of resolution, potentially providing even further insights, both independently and synergistically, into the spatial relationships between conspecifics.

Assessing and preserving population complexity is crucial for maintaining healthy marine populations, it not only influences a species' distribution, adaptive potential, persistence and resilience to harvesting, but defines the appropriate scale at which stocks ought to be managed. The literature generally implies that our current understanding of spatial structure and connectivity in marine populations is limited and many exploited populations are potentially mismanaged at the spatial level. However, through the constant improvement of traditional methods and

the development of novel ones, coupled with the synergistic advantages derived from integration, our understanding of spatial linkages and partitioning in marine populations is continuing to grow, along with our ability to successfully manage and conserve marine resources.

Literature Cited

Arkhipkin, A. I., Campana, S. E., Fitzgerald, J. and Thorrold, S. R. (2004). Spatial and temporal variation in elemental signatures of statoliths from the Patagonian longfin squid (*Loligo gahi*). *Canadian Journal of Fisheries and Aquatic Sciences* 61: 1212-1224.

Arreguín-Sánchez, F., Solís-Ramirez, M. J. S. and González de la Rosa, M. E. (2000). Population dynamics and stock assessment for *Octopus maya* (Cephalopoda: Octopodidae) fishery in the Campeche Bank, Gulf of Mexico. *Revista de Biologica Tropical* 48: 323-331.

Ashford, J. R., Arkhipkin, A. I. and Jones, C. M. (2006). Can the chemistry of otolith nuclei determine the population structure of Patagonian toothfish *Dissostichus eleginoides*? *Journal of Fish Biology* 69: 708-721.

Avise, J. C. (2004). *Molecular markers, natural history and evolution*. Massachusetts, Sinauer Associates Publishers.

Becker, B. J., Fodrie, F. J., McMillan, P. A. and Levin, L. A. (2005). Spatial and temporal variation in trace elemental fingerprints of mytilid mussel shells: a precursor to invertebrate larval tracking. *Limnology and Oceanography* 50: 48-61.

Becker, B. J., Levin, L. A., Fodrie, F. J. and McMillan, P. A. (2007). Complex larval connectivity patterns among marine invertebrate populations. *Proceedings of the National Academy of Sciences* 104: 3267-3272.

Begg, G. A. and Waldman, J. R. (1999). An holistic approach to fish stock identification. *Fisheries Research* 43: 35-44.

Bizikov, V. A. (2004). The shell in Vampyropoda (Cephalopoda): morphology, functional role and evolution. *Ruthenica* 3: 1-88.

Boletzky, S. v. (2003). Biology of early life stages in cephalopod molluscs. *Advances in Marine Biology* 44: 143-203.

Boyle, P. R. and Boletzky, S. v. (1996). Cephalopod populations: definition and dynamics. *Philosophical Transactions of the Royal Society of London Series B* 351: 985-1002.

Boyle, P. R. and Rodhouse, P. G. (2005). *Cephalopods: ecology and fisheries*. Oxford, Blackwell Science.

Bradbury, I. R., Campana, S. E. and Bentzen, P. (2008). Estimating contemporary early life-history dispersal in an estuarine fish: integrating molecular and otolith elemental approaches. *Molecular Ecology* 17: 1438-1450.

Bradbury, I. R., Laurel, B., Snelgrove, P. V. R., Bentzen, P. and Campana, S. E. (2008). Global patterns in marine dispersal estimates: the influence of geography, taxonomic category and life history. *Proceedings of the Royal Society B: Biological Sciences* 275: 1803-1809.

Bradbury, I. R., Laurel, B. J., Robichaud, D., Rose, G. A., Snelgrove, P. V. R., Gregory, R. S., Cote, D. and Windle, M. J. S. (2008). Discrete spatial dynamics in a marine broadcast spawner: re-evaluating scales of connectivity and habitat associations in Atlantic cod (*Gadus morhua* L.) in coastal Newfoundland. *Fisheries Research* 91: 299-309.

Bradbury, I. R. and Snelgrove, P. V. R. (2001). Contrasting larval transport in demersal fish and benthic invertebrates: the roles of behaviour and advective processes in determining spatial pattern. *Canadian Journal of Fisheries and Aquatic Sciences* 58: 811-823.

Brierley, A. S., Thorpe, J. P., Pierce, G. J., Clarke, M. R. and Boyle, P. R. (1995). Genetic variation in the neritic squid *Loligo forbesi* (Myopsida: Loliginidae) in the northeast Atlantic Ocean. *Marine Biology* 122: 79-86.

Bruce, B., Griffin, D. and Bradford, R. (2007). *Larval transport and recruitment processes of southern rock lobster*. Hobart, CSIRO Marine and Atmospheric Research.

Buresch, K. M., Gerlach, G. and Hanlon, R. T. (2006). Multiple genetic stocks of longfin squid *Loligo pealeii* in the NW Atlantic: stocks segregate inshore in summer, but aggregate offshore in winter. *Marine Ecology Progress Series* 310: 263-270.

Bustamante, P., Teyssie, J. L., Danis, B., Fowler, S. W., Miramand, P., Cotret, O. and Warnau, M. (2004). Uptake, transfer and distribution of silver and cobalt in tissues of the common cuttlefish *Sepia officinalis* at different stages of its life cycle. *Marine Ecology Progress Series* 269: 185-195.

Cabranes, C., Fernandez-Rueda, P. and Martínez, J. L. (2008). Genetic structure of Octopus vulgaris around the Iberian Peninsula and Canary Islands as indicated by microsatellite DNA variation. *ICES Journal of Marine Science* 65: 12-16.

Caddy, J. F. and Rodhouse, P. G. (1998). Cephalopod and groundfish landings: evidence for ecological change in global fisheries? *Reviews in Fish Biology and Fisheries* 8: 431-444.

Campana, S. (1999). Chemistry and composition of fish otoliths: pathways, mechanisms and applications. *Marine Ecology Progress Series* 188: 263-297.

Campana, S. E. and Thorrold, S. R. (2001). Otoliths, increments, and elements: keys to a comprehensive understanding of fish populations? *Canadian Journal of Fisheries and Aquatic Sciences* 58: 30-38.

Campana, S. E., Thorrold, S. R., Jones, C. M., Günther, D., Tubrett, M., Longerich, H., Jackson, S., Halden, N. M., Kalish, J. M., Piccoli, P., de Pontual, H., Troadec, H., Panfili, J., Secor, D. H., Severin, K. P., Sie, S. H., Thresher, R., Teesdale, W. J. and Campbell, J. L. (1997). Comparison of accuracy, precision, and sensitivity in elemental assays of fish otoliths using the electron microprobe, proton-induced X-ray emission, and laser ablation inductively coupled plasma mass spectrometry. *Canadian Journal of Fisheries and Aquatic Sciences* 54: 2068-2079.

Carvalho, G. R. and Hauser, L. (1994). Molecular genetics and the stock concept in fisheries. *Reviews in Fish Biology and Fisheries* 4: 326-350.

Casu, M., Maltagliati, F., Meloni, M., Casu, D., Cossu, P., Binelli, G., Curini-Galletti, M. and Castelli, A. (2002). Genetic structure of *Octopus vulgaris* (Mollusca, Cephalopoda) from the Mediterranean Sea as revealed by a microsatellite locus. *Italian Journal of Zoology* 69: 295-300.

Chapuis, M. and Estoup, A. (2007). Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution* 24: 621–631.

Chistiakov, D. A., Hellemans, B. and Volckaert, F. A. M. (2005). Microsatellites and their genomic distribution, evolution, function and applications: a review with special reference to fish genetics. *Aquaculture* 255: 1-29.

Cimmaruta, R., Mattiucci, S. and Nascetti, G. (2007). Integrating genetic and parasitological approaches in the frame of multidisciplinary fish stock analysis. *Parassitologia* 49: 119-123.

Clarke, M. R. (1996). The role of cephalopods in the world's oceans: general conclusions and the future. *Philosophical Transactions of the Royal Society of London Series B* 351: 1105-1112.

Cowen, R. K., Lwiza, K. M. M., Sponaugle, S., Paris, C. B. and Olson, D. B. (2000). Connectivity of marine populations: open or closed? *Science* 287: 857-859.

Crawford, C. M., Edgar, G. J. and Cresswell, G. (2000). The Tasmanian region. *Seas at the millennium* Shepard, C. and Zann, L. P., (eds). Netherlands, Elsevier. pp: 647-660.

Cresswell, G. (2000). Currents of the continental shelf and upper slope of Tasmania. *Papers and proceedings of the royal society of Tasmania* 133: 21-30.

Dillane, E., Galvin, P., Coughlan, J., Lipinski, M. R. and Cross, T. F. (2005). Genetic variation in the lesser flying squid *Todaropsis eblanae* (Cephalopoda, Ommastrephidae) in east Atlantic and Mediterranean waters. *Marine Ecology Progress Series* 292: 225-232.

Doubleday, Z. A., Semmens, J. M., Pecl, G. T. and Jackson, G. D. (2006). Assessing the validity of stylets as ageing tools in *Octopus pallidus*. *Journal of Experimental Marine Biology and Ecology* 338: 35-42.

Elsdon, T. S. and Gillanders, B. M. (2004). Fish otolith chemistry influenced by exposure to multiple environmental variables. *Journal of Experimental Marine Biology and Ecology* 313: 269-284.

Elsdon, T. S. and Gillanders, B. M. (2005). Strontium incorporation into calcified structures: separating the effects of ambient water concentration and exposure time. *Marine Ecology Progress Series* 285: 233-243.

Elsdon, T. S., Wells, B. K., Campana, S. E., Gillanders, B. M., Jones, C. M., Limburg, K. E., Secor, D. H., Thorrold, S. R. and Walther, B. D. (2008). Otolith chemistry to describe movements and life-history parameters of fishes: hypotheses, assumptions, limitations and inferences. *Oceanography and Marine Biology: an Annual Review* 46: 297-330.

FAO. (2008). *Global Production Statistics*, *1950 - 2005*. FAO. Accessed: 19 May 2008. Available from internet: http://www.fao.org/fishery/statistics/global-production.

Farnham, I. M., Singh, A. K., Stetzenbach, K. J. and Johannesson, K. H. (2002). Treatment of nondetects in multivariate analysis of groundwater geochemistry data. *Chemometrics and Intelligent Laboratory Systems* 60: 265-281.

Feyrer, F., Hobbs, J., Baerwald, M., Sommer, T., Yin, Q. Z., Clark, K., May, B. and Bennett, W. (2007). Otolith microchemistry provides information

complementary to microsatellite DNA for a migratory fish. *Transactions of the American Fisheries Society* 136: 469-476.

Forrester, G. and Swearer, S. (2002). Trace elements in otoliths indicate the use of open-coast verses bay nursery habitats by juvenile California halibut. *Marine Ecology Progress Series* 241: 201-213.

Fowler, S. W., Gillanders, B. M. and Hall, K. C. (2005). Relationships between elemental concentration and age from otoliths of adult snapper (*Pagrus auratus*, Sparidae). *Marine and Freshwater Research* 56: 661-676.

Francis, R. C., Hixon, M. A., Clarke, M. E., Murawski, S. A. and Ralston, S. (2007). Ten commandments for ecosystem-based fisheries scientists. *Fisheries* 32: 217-233.

Frank, K. T. and Brickman, D. (2000). Allee effects and compensatory population dynamics within a stock complex. *Canadian Journal of Fisheries and Aquatic Sciences* 57: 513-517.

Gaffney, P. M. (2000). Molecular tools for understanding population structure in Antarctic species. *Antarctic Science* 12: 288-296.

Galat, A., Koput, J. and Popowicz, J. (1979). Analyses of infrared amide bands of chitin. *Acta Biochimica Polonica* 26: 303-308.

Gillanders, B. M. (2002). Connectivity between juvenile and adult fish populations: do adults remain near their recruitment estuaries? *Marine Ecology Progress Series* 240: 215-223.

Gillanders, B. M. (2005). Using elemental chemistry of fish otoliths to determine connectivity between estuarine and coastal habitats. *Estuarine, Coastal and Shelf Science* 64: 47-57.

Goudet, J. (1995). Fstat (version 1.2): a computer program to calculate F-statistics. *Journal of Heredity* 86: 485-486.

Greatorex, E. C., Jones, C. S., Murphy, J., Key, L. N., Emery, A. M. and Boyle, P. R. (2000). Microsatellite markers for investigating population structure in *Octopus vulgaris* (Mollusca: Cephalopoda). *Molecular Ecology* 9: 641-642.

Grewe, P. M., Smolenski, A. J. and Ward, R. D. (1994). Mitochondrial DNA diversity in Jackass Morwong (*Nemadactylus macropterus*: Teleostei) from Australian and New Zealand waters. *Canadian Journal of Fisheries and Aquatic Sciences* 51: 1101-1109.

Grubert, M. A. and Wadley, V. A. (2000). Sexual maturity and fecundity of *Octopus maorum* in southeast Tasmania. *Bulletin of Marine Science* 66: 131-142.

Grubert, M. A., Wadley, V. A. and White, R. W. G. (1999). Diet and feeding strategy of *Octopus maorum* in southeast Tasmania. *Bulletin of Marine Science* 65: 441-451.

Hamer, P. A., Jenkins, G. P. and Gillanders, B. M. (2005). Chemical tags in otoliths indicate the importance of local and distant settlement areas to populations of a temperate sparid, *Pagrus auratus*. *Canadian Journal of Fisheries and Aquatic Sciences* 62: 623-630.

Hauser, L., Adcock, G. J., Smith, P. J., Bernal Ramirez, J. H. and Carvalho, G. R. (2002). Loss of microsatellite diversity and low effective population size in an overexploited population of New Zealand snapper (*Pagrus auratus*). *Proceedings of the National Academy of Sciences* 99: 11742-11747.

Hermosilla, C. (2004). *Morphological and genetic variation between populations of red octopus Enteroctopus megalocyathus (Cephalopoda, Octopoda) in southern Chile and Argentina* Degree thesis. Universidad Austral de Chile, Santiago.

Highsmith, R. C. (1985). Floating and algal rafting as potential dispersal mechanisms in brooding invertebrates. *Marine Ecology Progress Series* 25: 169-179.

Ikeda, Y., Nobuaki, A., Sakamoto, W., Mitsuhashi, M. and Yoshida, K. (1999). Preliminary report on PIXE analysis for trace elements of *Octopus dofleini* statoliths. *Fisheries Science* 65: 161-162.

Jackson, G. D. (1994). Application and future potential of statolith increment analysis in squids and sepioids. *Canadian Journal of Fisheries and Aquatic Sciences* 51: 2612-2625.

Jones, B. G., Chenhall, B. E., Debretsion, F. and Hutton, A. C. (2003). Geochemical comparisons between estuaries with non-industrialised and industrialised catchments: the Huon and Derwent River estuaries, Tasmania. *Australian Journal of Earth Sciences* 50: 653–667.

Jones, G. P., Milicich, M. J., Emslie, M. J. and Lunow, C. (1999). Self-recruitment in a coral reef fish population. *Nature* 402: 802-804.

Jones, G. P., Planes, S. and Thorrold, S. R. (2005). Coral reef fish larvae settle close to home. *Current Biology* 15: 1314-1318.

Kassahn, K. S., Donnellan, S., Fowler, S. W., Hall, K. C., Adams, M. and Shaw, P. W. (2003). Molecular and morphological analyses of the

cuttlefish *Sepia apama* indicate a complex population structure. *Marine Biology* 143: 947-962.

Kritzer, J. P. and Sale, P. F. (2004). Metapopulation ecology in the sea: from Levin's model to marine ecology and fisheries science. *Fish and Fisheries* 5: 131-140.

Lambert, W. J., Todd, C. D. and Thorpe, J. P. (2003). Genetic population structure of two intertidal nudibranch molluscs with contrasting larval types: temporal variation and transplant experiments. *Marine Biology* 142: 461.

Leporati, S. C., Semmens, J. M. and Pecl, G. T. (2008). Determining the age and growth of wild octopus using stylet increment analysis. *Marine Ecology Progress Series* 367: 213-222.

Leporati, S. C., Semmens, J. M. and Pecl, G. T. (2008). Reproductive status of *Octopus pallidus*, and its relationship to age and size. *Marine Biology* 155: 375–385.

Levin, L. A. (2006). Recent progress in understanding larval dispersal: new directions and digressions. *Integrative and Comparitive Biology* 46: 282-297.

Lipinski, M. R. (1998). Cephalopod life cycles: patterns and exceptions. *South African Journal of Marine Science* 20: 439-447.

Lipinski, M. R., Przybylowicz, W. J., Durholtz, M. D. and Mesjasz-Przybylowicz, J. (1997). Quantitative micro-PIXE mapping of squid statoliths. *Nuclear Instruments & Methods in Physics Research Section B - Beam Interactions with Materials and Atoms* 130: 374-380.

Lombarte, A., Rufino, M. M. and Sánchez, P. (2006). Statolith identification of Mediterranean Octopodidae, Sepiidae, Loliginidae, Ommastrephidae and Enoploteuthidae based on warp analysis. *Journal of the Marine Biological Association of the United Kingdom* 86: 767-771.

MacKenzie, K. (2002). Parasites as biological tags in population studies of marine organisms: an update. *Parasitology* 124: 153-163.

Maltagliati, F., Belcari, P., Casu, D., Casu, M., Sator, P., Vargiu, G. and Castelli, A. (2002). Allozyme genetic variability and gene flow in *Octopus vulgaris* (Cephalopoda, Octopodidae) from the Mediterranean Sea. *Bulletin of Marine Science* 71: 437-486.

Miller, J. A., Banks, M. A., Gomez-Uchida, D. and Shanks, A. L. (2005). A comparison of population structure in black rockfish (*Sebastes melanops*) as determined with otolith microchemistry and

microsatellite DNA. *Canadian Journal of Fisheries and Aquatic Sciences* 62: 2189-2198.

Milton, D. A. and Chenery, S. R. (2001). Can otolith chemistry detect the population structure of the shad hilsa *Tenualosa ilisha*? Comparison with the results of genetic and morphological studies. *Marine Ecology Progress Series* 222: 239-251.

Mokgalaka, N. S. and Gardea-Torresdey, J. L. (2006). Laser ablation inductively coupled plasma mass spectrometry: principles and applications. *Applied Spectroscopy Reviews* 41: 131-150.

Moltschaniwskj, N. A. (2004). Understanding the process of growth in cephalopods. *Marine and Freshwater Research* 55: 379-386.

Morin, P. A., Luikart, G., Wayne, R. K. and group., t. S. w. (2004). SNPs in ecology, evolution and conservation. *Trends in Ecology & Evolution* 19: 208-216

Murphy, J. M., Baguerias, E., Key, L. N. and Boyle, P. R. (2002). Microsatellite DNA markers discriminate between two *Octopus vulgaris* (Cephalopoda: Octopoda) fisheries along the northwest African coast. *Bulletin of Marine Science* 71: 545-553.

Napoleão, P., Sousa Reis, C., Alves, L. C. and Pinheiro, T. (2005). Morphological characterisation and elemental distribution of *Octopus vulgaris* Cuvier, 1797 vestigial shell. *Nuclear Instruments and Methods in Physics Research B* 231: 345-349.

Nathan, R. (2001). The challenges of studying dispersal. *Trends in Ecology & Evolution* 16: 481-483.

Norman, M. (2000). *Cephalopods - a world guide*. Hackenheim, Conchbooks.

Norman, M. and Reid, A. (2000). *A guide to squid, cuttlefish and octopuses of Australasia*. Melbourne, Goulde League of Australia & CSIRO Publishing.

Oosthuizen, A., Jiwaji, M. and Shaw, P. (2004). Genetic analysis of the *Octopus vulgaris* population on the coast of South Africa. *South African Journal of Science* 100: 603-607.

Oosthuizen, A. and Smale, M. J. (2003). Population biology of *Octopus vulgaris* on the temperate south-eastern coast of South Africa. *Journal of the Marine Biological Association of the United Kingdom* 83: 535-541.

Orsi Relini, L., Mannini, A., Fiorentino, F., Palandri, G. and Relini, G. (2006). Biology and fishery of *Eledone cirrhosa* in the Ligurian Sea. *Fisheries Research* 78: 72-88.

Palumbi, S. R. (1995). Using genetics as an indirect estimator of larval dispersal. *Ecology of Marine Invertebrate Larvae*. McEdward, L., (ed) Boca Raton, CRC Press. pp: 369-387.

Palumbi, S. R. (2004). Marine reserves and ocean neighbourhoods: the spatial scale of marine populations and their management. *Annual Review of Environment and Resources* 29: 31-68.

Peakall, R. and Smouse, P. E. (2006). GENALEX 6: genetic analysis in Excel, population genetic software for teaching and research. *Molecular Ecology Notes* 6: 288-295.

Pearce, N. J. G., Perkins, W. T., Westgate, J. A., Gorton, M. P., Jackson, S. E., Neal, C. L. and Chernery, S. P. (1997). A compilation of new and published major and trace element data for NIST SRM 610 and NIST SRM 612 glass reference materials. *Geostandards Newsletter* 21: 115-144.

Pearse, D. E. and Crandall, K. A. (2004). Beyond F_{ST}: analysis of population genetic data for conservation. *Conservation Genetics* 5.

Pecl, G. T., Tracey, S. R., Semmens, J. M. and Jackson, G. D. (2006). Use of acoustic telemetry for spatial management of southern calamary *Sepioteuthis australis*, a highly mobile inshore squid species. *Marine Ecology Progress Series* 328: 1-15.

Perez-Losada, M., Guerra, A., Carvalho, G. R., Sanjuan, A. and Shaw, P. W. (2002). Extensive population subdivision of the cuttlefish *Sepia officinalis* (Mollusca: Cephalopoda) around the Iberian Peninsula indicated by microsatellite DNA variation. *Heredity* 89: 417-424.

Pierce, G. J., Thorpe, J. P., Hastie, L. C., Brierley, A. S., Guerra, A., Boyle, P. R., Jamieson, R. and Avila, P. (1994). Geographic variation in *Loligo forbesi* in northeast Atlantic Ocean: analysis of morphometric data and tests of causal hypotheses. *Marine Biology* 119: 541-547.

Przybylowicz, W. J., Mesjasz-Przybylowicz, J., Pineda, C. A., Churms, C. L., Springhorn, K. A. and Prozesky, V. M. (1999). Biological applications of the NAC nuclear microprobe. *X-Ray Spectrometry* 28: 237-243.

Puebla, O., Sévigny, J. M., Sainte-Marie, B., Brêthes, J. C., Burmeister, A., Dawe, E. G. and Moriyasu, M. (2008). Population genetic structure of the snow crab (*Chionoecetes opilio*) at the Northwest Atlantic scale. *Canadian Journal of Fisheries and Aquatic Sciences* 65: 425-436.

Purcell, J. F., Cowen, R. K., Hughes, C. R. and Williams, D. A. (2006). Weak genetic structure indicates strong dispersal limits: a tale of two coral

reef fish. *Proceedings of the Royal Society B: Biological Sciences* 273: 1483-1490.

Raimundo, J., Caetano, M. and Vale, C. (2004). Geographical variation and partition of metals in tissues of *Octopus vulgaris* along the Portuguese coast. *Science of the Total Environment* 325: 71-81.

Raymond, M. and Rousset, F. (1995). GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86: 248-249.

Reichow, D. and Smith, M. J. (2001). Microsatellites reveal high levels of gene flow among populations of the California squid *Loligo opalescens*. *Marine Ecology* 10: 1101-1109.

Rice, W. R. (1989). Analyzing tables of statistical significance. *Evolution* 43: 223-225.

Ridgway, K. R. (2004). The 5500-km-long boundary flow off western and southern Australia. *Journal of Geophysical Research* 109: C10016 (article no.).

Ridgway, K. R. (2007). Seasonal circulation around Tasmania: an interface between eastern and western boundary dynamics. *Journal of Geophysical Research* 112: C04017 (article no.).

Rigby, P. R. and Sakurai, Y. (2005). Multidimensional tracking of giant Pacific octopuses in northern Japan reveals unexpected foraging behaviour. *Marine Technology Society Journal* 39: 64-67.

Rinaudo, M. (2006). Chitin and chitosan: properties and applications. *Progress in Polymer Science* 31: 603-632.

Rocha, F. J. and Vega, M. A. (2003). Overview of cephalopod fisheries in Chilean waters. *Fisheries Research* 60: 151-159.

Rodhouse, P. G. and Nigmatullin, C. M. (1996). Role as consumers. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* 351: 1003-1022.

Rozen, S. and Skaletsky, H. J. (2000). *Primer3 on the WWW for general users and for biologist programmers*. Totowa, Humana Press.

Ruttenberg, B. I., Hamilton, S. L., Hickford, M. J. H., Paradis, G., Sheehy, M. S., Standish, J. D., Ben-Tzvi, O. and Warner, R. R. (2005). Elevated levels of trace elements in cores of otoliths and their potential for use as natural tags. *Marine Ecology Progress Series* 297: 273-281.

Ryan, C. G. (1995). The nuclear microprobe as a probe of earth structure and geological processes. *Nuclear Instruments & Methods in Physics Research Section B - Beam Interactions with Materials and Atoms* 104: 377-394.

Ryan, C. G. (2000). Quantitative trace element imaging using PIXE and the nuclear microprobe. *International Journal of Imaging Systems and Technology* 11: 219-230.

Ryan, C. G. (2001). Developments in Dynamic Analysis for quantitative PIXE true elemental imaging. *Nuclear Instruments & Methods in Physics Research Section B - Beam Interactions with Materials and Atoms* 181: 170-179.

Ryan, C. G., van Achterbergh, E., Yeats, C. J., Win, T. T. and Cripps, G. (2002). Quantitative PIXE trace element imaging of minerals using the new CSIRO-GEMOC Nuclear Microprobe. *Nuclear Instruments & Methods in Physics Research Section B - Beam Interactions with Materials and Atoms* 189: 400-407.

Ryman, N., Utter, F. and Laikre, L. (1995). Protection of intraspecific biodiversity of exploited fishes. *Reviews in Fish Biology and Fisheries* 5: 417-446.

Sale, P. F., Cowen, R. K., Danilowicz, B. S., Jones, G. P., Kritzer, J. P., Lindeman, K. C., Planes, S., Polunin, N. V. C., Russ, G. R., Sadovy, Y. J. and Steneck, R. S. (2005). Critical science gaps impede use of no-take fishery reserves. *Trends in Ecology & Evolution* 20: 74-80.

Semmens, J. M., Pecl, G. T., Gillanders, B. M., Waluda, C. M., Shea, E. K., Jouffre, D., Ichii, T., Zumholz, K., Katugin, O. L., Leporati, S. C. and Shaw, P. W. (2007). Approaches to resolving cephalopod movement and migration patterns. *Reviews in Fish Biology and Fisheries* 17: 401-423

Shaw, P. W. (2002). Past, present and future applications of DNA-based markers in cephalopod biology: workshop report. *Bulletin of Marine Science* 71: 67-78.

Shaw, P. W., Arkhipkin, A. I., Adcock, G. J., Burnett, W. J., Carvalho, G. R., Scherbich, J. N. and Villegas, P. A. (2004). DNA markers indicate that distinct spawning cohorts and aggregations of Patagonian squid, *Loligo gahi*, do not represent genetically discrete subpopulations. *Marine Biology* 144: 961-970.

Shaw, P. W., Pierce, G. J. and Boyle, P. R. (1999). Subtle population structuring within a highly vagile marine invertebrate, the veined

squid *Loligo forbesi*, demonstrated with microsatellite DNA markers. *Molecular Ecology* 8: 407-417.

Smith, C. D., Groeneveld, J. C. and Maharaj, G. (2006). The life history of the giant octopus *Octopus magnificus* in South African waters. *African Journal of Marine Science* 28: 561-568.

Stephenson, R. L. (1999). Stock complexity in fisheries management: a perspective of emerging issues related to population sub-units. *Fisheries Research* 43: 247-249.

Stranks, T. N. (1988). Redescription of *Octopus pallidus* (Cephalopoda: Octopodidae) from south-east Australia. *Malacologia* 29: 275-287.

Stranks, T. N. (1996). Biogeography of *Octopus* species (Cephalopoda: Octopodidae) from southeastern Australia. *American Malacological Bulletin* 12: 145-151.

Stranks, T. N. (1998). The systematic and nomenclatural status of the Octopodinae described from Australia (Mollusca: Cephalopoda). *Smithsonian Contributions to Zoology* 586: 529-547.

Sunnucks, P. (2000). Efficient genetic markers for population biology. *Trends in Ecology & Evolution* 15: 199-203.

Swain, D. and Foote, C. (1999). Stocks and chameleons: the use of phenotypic variation in stock identification. *Fisheries Research* 43: 113-128.

Swan, S. C., Geffen, A. J., Morales-Nin, B., Gordon, J. D. M., Shimmield, T., Sawyer, T. and Massutí, E. (2006). Otolith chemistry: an aid to stock separation of *Helicolenus dactylopterus* (bluemouth) and *Merluccius merluccius* (European hake) in the Northeast Atlantic and Mediterranean. *ICES Journal of Marine Science* 63: 504-513.

Teske, P. R., Oosthuizen, A., Papadopoulos, I. and Barker, N. P. (2007). Phylogeographic structure of *Octopus vulgaris* in South Africa revisited: identification of a second lineage near Durban harbour. *Marine Biology* 151: 2119-2122.

Thorpe, J. P., Sole-Cava, A. M. and Watts, P. C. (2000). Exploited marine invertebrates: genetics and fisheries. *Hydrobiologia* 420: 165-184.

Thorrold, S. R., Jones, G. P., Planes, S. and Hare, J. A. (2006). Transgenerational marking of embryonic otoliths in marine fishes using barium stable isotopes. *Canadian Journal of Fisheries and Aquatic Sciences* 63: 1193-1197.

Thorrold, S. R., Latkoczy, C., Swart, P. K. and Jones, C. S. (2001). Natal homing in a marine fish metapopulation. *Science* 291: 297-299.

Thresher, R. (1999). Elemental composition of otoliths as a stock delineator in fishes. *Fisheries Research* 43: 165-204.

Tomczak, M. (1981). Bass Strait water intrusion in the Tasman Sea and mean temperature-salinity curves. *Australian Journal of Marine and Freshwater Science* 32: 699-708.

Tracey, S. R., Smolenski, A. and Lyle, J. M. (2007). Genetic structuring of *Latris lineata* at localized and transoceanic scales. *Marine Biology* 152: 119-128.

Triantafillos, L., Jackson, G. D., Adams, M. and McGrath Steer, B. L. (2004). An allozyme investigation of the stock structure of arrow squid *Nototodarus gouldi* (Cephalopoda: Ommastrephidae) from Australia. *ICES Journal of Marine Science* 61: 829-835.

Truett, G. E., Heeger, P., Mynatt, R. L., Truett, A. A., Walker, J. A. and Warman, M. L. (2000). Preparation of PCR-quality mouse genomic DNA with hot sodium and Tris (HotSHOT). *Biotechniques* 29: 52-54.

Van Oosterhout, C., Hutchinson, W. F., Wills, D. P. M. and Shipley, P. (2004). MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4: 535-538.

Vega, M. A., Rocha, F. J., Guerra, A. and Osorio, C. (2002). Morphological differences between the Patagonian squid *Loligo gahi* populations from the Pacific and Atlantic oceans. *Bulletin of Marine Science* 71: 903-913.

Villanueva, R. (1995). Experimental rearing and growth of planktonic *Octopus vulgaris* from hatching to settlement. *Canadian Journal of Fisheries and Aquatic Sciences* 52: 2639-2650.

Villanueva, R. and Bustamante, P. (2006). Composition in essential and non-essential elements of early stages of cephalopods and dietary effects on the elemental profiles of *Octopus vulgaris* paralarvae. *Aquaculture* 261: 225-240.

Waples, R. S. (1998). Separating the wheat from the chaff: patterns of genetic differentiation in high gene flow species. *Journal of Heredity* 89: 438-450.

Waples, R. S. and Gaggiotti, O. (2006). What is a population? An empirical evaluation of some genetic methods for identifying the number of

gene pools and their degree of connectivity. *Molecular Ecology* 15: 1419-1439.

Warner, R. R., Swearer, S. E., Caselle, J. E., Sheehy, M. and Paradis, G. (2005). Natal trace-elemental signatures in the otoliths of an open-coast fish. *Limnology and Oceanography* 50: 1529-1542.

Weir, B. S. and Cockerham, C. C. (1984). Estimating F-statistics for the analysis of population structure. *Evolution* 38: 1358-1370.

White, J. (2007). *First field estimates of age, growth and age at maturation for a merobenthic octopus species*. Honours thesis. University of Tasmania, Hobart.

Wilke, T. and Davis, G. M. (2000). Intraspecific mitochondrial sequence diversity in *Hydrobia ulvae* and *Hydrobia ventrosa* (Hydrobiidae: Rissooidea: Gastropoda): do their different life histories affect biogeographic patterns and gene flow? *Biological Journal of the Linnean Society* 70: 89-105.

Wolfram, K., Mark, F. C., Uwe, J., Lucassen, M. and Pörtner, H. O. (2006). Microsatellite DNA variation indicates low levels of genetic differentiation among cuttlefish (*Sepia officinalis* L.) populations in the English Channel and the Bay of Biscay. *Comparative Biochemistry and Physiology*, *Part D* 1: 375-383.

Zacherl, D. C. (2005). Spatial and temporal variation in statolith and protoconch trace elements as natural tags to track larval dispersal. *Marine Ecology Progress Series* 290: 145-163.

Zacherl, D. C., Paradis, G. and Lea, D. W. (2003). Barium and strontium uptake into larval protoconchs and statoliths of the marine neogastropod *Kelletia keletii*. *Geochimica et Cosmochimica Acta* 67: 4091-4099.

Zane, L., Bargelloni, L. and Patarnello, T. (2002). Strategies for microsatellite isolation: a review. *Molecular Ecology* 11: 1-16.

Zenitani, H., Kono, N. and Arai, N. (2003). Preliminary report on PIXE analysis for trace elements of *Engraulis japonicus* otoliths. *Fisheries Science* 689: 210-212.

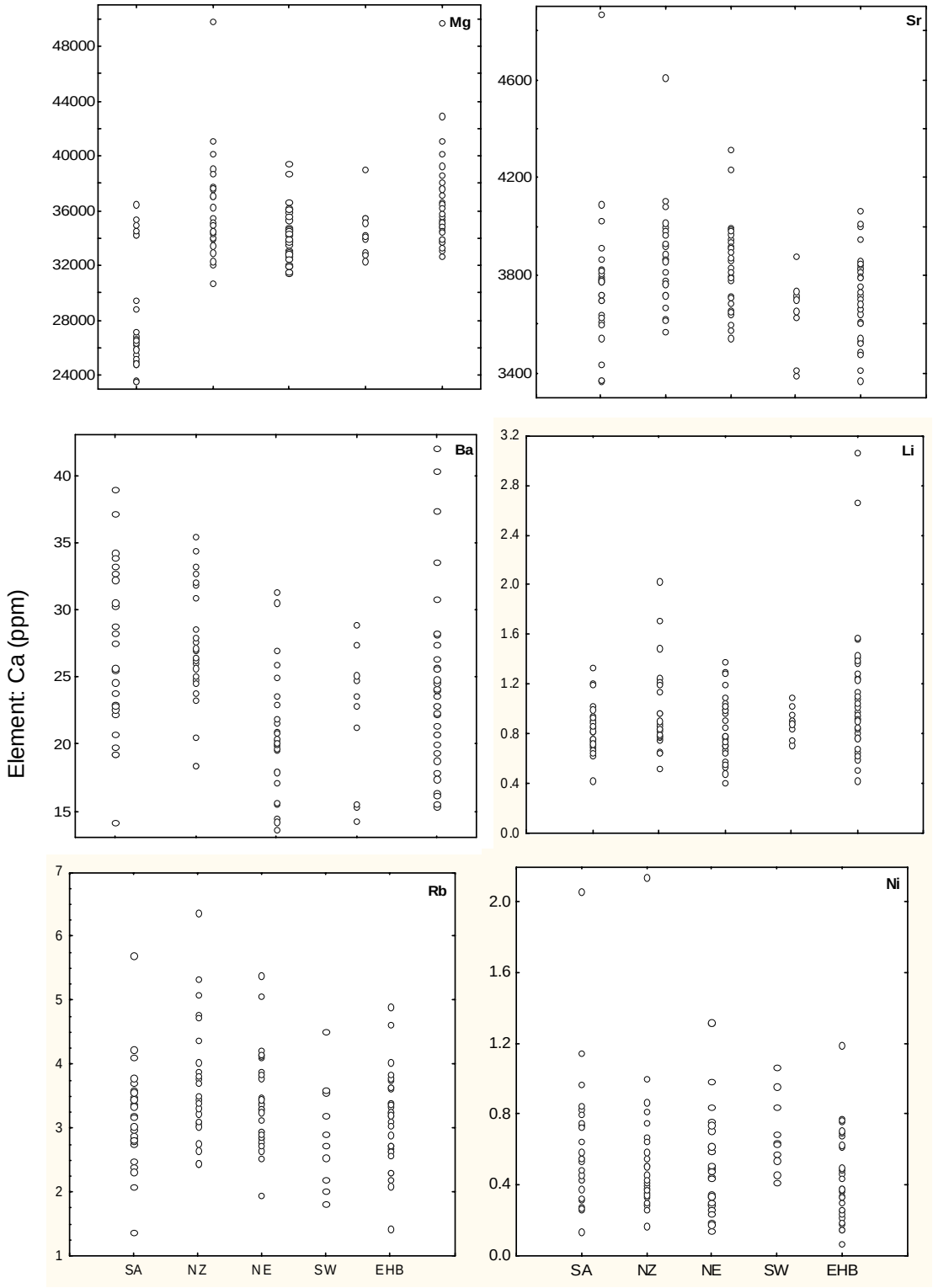
Ziegler, P. E., Lyle, J. M., Pecl, G. T., Moltschaniwskj, N. A. and Haddon, M. (2007). Tasmanian scalefish fishery 2006. Hobart, Tasmanian Aquaculture and Fisheries Institute.

Zumholz, K., Hansteen, T. H., Klügel, A. and Piatkowski, U. (2006). Food effects on statoliths composition of the common cuttlefish (*Sepia officinalis*). *Marine Biology* 150: 237-244.

Zumholz, K., Hansteen, T. H., Piatkowski, U. and Croot, P. L. (2007). Influence of temperature and salinity on the trace element incorporation into statoliths of the common cuttlefish (*Sepia officinalis*). *Marine Biology* 151: 1321-1330

Zumholz, K., Klügel, A., Hansteen, T. and Piatkowski, U. (2007). Statolith microchemistry traces the environmental history of the boreoatlantic armhook squid *Gonatus fabricii*. *Marine Ecology Progress Series* 333: 195-204.

Appendix I



Individual variability in Mg, Sr, Ba, Li, Rb, and Ni concentrations for each site. SA: South Australia; NZ: New Zealand; NE: northeast Tasmania; SW: southwest Tasmania; EHB: Eaglehawk Bay.

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