The Behavioural and Molecular Ecologies of the Southern Blue-Ringed Octopus, *Hapalochlaena maculosa* (Cephalopoda: Octopodidae)

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SUMMARY

The cephalopods (Mollusca: Cephalopoda) provide a unique animal group for studying the mechanisms and genetic consequences of sexual selection. This is because: i) both males and females can be selective of their mates; ii) males can employ complex phenotypic-conditional mating strategies to secure copulations; iii) promiscuity of both sexes is widespread across this taxon despite no paternal care or resource provisioning by males for the females they mate with; and iv) females store sperm from multiple males until egg-laying, suggesting that sperm competition and cryptic female choice might be strong determinants of resulting fertilisation patterns. Additionally, nearly all cephalopods are relatively short-lived and invest heavily into their reproductive cycles. These characteristics suggest that sexually selected traits and behaviours can evolve rapidly within some cephalopods, making these taxa useful models for the examination animal mating system evolution and exploring mechanisms of speciation based on assortative mating, and pre- or postzygotic reproductive isolation.

The southern blue-ringed octopus (*Hapalochlaena maculosa*) is an endemic Australian octopod that displays several distinctive life-history traits making it an ideal study species for addressing hypotheses related to sexual selection and population divergence. This species has a seven-month life cycle, ending in a synchronous semelparous breeding season. Gametes are limiting for *H. maculosa*, with males and females possessing approximately 50 spermatophores or eggs per individual respectively. The females hold their small egg-clutches in their arms to protect and clean them until the time of hatching. The young are direct-developing, and so there is no planktonic dispersal phase. Together, these aspects of life history in *H. maculosa* suggest both that ensuring offspring quality might be particularly important for this species, and that short generation times with no larval dispersal might lead to rapid divergence of heritable traits and behaviours among geographically distant populations.

The present study addressed the mating behaviour and genetic structuring of *H. maculosa* by combining investigations of four separate components of behavioural and molecular ecology in this species. Precopulatory mate choice behaviours were investigated through focal animal observations in the laboratory. Postcopulatory fertilisation processes were assessed through paternity analyses using genotyped

candidate parents. The roles of olfaction and social recognition were investigated by measuring the response of *H. maculosa* to conspecifics odours and comparing these responses to subsequent mate choice behaviours. Additionally, the broad-scale genetic structuring of *H. maculosa* was examined by obtaining 248 samples from across its geographic range, and using 17,523 single-nucleotide polymorphisms to identify patterns of population diversity, connectivity and local adaptation.

Focal animal observations showed no indication that females preferred to mate with males that displayed specific morphology or behaviour. However, females that terminated copulations mated longer with larger males. There was no indication of male preference for any female phenotypic traits, but male behaviours were consistent with theories of sperm competition, in that they spent more time in copulation with novel females, and females that had recently mated with higher numbers of competing males. Males mounted other males as frequently as they mounted females. However, male-male mounts were shorter than male-female mounts, suggesting that they might not be able to discriminate the sex of conspecifics until after they attempted to copulate.

Paternity analyses revealed multiple paternity in all genotyped egg-clutches. There was no relationship between either copulation time or mating chronology and the relative paternity of the candidate fathers, suggesting that differences in copulation durations observed in the first study might be related to mate guarding rather than sperm-loading or removal. Paternity of embryos along egg strings suggested that sperm might get mixed in the female oviducal gland, and paternal shares corresponded to remaining sperm signatures in maternal oviducal glands, post-egg deposition, in nine of twelve egg-clutches. Together these findings indicated it is unlikely for female *H. maculosa* to have the mechanical capacity to cryptically favour fertilisation by particular sperm she is holding. However, in one of the three cases where paternity did not correlate to residual sperm precedence, post-hoc analysis revealed that the male siring less paternity than expected was the female's full-sibling brother. This result anecdotally suggested that chemical processes might favour fertilisation to genetically compatible gametes post-copulation.

During odour cue trials, both male and female *H. maculosa* were observed to detect conspecifics via chemical cues in the water. Females responded to chemical signals differently based on the sex of the detected conspecific, but consistent with the prevalence of male-male mounts in the first study, males showed no evidence of sex

discrimination using chemical cues. Females that reacted strongly to a male's odour were more likely to be unreceptive his copulation attempts one week later, and females spent less time in copulation with these males compared to males whose odour elicited a weaker response. This study concluded that response to conspecific odours might be related to agonistic behaviour and that females might react strongly to the odours of males they do not want to copulate with.

Broad-scale genetic analyses revealed that *H. maculosa* forms a clinal species pattern across its geographic distribution, from the southwest Australian coastline to Tasmania. The genetic divergence between *H. maculosa* sampled from distal ends of its range was consistent with the genetic differentiation observed between *H. maculosa* and its sister-taxon *H. fasciata*. However, the taxonomic identity of *H. maculosa* was maintained through small amounts of gene flow between adjacent populations across the entire species distribution. The genetic structuring of sampled populations was highly affected by both limited gene flow, due to its quick holobenthic life history, and strong patterns of local adaptation. This indicated that *H. maculosa* populations diverge rapidly and would be particularly susceptible to speciation if any barriers to dispersal and gene flow were to arise across its current species range. Diversity indices within populations indicated that individuals occupying the same habitat are highly related. Despite this pattern, indices also suggested that inbreeding might be rare in this species, strengthening findings in the third study that postcopulatory fertilisation patterns in *H. maculosa* might favour offspring to unrelated parents.

Collectively, studies carried out as part of this PhD, and included in this dissertation demonstrated that the unique life history of *H. maculosa* leads to a unique behavioural ecology. Limited gamete production and intense sperm competition have driven the development of dynamic male mating behaviours to ensure chances of fertilisation. Additionally, the lack of a dispersal phase resulting in high levels of interrelatedness within populations appear to have led to the large investment that *H. maculosa* puts towards promiscuity, and possibly postzygotic isolation, in order to ensure offspring sired to compatible partners. Further studies are required for verification of this hypothesis, however similar examples of ensuring genetic compatibility might help to explain the widespread occurrence of polyandry among the Cephalopoda.

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CHAPTER 1: General Introduction

Sexual selection is the intraspecific reproductive competition among individuals of the same sex, resulting in directional filtering of alleles between generations (Darwin, 1906; Bateson, 1983). This process can often lead to the development of phenotypic traits or behaviours that can enable individuals to maximise their reproductive output (West-Eberhard, 1983; Arnqvist & Rowe, 1995; Andersson & Simmons, 2006). However, due to anisogamy, which is the differential investment between males and females towards their gametes in most animal mating systems (Kodric-Brown & Brown, 1987), strategies for maximising reproductive success have diverged between the two sexes in most studied dioecious animal species (Arnqvist & Rowe, 1995; Chapman *et al.*, 2003). Males, which have relatively cheap and less-limiting gamete production, typically have reproductive outputs that are constrained primarily by the numbers of female eggs that they can successfully fertilise in a lifetime (Bateson, 1983; Kodric-Brown & Brown, 1987). Therefore, sexual selection will generally impose males to develop traits or behaviours that enable them to achieve more copulations with females, gain access to healthier and/or more fecund females and to gain better fertilisation success with the females they do mate with (Parker, 1970; Reinhold *et al.*, 2002; Huffard *et al.*, 2010). Contrastingly, females will by definition have more metabolically demanding and usually limited gamete production relative to males of the same species (Kodric-Brown & Brown, 1987). In this context, female reproductive success is typically limited by the resources they have access to and the quality of offspring that they can produce (Kirkpatrick, 1982; Kodric-Brown & Brown, 1987). Therefore, sexual selection in females usually favours traits or behaviours that enable them to obtain more resources to create higher numbers of healthy viable eggs, and/or to fertilise these eggs with sperm from higher quality and/or genetically compatible males (Jennions & Petrie, 1997; Tregenza & Wedell, 2000; Kokko *et al.*, 2003).

Sexual selection can have large impacts on the genetic structure of a species by constraining the extent to which different alleles in a population can pass from one generation to the next (Darwin, 1906; Wright, 1940; West-Eberhard, 1983). The roles of intra-sexual competition and mate choice behaviours in the development of secondary sexual characteristics have been well documented throughout the literature (Hamilton

& Zuk, 1982; Kirkpatrick, 1982; Andersson & Simmons, 2006; Clutton-Brock, 2007). However, intra-sexual competition and mate choice are themselves hereditary behaviours evolved from sexually selective pressures (Kirkpatrick, 1982; Jennions & Petrie, 1997; Gavrilets *et al.*, 2001; Kokko *et al.*, 2003; Charmantier & Sheldon, 2006). Therefore, sexually selected traits and behaviours, as well as the forces driving them, are plastic within a species (Jennions & Petrie, 1997). They can be affected by biological and physical factors such as changes to the environment (Heuschele *et al.*, 2009), predation (Hedrick & Dill, 1993; Franklin *et al.*, 2014), sensory mechanisms (Ryan, 1998), founder effects during migration to new habitats (Noor, 1999) and differences in the operational sex ratio ('OSR': Mitani *et al.*, 1996; Jirotkul, 1999). Where a species' range becomes large enough that gene flow is no longer homogenous among populations, or when the species range encompasses divergent environmental pressures this can lead to variation in mate choice behaviours within that species (Irwin, 2000; Brooks & Endler, 2001; Heuschele *et al.*, 2009). This can eventually lead to the evolution of new sexually selected traits or behaviours along the species range and possibly even speciation (Stratron & Uetz, 1981; West-Eberhard, 1983; Verrell & Arnold, 1989; Hill, 1994).

The cephalopods (Mollusca: Cephalopoda) are a class of marine invertebrates that provide an interesting and unique animal model for investigating the behaviours and genetic consequences imposed by sexual selection for several reasons. Both male and female promiscuity are widespread across all studied taxa in this class (Mangold, 1987; Hanlon & Messenger, 1998). Despite the prevalence of polyandry, males are not known to provide any resources or paternal care (Mangold, 1987; Hanlon & Messenger, 1998), suggesting that multiple mating by females might be maintained through a currently unidentified selective advantage (c.f. Squires *et al.*, 2012), possibly to do with maximisation of brood quality (Kirkpatrick, 1982). Precopulatory female selection of male partners has been observed in some species (Hall & Hanlon, 2002; Wada *et al.*, 2005a). However, all female cephalopods possess a mechanism for storing sperm from the males they mate with (Froesch & Marthy, 1975; Mangold, 1987; Perez *et al.*, 1990; Hanlon *et al.*, 1999; Hoving *et al.*, 2010a; Hoving *et al.*, 2010b; Bush *et al.*, 2012; Cuccu *et al.*, 2014), suggesting that postcopulatory processes might also be crucial in determining which males gain successful fertilisation of female eggs. In addition, male spermatozoa are encased into a finite number of spermatophores (Mann *et al.*, 1970; Wodinsky, 2008). These discrete sperm packages are transferred to females individually, and

signify that males might invest heavily into their gamete production (Mann *et al.*, 1970; Wodinsky, 2008). This form of male gamete packaging is relatively rare among the animal kingdom (Mann, 1984), and suggests that strategic male allocation of gametes and/or male mate choice might be relatively more important in cephalopod mating systems than in other taxa where traditional anisogamy exists (Mann *et al.*, 1970; Kodric-Brown & Brown, 1987). Finally, cephalopods also have sophisticated neural systems and unique sensory mechanisms (discussed further below), that can enable complex mating behaviours that can parallel that of higher-order studied vertebrate mating systems (Corner & Moore, 1981; Mather & Anderson, 1993; Hanlon *et al.*, 1994; Huffard, 2007; Mäthger & Hanlon, 2007; Mäthger *et al.*, 2009).

The southern blue-ringed octopus (*Hapalochlaena maculosa*) presents an ideal study species for addressing hypotheses related to sexual selection in the Cephalopoda. This is due to several aspects of life history in *H. maculosa*, particularly relating to what is known so far of its unique reproductive biology. Both males and females of this species mate with multiple partners within a single, terminal breeding season (Tranter & Augustine, 1973). Like all octopuses (Cephalopoda: Octopodidae), the females hold sperm from these males until egg-laying, when fertilisation occurs (Tranter & Augustine, 1973). Copulations in this species have much longer durations than observed copulations reported across other cephalopod taxa (see review in Hanlon & Messenger, 1998), suggesting that male sperm competition behaviours, such as sperm loading (Parker, 1990), sperm removal (Birkhead & Hunter, 1990) or monopolization of females (Birkhead *et al.*, 1989) might be important for male reproductive success in this species. *Hapalochlaena maculosa* copulations have been observed to be terminated by both males and females in the laboratory (Morse *et al.*, 2015), suggesting that either sex can regulate their time and/or potential gamete investment during copulation. Male *H. maculosa* produce approximately fifty spermatophores in a lifetime, which is equivalent to the numbers of eggs born by females (Tranter & Augustine, 1973). This implies that strategic spermatophore allocation might be particularly important for males of this species. Finally, because *H. maculosa* has a brief seven-month life cycle with a synchronous, predictable and terminal breeding season (Tranter & Augustine, 1973), it is feasible to obtain sufficient numbers of sexually mature adults for simultaneous study, and to assess multiple generations in the laboratory within a reasonable timeline.

In addition to the above unique life history traits of *H. maculosa*, it also provides an ideal species for examining micro-evolutionary processes (e.g. selection, drift and gene flow) that contribute to the genetic divergence of populations over a large spatial scale. This is because *H. maculosa* has no planktonic larval phase (Tranter & Augustine, 1973), making it one of relatively few holobenthic marine organisms (see prevalence of biphasic life histories among marine invertebrates in Thorson, 1950). Throughout its life cycle, *H. maculosa* is capable of swimming only very short distances via jet propulsion from the siphon (Tranter & Augustine, 1973). Due to its seemingly limited dispersal capacity, this species is possibly more vulnerable to habitat fragmentation and reproductive isolation between local populations (Slatkin, 1973). Despite this, *H. maculosa* is widespread along the southern coastline of Australia (Jereb *et al.*, 2014), and might reach into subtropical waters on Australia's west coast. However, there is debate whether this west coast part of the distribution belongs to an undescribed sister-taxon that is morphologically similar (Norman, 2000). This broad range of a taxon that has inferably limited gene flow suggests that differences in environmental pressures might lead to unique local adaptations and possibly speciation within different regions of the *H. maculosa* distribution.

The limited dispersal ability of *H. maculosa* also poses the question of how this species might contend with inbreeding. Short dispersal distances from natal sites would suggest that many individuals occupying the same habitat might be closely related. One way that inbreeding avoidance could be facilitated in *H. maculosa* would be for individuals to selectively mate with less related conspecifics through precopulatory choice (Pusey & Wolf, 1996; Tregenza & Wedell, 2000). Another method would be for male and female *H. maculosa* to mate with multiple partners within their single breeding season, and to use postcopulatory mechanisms to bias fertilization to compatible gametes (Zeh & Zeh, 1996, 1997; Tregenza & Wedell, 2002). Either one of these processes could potentially be a mechanism for inbreeding avoidance in *H. maculosa*. However, very little is currently known about the mating habits or any intra-specific interactions within this species (c.f. Tranter & Augustine, 1973).

The combination of female promiscuity and sperm storage in the *H. maculosa* mating system strongly suggests that postcopulatory mechanisms might play an important role in determining the reproductive output of an individual in this species. Biological processes that can affect fertilisation after mating can generally be divided

into sperm competition (Parker, 1970) and cryptic female choice ('CFC': Eberhard, 1996), depending on whether the mechanism is employed by the male or the female. Several modes of sperm competition could potentially affect fertilisation patterns in *H. maculosa*. Some of these include: 'Sperm-loading' or increased sperm contributions by males (Parker, 1990), the removal of previous males' sperm by subsequent males (Birkhead & Hunter, 1990), differential motility of male spermatozoa (Birkhead *et al.*, 1999), and mate guarding to reduce sperm from competing males (Birkhead *et al.*, 1989). These processes could theoretically evolve in *H. maculosa* if they result in a greater reproductive success for the males who perform these behaviours. Additionally, mechanisms of sperm competition can also be selected for among females, if the traits or behaviours employed by males to gain better rates of fertilisation can be inherited by their sons (Yasui, 1997; Kokko *et al.*, 2003).

Cryptic female choice is the postcopulatory bias of sperm use for egg fertilisation by females (Eberhard, 1996). This can be helpful for females to increase the chances of their offspring being sired to genetically fitter and/or more genetically compatible males (Eberhard, 1996; Zeh & Zeh, 1996, 1997; Tregenza & Wedell, 2000; Mays Jr. & Hill, 2004). Octopuses present an ideal taxonomic group for investigating mechanisms and consequences of CFC. Female octopuses possess paired, muscular and innervated oviducal glands, where sperm is stored until fertilisation (Froesch & Marthy, 1975). This means that CFC could potentially occur through mechanical processes involving the female selectively pumping different sperm to the eggs during fertilisation. Alternatively, it could also take place through chemical processes that might favour the longevity or fertilisation of sperm from specific males over others (Eberhard, 1996). Although sperm competition in cephalopods has received a lot of attention in the current literature (Cigliano, 1995; Hanlon *et al.*, 1997; Hall & Hanlon, 2002; Shaw & Sauer, 2004; Wada *et al.*, 2005b; Buresch *et al.*, 2009; Wada *et al.*, 2010; Squires *et al.*, 2015; Naud *et al.*, 2016), the role of CFC is not well-understood in cephalopods (c.f. Buresch *et al.*, 2009; Squires *et al.*, 2015; Naud *et al.*, 2016) and has not yet been investigated within octopuses.

In order to strengthen the understanding of the behavioural ecology of a species, it is additionally necessary to consider its cognition and sensory systems. As *H. maculosa* often live in dark, turbid environments and are nocturnal (Morse *et al.*, 2015), chemoreception may be an important sensory mechanism for this species and its ability to interact with conspecifics. Octopuses can detect chemical signals in the water through

olfactory organs located close to their eyes and can also 'taste' chemical cues upon contact with objects using chemoreceptor cells on their suckers (Budelmann, 1996). These sensory mechanisms aid octopuses in the detection of prey items (Wells, 1963; Chase & Wells, 1986). However, recent laboratory trials with the California two-spot octopus (*Octopus bimaculoides*) revealed that this octopus can detect conspecifics from a distance based on chemical cues, and can also discriminate the sex of these detected individuals (Walderon *et al.*, 2011). This raises the possibility that some octopuses might be able to use chemoreception for locating mates, and possibly in discriminating between potential mates and/or individual recognition. Despite the hypothesis that olfaction be an important mode of sensory in cephalopod mating interactions, the roles that chemical cues and chemosensory play in mate choice behaviour have not yet been investigated within any cephalopod taxa.

Knowledge of the behavioural ecology of *H. maculosa* is currently limited for a variety of reasons. Firstly, it has historically been difficult to find this species in substantial numbers to gain meaningful sample sizes for empirical studies. Secondly, this species is nocturnal (Morse *et al.*, 2015), making it difficult to make observations in the field. Also, until recently molecular techniques were both costly and limited in their capacity to detect differences in selective signatures between habitat types or regions (Peterson *et al.*, 2012). In addition to innovating novel trapping methods to obtain animals and using infrared cameras make detailed behavioural observations within a laboratory setting, this study incorporated recently developed Single Nucleotide Polymorphism (SNP) markers to analyse connectivity and selection patterns of *H. maculosa* and possible sister-taxa across their geographic distributions. The recent integration of full genome-wide SNP loci into ecological and evolutionary research has shown that the genotyping by sequencing methodology is becoming a far more comprehensive and efficient way of addressing these types of questions related to the molecular ecology of a species (Angeloni *et al.*, 2012; DeFaveri *et al.*, 2013; Miller *et al.*, 2014). SNPs are highly abundant across genomes, enabling genomic patterns to be analysed with a much finer resolution than with traditional markers (Davey *et al.*, 2011; Angeloni *et al.*, 2012; DeFaveri *et al.*, 2013; Miller *et al.*, 2014). These high genomic density markers allow for detailed studies of population diversity, divergence and adaptive radiation to be easily addressed at both broad and fine scales in non-model

organisms such as *H. maculosa* (Nielsen *et al.*, 2005; Jones *et al.*, 2012; Johnston *et al.*, 2014; Larson *et al.*, 2014; Leaché *et al.*, 2014)

This thesis is comprised of four separate studies, each investigating a distinct component of the behavioural and molecular ecologies of *H. maculosa*. The first study aimed to observe the extent to which precopulatory choice impacted sexual selection in this species by experimentally examining the mating behaviours of *H. maculosa* in a laboratory setting. The subsequent study aimed to identify mechanisms of postcopulatory sexual selection by assessing patterns of sperm competition and the capacity of female *H. maculosa* to use CFC to bias the paternity of her offspring. This aim was addressed by using genetic markers to examine the paternity of female egg clutches after laboratory pairings with genotyped candidate fathers, and to compare these paternities to residual sperm signatures in female oviducal glands after egg-laying. To determine the possible role of olfaction and social recognition in the *H. maculosa* mating system, the third study measured the reactions of this species to the odours of conspecifics, and compared these patterns to mate choice behaviours observed in follow-up focal animal studies. Finally, the fourth study explored the micro-evolutionary processes shaping the genetic structure of the *H. maculosa* species by identifying its broad-scale genetic diversity, connectivity and local adaptation signatures along its entire geographic range. By combining the findings from these four separate studies, this thesis specifically aimed to describe how sexual selection might impact the behaviour and genetic structure of *H. maculosa,* and more broadly to provide insight to the potential roles of polyandry, sperm competition, CFC, chemical cues and population structure within cephalopod mating systems.

CHAPTER 2: A Review of the Current Knowledge on the Processes of Sexual Selection among the Cephalopoda

2.1: INTRODUCTION

Sexual selection is the competition within one or both sexes of a species towards optimising individual reproductive success (Darwin, 1906; Bateson, 1983). The resulting disparity in reproductive outcomes among individuals in a species can lead to the development of specific behaviours and/or phenotypic traits that can enable individuals who display them to increase their genetic contribution to subsequent generations (West-Eberhard, 1983; Andersson & Simmons, 2006). Anisogamy, which is the differential investment between males and females towards their gametes in most animal mating systems (Kodric-Brown & Brown, 1987), typically results in conflicting strategies for enhancing reproductive output between males and females of the same species (Chapman *et al.*, 2003). Females, which have a relatively higher investment towards their gametes, generally have reproductive capacities that are limited by the resources they have access to (Bateson, 1983; Kodric-Brown & Brown, 1987). Meanwhile males, which are usually less limited by their gamete production, are primarily limited by the number of female gametes they can successfully fertilise (Kodric-Brown & Brown, 1987). Therefore, where anisogamy exists sexual selection can impose females to evolve mechanisms by which they can obtain more resources to create higher numbers of healthy viable eggs, and/or to fertilise these eggs with sperm from higher quality and/or genetically compatible males (Kirkpatrick, 1982; Kodric-Brown & Brown, 1987; Tregenza & Wedell, 2000; Kokko *et al.*, 2003). Dissimilarly, sexual selection will often drive males of a species to develop traits or behaviours that enable them to achieve more copulations with females, to mate with healthier more fecund females and to attain greater fertilisation success with the females they mate with (Parker, 1970; Kodric-Brown & Brown, 1987; Reinhold *et al.*, 2002).

The cephalopods (Mollusca: Cephalopoda) are a class of invertebrates that might provide a different type of model for studying the mechanisms and impacts of sexual selection. This is due to the spermatozoa of male cephalopods being encased in a finite number of discrete spermatophores that have to be transferred to the female individually (Mann *et al.*, 1970). Depending on species, males may or may not be able to

regenerate spermatophores (Anderson *et al.*, 2002). In species where males can regenerate spermatophores, the time and energy needed to do so can potentially limit the number of females they can successfully mate with during a spawning period (Mann *et al.*, 1970; Anderson *et al.*, 2002). The constraint of having a fixed or limited male reproductive capacity might lead to a higher investment by males towards their gametes, and could present a system where both male and female mate selection might be important to the reproductive success of individuals within species (e.g. Huffard *et al.*, 2008a, 2010). Additionally, several other cephalopod characteristics make this a unique and interesting class of animals for studying the processes of sexual selection. Male and female promiscuity are reported across this class (Mangold, 1987; Hanlon & Messenger, 1998), and in at least one example females obtain more mates than males on average (algae octopus, *Abdopus aculeatus*: Huffard, 2005). Males are known to employ size-conditional mating strategies (Hanlon *et al.*, 1997; Hall & Hanlon, 2002; Huffard *et al.*, 2008a). Females in some species are known to be selective of mates (Hall & Hanlon, 2002; Wada *et al.*, 2005a), can store sperm (Perez *et al.*, 1990; Hanlon *et al.*, 1999; Morse, 2008; Hoving *et al.*, 2010b; Bush *et al.*, 2012; Cuccu *et al.*, 2014) and can potentially be selective about which sperm they use (Naud *et al.*, 2004; Shaw & Sauer, 2004; Buresch *et al.*, 2009; Sato *et al.*, 2013). Females receive no resources or parental care from the males they mate with (Mangold, 1987; Hanlon & Messenger, 1998; but see possible spermatophore consumption in Wegener *et al.*, 2013) suggesting that male quality and/or genomic compatibility might be important factors in female mate selection (as observed in other animals: Jennions & Petrie, 1997; Tregenza & Wedell, 2000; Kokko *et al.*, 2003). Furthermore, cephalopods' capacity for complex behavioural and visual displays might enable courtship and/or discretion of potential mates (Corner & Moore, 1981; Hanlon *et al.*, 1994; Huffard, 2007; Mäthger & Hanlon, 2007; Mäthger *et al.*, 2009).

Hanlon and Messenger (1998) provided an excellent summary of cephalopod sexual selection in their chapter on *Reproductive Behaviour*. However, since the time of this publication: a) Additional information has come available on the reproductive biology of several deep-sea cephalopods through more specimens taken by trawling nets and observations made via remotely operated vehicles (ROV); b) There have been considerably more field observations of sepiid cuttlefish (Sepioidea: Sepiidae), loliginid squid (Teuthoidea: Loliginidae) and octopus (Octopoda: Octopodidae, see Appendix 2.1), enabling a deeper understanding of the natural copulatory behaviour of these clades in

the wild; and c) The relatively recent integration of molecular markers into the research of cephalopod mating systems has made significant contributions to the understanding of relative reproductive success and mechanisms of sperm competition within sepiids and loliginids. This review covers these recent advances and aims to summarise the current understanding of how sexual selection operates in cephalopod mating systems, with the intention of prompting future directions for investigations in this area.

There is still much debate over the phylogeny of cephalopod taxa due to a limited fossil record and conflicting ordinal-level classifications based on different molecular methods (Voss, 1977; Berthold & Engeser, 1987; Young *et al.*, 1998; Strugnell *et al.*, 2005). For consistency, this review follows the phylogeny and nomenclature stated in Strugnell *et al.* (2005), as at the time of writing, their study provides the most comprehensive molecular analysis of the evolutionary relationships among extant coleoid cephalopods (Cephalopoda: Coleoidea). This review is divided into three sections following the introduction, and each section is further subdivided by either taxonomic order or family based on the above stated nomenclature. Section '*2.2: Reproductive Biology of Cephalopods*' covers how reproduction takes place in each of the five cephalopod orders (see Appendix 2.1; Voss, 1977; Strugnell *et al.*, 2005). Section '*2.3: Precopulatory Behaviour in Coastal Cephalopods*' addresses the mechanisms and behaviours that might lead to differential copulatory rates within the mating systems of three shallow-water cephalopod families. Section '*2.4: Postcopulatory Sexual Selection in Coastal Cephalopods*' summarises the current knowledge of postcopulatory processes that might lead to differential fertilisation success among studied male cephalopods. It is necessary that the third and fourth sections of this review focus primarily on families of shallow, coastal examples of decapods (Sepiidae, Loliginidae) and octopus (Octopodidae) because current knowledge of most deep-sea and pelagic cephalopods is still mostly confined to measurements of dead specimens and limited ROV observations. This review concludes with a final section '*2.5: Conclusions and Suggested Areas for Future Research*', which will highlight some gaps in the current knowledge of cephalopod mating systems that might serve as feasible and productive topics for investigation in the near future.

2.2: REPRODUCTIVE BIOLOGY OF CEPHALOPODS

2.2.1: General Life History in Cephalopoda

Shallow-water coastal cephalopods are typically known for having relatively fast growth rates and short life-cycles ended with a terminal spawning season (Mangold, 1987; Hanlon & Messenger, 1998). By contrast, many deep-sea and pelagic taxa have developed a more protracted spawning period (Rocha *et al.*, 2001). Difficulty in finding mates, small egg-clutches due to limitation of resources and low offspring survival, as well as stable environmental conditions with reduced predation of adults have each been hypothesised as selective pressures towards increased parental investment and multiple spawning events for cephalopod taxa occupying deep-sea habitats (Mangold, 1987; Rocha *et al.*, 2001; Hoving *et al.*, 2015). Some life history characteristics of deep-sea and oceanic cephalopods include relatively longer life-cycles, prolonged embryonic development within larger eggs, maternal care of egg masses, intermittent or continuous spawning over a terminal breeding season and/or iteroparity throughout the adult phase (Villanueva, 1992; Seibel *et al.*, 2000; Rocha *et al.*, 2001; Hoving *et al.*, 2004; Barratt *et al.*, 2007; Laptikhovsky *et al.*, 2007; Hoving *et al.*, 2008; Laptikhovsky *et al.*, 2008; Arnold, 2010; Bush *et al.*; Hoving *et al.*, 2015).

This latter mode of life history is exemplified by the nautiloids (Cephalopoda: Nautiloidea), which are expected to live more than twenty years and spawn continuously once sexually mature (Mikami & Okutani 1977; Saunders 1984; Arnold 1987). Nautiloids are taxonomically distinct from other cephalopods in that they are the only extant representatives of ectocochleate cephalopods (See Appendix 2.1; Voss, 1977). However, several coleoid taxa (Cephalopoda: Coleoidea) are also reported to spawn over multiple seasons. These taxa include: several oegopsid squids (Teuthoidea: Oegopsida, see Appendix 2.1; Harman *et al.*, 1989; Hoving *et al.*, 2004), *Opisthoteuthis* spp. (Octopoda: Opisthoteuthidae, see Appendix 2.1; Villanueva, 1992), *Graneledone* spp. (Octopoda: Octopodidae; Bello, 2006; Guerra *et al.*, 2012), *Octopus chierchiae* (Octopoda: Octopodidae; (Rodaniche, 1984) and the currently undescribed larger Pacific striped octopus ('LPSO'; Octopoda: Octopodidae) which has a continuous spawning phase (Caldwell *et al.*, 2015). These taxa, with the exceptions of *O. chierchiae* and LPSO are all either deep-sea or pelagic cephalopods. Some of the larger oegopsid squid and the giant Pacific octopus (*Enteroctopus dofleini*) have relatively slower growth rates and are estimated to live for two to five years (Hartwick, 1983; Hoving & Robison, 2017).

However the rest of the coleoid cephalopods have life-spans of only seven months to two years, and in the case of terminal spawners, die shortly after breeding (Mangold, 1987; Hanlon & Messenger, 1998).

2.2.2: Reproductive Biology in Sepioidea

Within the Sepioidea, mating behaviour has been described for several sepiid (cuttlefish) and sepiolid (dumpling squid; Sepioidea: Sepiolidae) species. In sepiids, fertilisation is external (Mangold, 1987). Eggs are fertilised by sperm either stored in females' seminal receptacles, located ventral to their buccal membrane, or from recently deposited spermatophores on females arms and/or buccal areas (Naud *et al.*, 2005). Copulation takes place in the head to head position. Pairs face each other, intertwine arms and males use their hectocotylised fourth left arms to transfer spermatophores from their funnel to females' seminal receptacles and/or directly onto females' buccal areas. Males then use the hectocotylus to break open spermatophores, and possibly to manipulate their placement on the female (Hanlon *et al.*, 1999). In sepiolids, fertilisation is internal (Hoving *et al.*, 2008; Hoving *et al.*, 2009; Rodrigues *et al.*, 2009; Squires *et al.*, 2013). Males usually use their arms to latch onto females' necks (Hoving *et al.*, 2008; Rodrigues *et al.*, 2009; Squires *et al.*, 2013), or in the case of *Rossia pacifica* grasp females from a parallel position (Brocco, 1971). In most cases males then use their hectocotylised first left arms to transfer spermatangia to inside females' mantle cavities where sperm is stored in posterior pouch-like receptacles (Hoving *et al.*, 2008; Rodrigues *et al.*, 2009; Squires *et al.*, 2013). *Rossia moelleri* is an interesting exception. Males of this species are known to implant spermatangia into females' external mantle tissue (Hoving *et al.*, 2009). These authors suggest that a combination of mechanical and chemical processes aid the spermatangia to enter through females' skin to their oviducts autonomously.

Sepioids tend to lay comparatively fewer and larger eggs than most other coleoid cephalopods (Natsukari, 1970; Rocha *et al.*, 2001; Laptikhovsky *et al.*, 2003; Laptikhovsky *et al.*, 2008; Squires *et al.*, 2013). Large sepiids are predicted to lay up to 8,000 eggs over a lifetime of intermittent spawning (Laptikhovsky *et al.*, 2003). Fecundity in sepiolids has been recorded up to 646 eggs in captive southern dumpling squid, *Euprymna tasmanica* (Squires *et al.*, 2013), and has been predicted to be as high as approximately 800 eggs, based on ripe egg counts in *Rossia macrosoma* (Laptikhovsky

et al., 2008). Pygmy squids (Sepioidea: Idiosepiidae, based on phylogenetic placement in Strugnell *et al.*, 2005) are reported to only lay between 25 - 64 eggs, usually over two closely timed spawning events (Natsukari, 1970). The potential fecundity of *Spirula* spp. (Sepioidea: Spirulidae) is still currently unknown, as no egg or oocyte counts for this mono-generic family have been published within available literature.

Female sepioids are not known to physically guard their eggs (Hanlon & Messenger, 1998), but several mechanisms for hiding eggs are employed across sepioid taxa. Where known, all sepioids attach their eggs to some form of substrate (Adamo *et al.*, 2000; Hall & Hanlon, 2002; Laptikhovsky *et al.*, 2008; Squires *et al.*, 2013). *Sepia officinalis* and many sepiolids lay opaque eggs, often darkened with ink to minimise detection by predators (Boletzky *et al.*, 2006; Rodrigues *et al.*, 2009; Squires *et al.*, 2013). *Sepia esculenta* and *Euprymna scolopes* achieve the same result by attaching sand and rubble to eggs with a sticky exterior (Arnold *et al.*, 1972; Natsukari & Tashiro, 1991). *Sepia latimanus* and *Sepia pharaonis* hide their eggs in coral crevices, possibly to help guard them against predatory fish (Corner & Moore, 1981; Gutsall, 1989). Additionally, it has been hypothesised that bacterial communities, passed to the egg capsule from the mother's accessory nidamental gland, might help to protect sepioid eggs from fouling or harmful microbes (Collins *et al.*, 2012). All sepioid larvae resemble the adult forms upon hatching (Boletzky, 1987), apart for the phylogenetically controversial family of idiosepiids which are born without tentacles (Natsukari, 1970). Both adults and hatchlings of all other studied sepioids live on or near the seafloor, and this lack of a planktonic phase suggests that dispersal might be more limited in this order than in most other cephalopod taxa (Boletzky, 1987).

2.2.3: Reproductive Biology in Teuthoidea

The method of sperm transfer occurs in a variety of ways across the teuthoid taxa. In loliginid squid, pairs mate in both head to head and parallel positions, and males use their hectocotylised arms to place spermatophores in seminal receptacles near the females' mouths or inside females' mantle cavities near the distal ends of their oviducts (Hanlon *et al.*, 1997; Jantzen & Havenhand, 2003). Female *Lolliguncula brevis* have specialised pads on the inside of their mantle walls where males place spermatophores during parallel mating (Hanlon *et al.*, 1983). A third method of spermatophore placement has been observed during sneaker copulations (described in section 3.1.1) in

Loligo vulgaris. Sneaker males in this species have been observed to opportunistically place spermatophores directly into females' arms either on or near eggs that are about to be deposited onto an egg mass (Hanlon *et al.*, 2002).

Many oegopsid squid are known to copulate in manners similar to the above (Hanlon & Messenger, 1998), however some notable exceptions follow: In *Lycoteuthis lorigera* females can store spermatophores in dorsal pouches located on the neck (Hoving *et al.*, 2007). *Illex* spp. (Oegopsida: Ommastrephidae) are not known to have any seminal receptacle, and sperm are stored only inside spermatophore casings within females' mantle cavities (Durward *et al.*, 1980; Arkhipkin & Laptikhovsky, 1994). External spermatophore placement is also common in several species of deep-sea squids, including *Architeuthis* sp. (Hoving *et al.*, 2004), *Octopoteuthis deletron* (Hoving *et al.*, 2012), *Taningia danaei* (Hoving *et al.*, 2010b) and *Moroteuthis ingens* (Hoving & Laptikhovsky, 2007). This method of spermatophore placement has been suggested as a consequence of size dimorphism between the sexes, in that smaller males need to be able to mate quickly and escape from larger and potentially cannibalistic females (Hoving *et al.*, 2004). Like in *R. moelleri*, externally placed spermatophores in these oegopsid species enter through females' skin autonomously to achieve fertilisation (Hoving & Laptikhovsky, 2007).

Fecundity is generally quite high across teuthoids. Spawned egg counts in loliginid squid have ranged from 2,024 in *L. brevis* to 55,308 in *Doryteuthis pealei* (Hixon, 1980). However, oegopsid squid tend to have markedly higher fecundities (Mangold, 1987). The highest fecundities among teuthoids have been estimated based on oocyte counts, and are 1 – 6 million in *Dosidicus gigas* (Ehrhardt *et al.*, 1983) and 3- 6 million in *Architeuthis* sp. (Hoving *et al.*, 2004). These are both deep-water species, which as stated earlier, tend to have extended or multiple spawning events leading to higher fecundity than counterpart taxa in coastal or shallow-water habitats (Rocha *et al.*, 2001).

Loliginid squid deposit eggs on the substrate, either in single clutches or in communal egg masses (Hanlon *et al.*, 1997; Jantzen & Havenhand, 2003). Similar to sepioids, female loliginids also possess an accessory nidamental gland, and it is thought that this aids inoculating their eggs against harmful pathogens (Barbieri *et al.*, 1996). In terminal spawners (e.g. *Doryteuthis plei* and *D. pealei*), the females usually die shortly after egg deposition (McGowan, 1954; Roper, 1965), or in the case of intermittent spawners will re-join the shoal of adults (e.g. *Doryteuthis opalescens* in Hanlon *et al.*,

2004). Egg deposition and care are variable among the oegopsids. In deep-sea habitats where there are typically few hard surfaces enabling egg attachment, where known, most oegopsid squid lay eggs in neutrally buoyant egg masses that they let go into the water column (Guerra *et al.*, 2002; O'Shea *et al.*, 2004; Staaf *et al.*, 2008). An exception to this pattern is in the family Enoploteuthidae, where females lay single, buoyant egg-capsules (Young & Harman, 1985). Maternal egg care has been reported in *Bathyteuthis berryi* and *Gonatus onyx.* These species have been known to carry their egg masses in their arms and guard them throughout their development (Seibel *et al.*, 2000; Bush *et al.*, 2012). In the case of *G. onyx*, embryonic development is estimated to take up to nine months, and females will drop their two tentacles after egg deposition to better hold the egg mass with their eight arms (Seibel *et al.*, 2000). Larval morphology is highly variable among teuthoids, however all taxa studied to date are planktonic upon hatching (Boletzky, 1987).

2.2.4: Reproductive Biology in Vampyromorpha

The order Vampyromorpha fits phylogenetically within the superorder Octopodiformes (Strugnell *et al.*, 2005), and is represented by only a single extant species, *Vampyroteuthis infernalis* (Young *et al.*, 1998). This species occupies extreme depths (500 – 3,000 m, Seibel *et al.*, 1997), and ROV footage has never captured them mating. Therefore, knowledge of reproduction in *V. infernalis* is limited to observations made from dead specimens. *V. infernalis* males lack a hectocotylised appendage, and it is thought that they use their funnel to transfer spermatophores into females' spermathecae, which in this species are two sperm storage pits located beneath females' eyes (Pickford, 1949b). Single *V. infernalis* eggs have been found drifting freely in open waters, suggesting that females might deposit eggs singly into the water column (Pickford, 1949a). Examination of oocyte development and numbers in dead specimens indicate that female *V. infernalis* have multiple spawning events throughout their lifetime, and can have potential fecundity up to 20,711 (Hoving *et al.*, 2015). The paralarvae of *V. infernalis* resemble adults except for that they have a set of oblique fins, which later get reabsorbed as the adult fins grow in (Young & Vecchione, 1999). The paralarvae can swim freely in deep water habitats, however it is not known whether hatchlings have a free-drifting phase before metamorphosing into the described paralarval form (Young & Vecchione, 1999).

2.2.5: Reproductive Biology in Octopoda

Egg fertilisation in the incirrate octopods (Octopoda: Incirrata, see Appendix 2.1) is always internal (Froesch & Marthy, 1975). The male hectocotylus, which is usually the third right arm (Robson, 1929), terminates in a specialised organ called a ligula (Wells & Wells, 1972). The ligula is composed of erectile tissue in some species (Thompson & Voight, 2003), and it is thought that this structure aids in spermatophore placement and/or removal of competing spermatophores (Voight, 1991; Cigliano, 1995). Males are hypothesised to use the ligula to reach inside the female's mantle aperture and presumably locate one of the two oviducts (Wells & Wells, 1972). Spermatophores are then passed from the male's terminal organ, which is inside the mantle, through the funnel and into the base of the hectocotylised arm (Wells & Wells, 1972). The spermatophores are carried through a ventral groove in the hectocotylus to the ligula using a wave of contractions along the arm (Wodinsky, 2008). The male then uses the ligula to place each spermatophore at one of the openings to the female's paired oviducts (Wells & Wells, 1972). This process happens either while the male is mounting the female's mantle (e.g. *Eledone* spp. in Orelli, 1962; and *Hapalochlaena* spp. in Tranter & Augustine, 1973; Overath & Boletzky, 1974), by the male reaching over to the female with the hectocotylus from a distance (e.g. *Octopus digueti* in Voight, 1991; and *A. aculeatus* in Huffard *et al.*, 2008a), or in a beak to beak mating position with the female at times enveloping the male in her web (LPSO in Caldwell *et al.*, 2015). Males of some species have been observed to use both mounting and reach strategies (*Octopus cyanea* in van Heukelem, 1966; and *Octopus tetricus* in Huffard & Godfrey-Smith, 2010), and might use the mounting position more often with females that are unreceptive (P. Morse personal observations with *O. tetricus*).

Osmotic pressure from exposure to seawater (Hanson *et al.*, 1973), and possibly mechanical rupture from the ligula, break open spermatophores and sperm is usually stored as spermatozoa inside the spermathecae of female's oviducal glands (Froesch & Marthy, 1975). However, in several deep-water octopuses (e.g. *Eledone* spp., *Graneledone macrotyla* and *Vulcanoctopus hydothermalis*) spermatangia migrate to the female's ovaries, where fertilisation occurs (Orelli, 1962; Perez *et al.*, 1990; González *et al.*, 2008; Guerra *et al.*, 2012). In the pelagic environment, where the likelihood of encountering a conspecific of the opposite sex to mate may be the relatively low, males in three genera of pelagic octopods, *Argonauta, Tremoctopus* and *Ocythoe*, have

hectocotyli that fill with sperm, get broken off and left inside the female's mantle cavity (Laptikhovsky & Salman, 2003). Males of the cirrate octopods (Octopoda: Cirrata) do not have a ligula, and it has not yet been established how copulation occurs (Mangold, 1987).

Fecundity is highly variable among incirrate octopods, and egg count estimates have ranged from approximately 30 in *Bathypolypus arcticus* (O'Dor & Malacaster, 1983) up to 700,000 in *O. cyanea* and *O. tetricus* (van Heukelem, 1966; Joll, 1976). Fecundity within the cirrate octopods has so far only been assessed for *Opisthoteuthis grimaldii,* and the maximum fecundity estimated in this species was 3,202 based on follicular sheath and remaining egg counts (Boyle & Daly, 2000). *Opisthoteuthis* spp. lay single eggs continuously throughout their adult life cycle, and there is no indication of parental care within these octopods (Villanueva, 1992; Daly *et al.*, 1998; Boyle & Daly, 2000). Incirrate octopods are unique among cephalopods in that all species of this suborder appear to display some form of extended egg care (Mangold, 1987; Hanlon & Messenger, 1998). Most female incirrate octopods attach eggs to hard substrates, usually inside dens or shelters, where they guard and clean the eggs until hatching (Hanlon & Messenger, 1998). This maternal behaviour has also been reported in two species of deep-sea octopuses, *Graneledone* sp. and *Benthoctopus* sp. during ROV observations (Voight & Grehan, 2000). These authors suggest, that in an environment with limited substrate, these octopuses aggregate around deep-sea rock outcrops as they begin their brooding phase.

Several other octopod species have ways of carrying their developing eggs with them. Several members of *Amphioctopus*, *Macrotritopus defilippi, Hapalochlaena maculosa* and *Wonderpus photogenicus*, which all live in sand or silt habitats, carry their eggs to the ventral aboral web, in line of water expelled from the funnel (Tranter & Augustine, 1973; Hanlon *et al.*, 1985; Huffard & Hochberg, 2005; Miske & Kirchhauser, 2006). The pelagic *Boliataena microtyla* carries its eggs and reportedly also their larvae within their arms (Young, 1972). *Tremoctopus* spp. carry their eggs using a calcified material that they secrete from their web and attach to their dorsal arms (Naef, 1928). *Vitreledonella richardi* carries its developing eggs and possibly newly hatched larvae within the female's mantle cavity (Joubin, 1933). The argonauts (Octopoda: Argonautidae) carry their eggs within their shell (Laptikhovsky & Salman, 2003). *Ocythoe* spp. have long winding oviducts, where embryos develop as they pass through (Naef, 1928), making the species of this genera the only known ovoviviparous

cephalopods. Upon hatching, octopod larvae are either benthic and resemble their adult forms (e.g. members of the subfamily Bathypolypodinae, Boletzky, 1987; and *H. maculosa*, Tranter & Augustine, 1973) or are planktonic (e.g. many *Octopus* spp., Boletzky, 1987).

2.2.6: Reproductive Biology in Nautilida

Distributional data have indicated that populations, where sampled, always have an Operational Sex Ratio (OSR) biased towards males (1:3 in Saunders & Ward, 1987). Additionally, Haven (1977) who sampled a population of *Nautilus pompilius* year round to depths of 340m, found an increase in female catch rates between January and May. These data suggest there might be a seasonal migration of females in this species, possibly related to an annual breeding, feeding or spawning season.

There is relatively very little known about the reproductive habits of these animals in the wild. Aquarium observations have provided a basic understanding of copulatory behaviour in captive nautilids (*Nautilus macromphalus* in Mikami & Okutani, 1977; *N. pompilius* in Arnold, 2010). Successful copulations takes place by the male grasping the female with his tentacles and drawing the pair's mantle apertures together. The male then uses an enlarged labial tentacle, called a spadix, to push the female's buccal tentacles to the side and transfer one long spermatophore (~30 cm) to the female's organ of Valenciennes (Mikami & Okutani, 1977), an analogue to the seminal receptacle in teuthoids except that the spermatophores appear to remain intact within the organ of Valenciennes until the time of fertilisation (Arnold, 2010). The exact method of fertilisation is still not understood in nautilids. However, it has been hypothesised that the spermatophore(s) break during egg-laying and the spermatozoa migrate independently towards the oocyte micropyle(s) (Arnold, 2010).

Copulations have been reported to last as long as 30 hours (Mikami & Okutani, 1977), and females been observed as passive throughout the process. One interesting aspect of copulatory behaviour in nautilids is that males are frequently observed to bite the females on the mantle and shell during copulation (Arnold, 2010). The reason for this behaviour is still not understood. Bites were observed to leave marks on the shell, suggesting that these could theoretically be used as an indication of a female's mating and/or possibly egg-laying history (Arnold, 2010). However, males' response to and/or preference for females with different numbers of bite marks have not been assessed.

Arnold (2010) additionally indicates that male copulation attempts are extended to any object that shares a similar shape and size of another nautilid, and that male/male copulation attempts are common. This suggests that nautilids have difficulty recognising conspecifics and/or discriminating between sexes. This aspect of social naiveté might be related to living at extreme depths where the ability to find mates could be limiting to reproductive success. In this context, it is likely to be less costly for males to waste time and/or energy attempting unviable copulations, than to risk missing an opportunity to transfer gametes to a suitable mate.

In captivity, nautilids have been known to deposit eggs both singly and in small clusters on aquarium floors over an extended annual period, and to do so over multiple years (Carlson *et al.*, 1992; Arnold, 2010). The eggs' exteriors are tough, flexible and opaque white in colour (Mikami & Okutani, 1977). Embryonic development in nautilids takes from nine months to over a year (Arnold, 2010), and there have been no observations of maternal egg care. Upon hatching, juveniles appear like miniature adults and are immediately capable of actively swimming and feeding on cut-up pieces of prawn (Carlson *et al.*, 1992).

2.3: PRECOPULATORY BEHAVIOUR IN COASTAL CEPHALOPODS

2.3.1: Female Choice and Male/Male Competition

2.3.1.1: Sepiidae

Sepiids have highly promiscuous mating systems, and as mentioned in the previous section females will spawn multiple times throughout what is thought to be a terminal breeding season (Mangold, 1987). Copulations are opportunistic within most sepiid species (Hanlon & Messenger, 1998), resulting in the knowledge of reproductive behaviour in many species to be limited to observations in aquaria where behaviours are likely affected by inaccurate representations of OSR and/or females' inability to reject unwanted copulations. However, both *Sepia apama* and *S. latimanus* are known to have spawning aggregations in which males congregate around egg-laying sites in order to attempt copulations with spawning females (up to six individuals in *S. latimanus*, Corner & Moore, 1981); up to 1000s of individuals in *S. apama* (Hall & Hanlon, 2002). Field observations at these spawning sites have revealed detailed accounts of natural reproductive behaviour in these species.

The OSR observed at wild spawning assemblages are always male biased (4:1 – 11:1 in *S. apama*, Hall & Hanlon, 2002; ~3:1 - 4:1 in *S. latimanus*, Corner & Moore, 1981), consistent with both field and aquarium observations of females copulating with multiple males between egg-laying intervals (aquarium observation of *S. officinalis,* Adamo *et al.*, 2000; and *Sepiella japonica*, Wada *et al.*, 2006; field observations of *S. latimanus,* Corner & Moore, 1981; and *S. apama,* Hall & Hanlon, 2002) intense male/male aggression over females and frequent female rejection of males (Corner & Moore, 1981; Adamo *et al.*, 2000; Schnell *et al.*, 2015; notably, wild *S. apama* females rejected 70% of male copulation attempts in Hall & Hanlon, 2002). Hall and Hanlon (2002) report that within *S. apama* spawning sites, males engage in agonistic visual signalling using chromatophore patterning, moderate physical contact and occasional biting to compete for access to females. Typically, the larger males win these agonistic bouts and gain consort status with individual spawning females. The consort males escort females to egg-laying sites while copulating once, or occasionally twice, and meanwhile guarding her from other males. Although female rejections are common, these authors reported that the frequency of forced copulations was only 3%, suggesting that female discretion of males plays an important role in this mating system.

Smaller, lone males either try to locate lone females, challenge consort males in agonistic bouts for access to their females or attempt to gain sneaker copulations with already-paired females (Norman *et al.*, 1999; Hall & Hanlon, 2002). Sneaker copulations are obtained in one of three ways: i) sneaks either overtly follow a female and her consort, waiting for an opportunity when the male is distracted to attempt copulating with the female; ii) some sneaks remain hidden, often under rocks where females lay eggs, in order to attempt a concealed copulation; or iii) some of the smallest sneaks mimic female chromatophore patterning to avoid getting attacked by consort males as they get close enough to mate with the guarded female (Norman *et al.*, 1999; Hall & Hanlon, 2002). Similarly, in *Sepia plangon* which do not spawn in aggregations, some males have been observed to display female patterning on one half of the body that is exposed to a nearby male, while showing typical male patterning to a female with their other half of the body (Brown *et al.*, 2012).

In the field, *S. apama* females have been observed to accept or reject copulations with males regardless of size or mating strategy. Hall and Hanlon (2002) reported that females often rejected large males to later accept copulations with relatively smaller

males. Consort males gain more copulations with the females they guard (Hall & Hanlon, 2002; Naud *et al.*, 2004), and this is intuitively an advantageous strategy, as males have been reported to compete intensely for consort status. However, small males might still achieve a competitive copulatory success overall, by investing less time per female and thereby being able to copulate with more females. There is no direct evidence for courtship in sepiids, although a variety of chromatophore displays are very common during precopulatory behaviour (Corner & Moore, 1981; Boal, 1997; Norman *et al.*, 1999; Adamo *et al.*, 2000; Hall & Hanlon, 2002; Brown *et al.*, 2012; Schnell *et al.*, 2015). It is thought that these displays might be used in signalling agonistic intent and for sex recognition (Boal, 1997; Hall & Hanlon, 2002; Schnell *et al.*, 2015). However, as mentioned above, sneaker males have been observed to dishonestly display female colouration (Norman *et al.*, 1999; Hanlon *et al.*, 2005; Brown *et al.*, 2012). In *S. latimanus*, both chromatophore displays and various posturing are suggested as means of courtship (Corner & Moore, 1981). However, it is not clear whether females are more or less likely to copulate with males displaying different intensities of these displays, or whether these visual signals are used only to signal sex or agonistic intent like in other *Sepia* spp.

During laboratory choice trials, *S. officinalis* females also showed no preference for male size or social hierarchy. However, females interestingly spent more time with males that had copulated more recently (Boal, 1997). This finding probably suggests one of two things: a) That females show preference for a male trait or behaviour that has not yet been measured; or b) That females can discern males' mating history, possibly based on chemical cues, and are attracted to males that have already established a high copulatory success. In the former scenario, females could base their preference in mates based on chemical or visual cues that have not yet been assessed, and these will be speculated upon in section 2.3.3. If the latter case applies, then there could be a selective advantage for females to prefer males with higher copulatory success, because this is likely to result in them having sons which also have higher copulatory success than competing males. In this way a female preference for male promiscuity could be reinforced through achieving more grandchildren, and this would lead to a *Fisherian* run-away process (Kirkpatrick, 1982).

Overall, the fact that there is intense male competition for females during spawning and that female sepiids frequently reject male copulation attempts,

presumably based on cues other than size or hierarchy, support that female choice plays an important role in the differential reproductive success of male sepiids. However, it is still not certain what criteria females might use to discern between potential mates. Also, the details of female sperm storage, external fertilisation and vigilant mate guarding by consort males leading up to egg deposition all suggest that the timing and order of sperm placement are likely to influence the resulting fertilisation patterns, and this will be addressed in more detail within section 2.4.2.

2.3.1.2: Loliginidae

Loliginid squid are among the most social of the cephalopods, in that they hunt in shoals and all species mate in large spawning aggregations (Hanlon, 1998). Like in *S. apama*, these spawning aggregations have male-biased OSRs (1.4:1 in Hanlon *et al.*, 2002; 1:1 – 3:1 in Jantzen & Havenhand, 2003), there is a high turnover of mates for both males and females and there is intense male/male aggression over females (Hanlon *et al.*, 1997; Hanlon *et al.*, 2002; Jantzen & Havenhand, 2003). Currently, females of only one loliginid species are known to be selective of male partners. Wada *et al.* (2005a) observed *Sepioteuthis lessoniana* in the laboratory and reported that females rejected more than half of copulations attempted by small subordinate males, but rather chose to copulate in 95% of attempts by larger, more dominant males. Hanlon *et al.* (1994) provided an excellent account of different body patterning and postures employed by male *L. vulgaris* within spawning assemblages. These authors suggested that males of this species use courtship in the forms of both chromatophore patterning and by displaying enlarged testes, which are visible in this species through the mantle, to females. However, additional field and laboratory observations of mating behaviour in *Loligo* spp. have suggested that females are likely to accept copulations with all attempting males (Hanlon *et al.*, 2002; Shaw & Sauer, 2004), which questions the need for courtship behaviour. It is possible that, rather than for courtship, these body patterns and testis displays are used for sex identification within loliginid mating systems.

In both *Loligo* and *Sepioteuthis,* male/male aggression and dominance hierarchy greatly influence copulatory success among males (Hanlon *et al.*, 1994; Hanlon *et al.*, 1997; Hanlon *et al.*, 2002; Jantzen & Havenhand, 2003; Wada *et al.*, 2005a). Females of these genera usually arrive at spawning grounds already with a paired consort male,

and lone large males that are already waiting at egg-laying sites, frequently challenge paired males for consort status (Hanlon *et al.*, 1997; Hanlon *et al.*, 2002; Jantzen & Havenhand, 2003). These challenges take the form of intense visual signalling and occasionally fin beating. Both Hanlon *et al.* (2002), and Jantzen and Havenhand (2003) report high turnovers of consorts in *L. vulgaris* and *Sepioteuthis australis* respectively. Additionally, smaller sneaker males attempt sneaker copulations with already paired females, by quickly moving in between females and consort males and attempting to mate with females in a head to head position (Sauer *et al.*, 1997; Hanlon *et al.*, 2002). Sneaker males time their attempts for when females are about to deposit an egg capsule, and place spermatophores either onto females' arms or directly on egg capsules (Hanlon *et al.*, 2002). Similar to *S. apama*, Jantzen and Havenhand (2003) observed some *S. australis* sneaker males to mimic female body patterns in order to obtain sneaker copulations without prompting aggression from consort males.

Consort males in both genera place spermatophores internally, close to the opening of females oviducts, however this happens in a 'male parallel' position in *Loligo* spp. (Hanlon, 1998), while *S. australis* are observed to most often do this in an upturned position (Jantzen & Havenhand, 2003). Hanlon *et al.* (2002) report that *L. vulgaris* females arrive at spawning sites already having sperm in their receptacles from what are thought to be from previous head to head copulations. It is likely that females of both genera will copulate with males opportunistically outside of spawning aggregations, and store sperm until future egg depositions. Although females of most loliginid species have not been observed to be selective about which males they copulate with (Hanlon *et al.*, 1997; Hanlon *et al.*, 2002; van Camp *et al.*, 2004; c.f. Wada *et al.*, 2005a) the high frequency of multiple copulations between egg laying intervals (Hanlon *et al.*, 2002; Jantzen & Havenhand, 2003), differential male mating strategies with different methods of sperm placement (Hanlon *et al.*, 1997; Hanlon *et al.*, 2002), females' capacity to store sperm and to possibly be selective about which sperm they use during external fertilisation (Hanlon *et al.*, 2002; Shaw & Sauer, 2004) all suggest that sperm competition, and conceivably postcopulatory female choice could greatly influence male reproductive success in loliginid mating systems. These postcopulatory processes will be addressed within section 2.4.3.

2.3.1.3: Octopodidae

Octopus are different from cuttlefish and squid in that they are mostly solitary animals, with little to no social interactions outside of agonistic disputes over den space or mates (Cigliano, 1993; Huffard *et al.*, 2008a), cannibalism (Hanlon & Forsythe, 2008) and predominantly opportunistic copulations (Hanlon & Messenger, 1998; but not always in Huffard *et al.*, 2008a or Caldwell *et al.*, 2015). Within mating systems that have been observed in the field, OSR is considerably more balanced than is common in decapods (1:1 – 3.5:1 in *A. aculeatus,* Huffard, 2005; 0.34:1 – 1.8:1 in *Octopus hubbsorum,* Lopez-Uriarte & Rios-Jara, 2009). This might suggest that precopulatory choice could be important for both male and female mate selection within this family, and is consistent with observations that females of at least three species can initiate copulations with males (*O. cyanea*, Wells & Wells, 1972; *Hapalochlaena lunulata*, Cheng & Caldwell, 2000; and *H. maculosa*, Morse *et al.*, 2015). As mentioned previously, all recorded incirrate octopods are terminal spawners with the exceptions of *Graneledone* spp. (Bello, 2006; Guerra *et al.*, 2012), *O. chierchiae* (Rodaniche, 1984) and LPSO (Caldwell *et al.*, 2015).

There is limited evidence for sex recognition and courtship in octopuses. Cheng and Caldwell (2000) observed *H. lunulata* males to attempt copulations with other males as often as with females. However, *Octopus bimaculoides* are able to discriminate between different sexes of conspecifics based on odour cues (Walderon *et al.*, 2011). It has been suggested that some octopuses use behavioural cues for sex recognition and possibly courtship. Packard (1961) suggested that male *Octopus vulgaris* might display their proximal suckers, which are sexually dimorphic and bigger on males in this species, to signal their sex and obtain copulations with females. However in a follow-up study, males of this species were not observed to display their enlarged suckers to females during laboratory copulations, and therefore there would have been no opportunity for females to assess this trait (Wells & Wells, 1972). Voight (1991) has suggested that the ligula might be used in courtship and influential to male copulatory success. Ligulae have species-specific morphology among octopuses, and in some species can be extraordinarily large compared to body size (e.g. *Bathypolypus bairdii*, Thompson & Voight, 2003). Voight (1991) reported male *O. digueti* to display their ligulae to females and make contact with females using their ligulae prior to copulation, however no evidence of true courtship was found. A tactile phase leading up to copulation has also been noted within in pairs of *O. vulgaris, O. cyanea* and *O. tetricus* during laboratory

observations (Wells & Wells, 1972; Morse, 2008), and it is possible that this behaviour enables female assessment of males' ligulae. Both *A. aculeatus* and *Amphioctopus marginatus* males have been observed in the field to display different chromatophore patterns to females before copulation (Huffard, 2007; Huffard & Godfrey-Smith, 2010), and in the case of *A. marginatus* these authors have suggested visual patterns could be used to recognise conspecifics and reinforce reproductive isolation between conspecific relatives. In addition to chromatophore displays, *A. aculeatus* pairs have been observed to synchronously perform a mantle bounce behaviour leading up to copulation and females of this species have been observed to change postures to what is called a "DACT display" to signal receptivity (Huffard, 2007).

It is likely that many of these behaviours are means of sex and/or species recognition but it is not clear if they are methods of courtship. Females of at least five species of octopus are known to frequently resist and/or reject male copulation attempts in laboratory conditions (*O. cyanea*, Wells & Wells, 1972; *O. digueti*, Voight, 1991; *O. tetricus*, Morse, 2008; *O. bimaculoides* Mohanty *et al.*, 2014; and *H. maculosa* Morse *et al.*, 2015). However, no investigations have yet compared female receptivity to varying forms or intensities of the above traits and behaviours. Additionally, most observations of octopus reproductive behaviour have taken place in the laboratory, where artificial measures of OSR, and confined spaces that limit females' ability to reject copulations, make it difficult to accurately assess potential female preferences and/or which males will achieve higher copulatory success within natural mating systems.

Currently, the most detailed description of octopus mating systems has come from field observations of *A. aculeatus* (Huffard, 2007; Huffard *et al.*, 2008a, 2010). These authors reported high levels of male/male aggression in the form of males competing over mate-guarding status with larger females. Larger males typically won most bouts and gained exclusive access copulating with their guarded female. Mate guarding males found dens close enough to the females that they could copulate by reaching their ligulae into the females' den without having to leave their own. Both males and females engaged in opportunistic copulations while foraging away from their dens, and smaller males attempted to gain sneaker copulations with guarded females by camouflaging themselves or hiding behind rocks to not instigate aggression from the guarding males. In this species, females were observed to accept copulations with nearly all males. However, due to competition among males, large mate-guarding males

obtained higher copulation rates within the studied populations (Huffard *et al.*, 2008a). Like in squid and cuttlefish, the high levels of female promiscuity, sperm storage and mate guarding all suggest, that in addition to differential copulatory rates, sperm competition most likely plays an influential role in male reproductive success within shallow-water octopus mating systems (section 2.4.4).

2.3.2: Differential Copulatory Success in Females

Currently, male preference of females and differential female copulatory rates have not been extensively noted within sepiid or loliginid taxa. Male *S. apama* have been observed to preferentially attempt copulations with unfamiliar females (Schnell *et al.*, 2015). However, this observation was probably more indicative of the males not wanting to waste additional spermatophores with females they had already mated with, and this behaviour did not necessarily result in differential copulatory rates among females within this mating system. Among the loliginids, one study reported that younger *S. australis* females laid more eggs than older females during one month of observations in aquaria (van Camp *et al.*, 2005). These authors have suggested that this might signify male preference in this species towards younger females. However, females' capacity for sperm storage and intermittent egg laying among loliginid squid (Hanlon & Messenger, 1998) means that many of the females might not have finished laying eggs during the duration of this study, and so currently there is no evidence to support the theory of male choice in this species.

Field observations of *A. aculeatus* have observed males to preferentially mate guard and copulate more with larger females, which are likely to have a higher egg-laying capacity than smaller females (Huffard *et al.*, 2008a). Males of this species were also observed to have longer bouts of male/male aggression over larger females, however were more likely to engage in competitive bouts over medium sized females which are less likely to soon be usurped by other larger males (Huffard *et al.*, 2010). Similar observations have been made of *O. bimaculoides* in the laboratory, where higher levels of male-male aggression were reported in the presence of immature females (Mohanty *et al.*, 2014). These authors have hypothesised that a first-male sperm precedence in fertilisation patterns could lead to a greater male investment toward mating with smaller or younger females in some *Octopus* mating systems. However, this hypothesis has yet to be verified through analyses of brood paternities.

Observations of male preference and differential female copulatory success in female octopuses, but not necessarily in decapods are likely related differences in OSR between these mating systems. In decapods where OSR is more heavily male biased (Hall & Hanlon, 2002; Jantzen & Havenhand, 2003), it is more likely that males might attempt copulations with every possible female they have access to. In shallow-water octopuses where the OSR is more balanced (Huffard, 2005; Lopez-Uriarte & Rios-Jara, 2009), male preference in females might be an important factor to the reproductive success of females.

2.3.3: The Roles of Signalling and Sensory in Precopulatory Mate Choice

2.3.3.1: Visual signalling

Cephalopods possess a unique system of neurally controlled chromatophores, leucophores, iridiophores and dermal muscles that allow them to rapidly change the colour, tone, pattern and texture of their skin (Packard & Hochberg, 1977; Mäthger & Hanlon, 2007). This ability helps to enable cephalopods to employ impressive crypsis behaviours for defence against potential predators (e.g. Huffard *et al.*, 2005). Additionally, several studies have identified cephalopods to use these pattern-changing abilities as a means of intra-specific signalling (Hanlon *et al.*, 1994; Boal *et al.*, 2004; Palmer *et al.*, 2006). As mentioned above, visual displays using various chromatophore patterns have been observed in spawning assemblages of sepiids and loliginid squid, as well as in precopulatory behaviours of octopuses (Corner & Moore, 1981; Hanlon *et al.*, 1994; Hall & Hanlon, 2002; Huffard, 2007; Huffard & Godfrey-Smith, 2010; Schnell *et al.*, 2015). It is so far postulated that visual signals might aid in sex and species recognition, and for displaying agonistic intent between con-specifics (Boal, 2006; Scheel *et al.*, 2016). However, no studies so far have shown a response of opposite sex receivers to these signals, which leaves the role of visual signalling in courtship unknown.

An important aspect of visual signalling in cephalopods is that most studied taxa are not able to discriminate between different wavelengths of light like in human colour vision (Messenger *et al.*, 1973; Mäthger *et al.*, 2009), but rather are sensitive to the angles in which light is travelling (Moody & Parriss, 1961; Saidel *et al.*, 1983; Shashar *et al.*, 1996). This is termed polarisation-sensitivity, and is common amongst invertebrates and has also been reported in some birds and fish (Cronin *et al.*, 2003). Polarisation-sensitivity is useful in deep-water environments where the wavelength spectrum of light

decreases with depth but properties of polarised light remain intact (Shashar & Cronin, 1996). The ability to discriminate polarised light properties likely helps cephalopods with both navigation and in locating crustacean prey-items that have highly polarised exoskeletons (Shashar & Cronin, 1996). However, cephalopods are also able to change the polarised patterns reflected from their skin using their chromatophores and iridiophores (Shashar *et al.*, 1996; Boal *et al.*, 2004). Because cephalopods use skin patterning for visual signalling (Palmer *et al.*, 2006), are polarisation-sensitive (Moody & Parriss, 1961) and have the ability to alter the polarised patterns reflected from their skin (Shashar *et al.*, 1996), this presents the very likely possibility that that cephalopods might have the capacity to use polarised signalling as a concealed means of intra-specific communication (Mäthger *et al.*, 2009).

Evidence for use of polarised signalling as a communication channel in cephalopods is still very limited. So far the only experiments assessing cephalopods' ability to communicate using polarised signalling have taken place with *S. officinalis*. Shashar *et al.* (1996) observed *S. officinalis* to respond differently to their own image in a mirror depending on whether or not the mirror distorted the reflectance of polarised light, suggesting that this species might send and respond to polarised signals. In a follow-up study incorporating imaging polarimeters, which can colour-code and assign numeric values to polarisation patterns, Boal *et al.* (2004) observed female *S. officinalis* to display more polarised patterns than males. However, neither the quantity nor the nature of these displays differed in response to the number or sex of conspecifics viewed by the displaying female. Additionally these authors observed females to have higher activity levels when viewing conspecifics through clear barriers than when separated by barriers that distorted the polarisation patterns of conspecifics.

The two above studies suggest that female *S. officinalis* are at least able to perceive and respond to polarised patterns of conspecifics. However, it is not currently known what type of information might be sent or received through polarised signals, or what benefit these signals might have for the signaller or receiver. So far the use of imaging polarimeters has not yet been incorporated into observing cephalopod interactions in the field, or in a context of investigating mate choice or potential courtship. As visual signalling has been observed as an important component of precopulatory behaviour in studied cephalopods, the further integration of imaging

polarimetry within field or laboratory mate choice studies would be likely to reveal substantially more information about cephalopod visual displays.

2.3.3.2: Chemoreception

Cuttlefish, squid and octopus can sense chemical stimuli both from a distance using olfactory organs close to the eyes, and upon contact with objects using chemoreceptor cells located on the lips and suckers (Budelmann, 1996). *S. officinalis* increase ventilation rates when exposed to seawater containing odour from conspecifics, suggesting that this species can detect other members of its species based on chemical stimuli from a distance (Boal & Marsh, 1998). However, *S. officinalis* does not display any change in approach behaviour based solely on odours from conspecifics of different sex or mating history (Boal & Golden, 1999). Therefore, it is currently not supported that distance chemoreception would play a role in sex identification or mate choice in this species. However, it has not yet been assessed whether chemical cues might influence female receptivity to approaching males. Distance chemoreception between conspecifics has not yet been investigated within the Loliginidae, however they definitely have the capacity to obtain information from chemical stimuli in the water (Lucero *et al.*, 1992). Tactile chemoreception of conspecific eggs has been investigated within *D. pealei,* and it is suggested that a pheromone present in egg capsules of this species triggers males to engage in male/male agonistic behaviour to compete over females (Buresch *et al.*, 2003; King *et al.*, 2003). It is likely that this mechanism is partially responsible for the synchronised spawning assemblages within loliginid taxa (Buresch *et al.*, 2003; King *et al.*, 2003).

Distance chemoreception could potentially play a role in the mating system of at least two octopus species. Laboratory trials with *O. bimaculoides* revealed that this species can detect conspecifics based on odour cues, and that ventilation rates of individuals were different depending on the sex of conspecifics that were detected (Walderon *et al.*, 2011). Similar studies with *H. maculosa* found that the change in female ventilation rates in response to male odours correlated with agonistic behaviour and the probability that the female would reject a copulation attempt from the detected male (Morse *et al.*, 2017). Therefore distance chemoreception might enable some octopuses to determine the sex of conspecifics, and possibly to locate and/or discriminate between potential mates. Octopuses also possess many more chemoreceptors per sucker than

decapods (10,000 cells per sucker in octopuses compared to ~100 cells in cuttlefish suckers, Budelmann, 1996). This is likely related to the way in which octopuses reach into holes and crevices while foraging for food (Budelmann, 1996). However, a neurological study in *O. vulgaris* has also identified that the olfactory lobes, responsible for processing the sensory of chemical stimuli, are integrated with parts of the brain that regulates signal molecules involved in reproductive behaviours as well as feeding (Polese *et al.*, 2015). As mentioned previously in text, a tactile phase prior to copulation has been observed in *O. vulgaris* and *O. cyanea* (Wells & Wells, 1972), *O. digueti* (Voight, 1991) and *O. tetricus* (Morse, 2008). It is feasible that because octopuses have well-developed tactile chemoreception, that this could be used by some species to identify species, sex or possibly relatedness and/or quality or potential mates. As yet, the role of tactile chemoreception in mate choice has not been investigated within any cephalopod mating systems.

2.4: POSTCOPULATORY SEXUAL SELECTION IN COASTAL CEPHALOPODS

2.4.1: The Role of Sperm Competition in Sexual Selection

The aspects female promiscuity and sperm storage strongly suggest that postcopulatory processes take an influential role in sexual selection within cephalopod mating systems. The previous section addressed how different traits or behaviours can lead to differential copulatory rates within species. However reproductive success is based on the quantity of alleles passed on to future generations, and in highly promiscuous mating systems where females store sperm from multiple males in between egg laying intervals, copulatory rates alone will not necessarily determine the reproductive success of individuals. The differential fertilisation success between males that have copulated with the same female is referred to as sperm competition (Parker, 1970). Sperm competition can impact the relative reproductive success of males if certain morphological traits or behaviours can help some males to achieve increased fertilisation success (Parker, 1970). Sperm competition can also affect the reproductive success of females if fertilisation can be biased towards males that are more genetically compatible (Zeh & Zeh, 1996, 1997; Tregenza & Wedell, 2000; Mays Jr. & Hill, 2004), or if whichever trait or behaviour used by males to achieve higher fertilisation success can be inherited by their sons (Yasui, 1997; Kokko *et al.*, 2003).

A multitude of factors can affect sperm competition in animal mating systems. Several of these include: The numbers of males contributing sperm to a female (Parker, 1990), the relative contributions of sperm provided by each male (Parker, 1990), removal of previous males' sperm by subsequent male partners (Birkhead & Hunter, 1990), preferential locations for sperm placement (Naud *et al.*, 2005), differential sperm motility (Birkhead *et al.*, 1999), cryptic female choice (CFC) of sperm (Eberhard, 1996), and differential longevity of sperm and/or stratification of sperm within sperm storage receptacles that can lead to differences in fertilisation success based on the order of copulation by competing males (Birkhead & Hunter, 1990; Naud & Havenhand, 2006; Squires *et al.*, 2015; Hirohashi *et al.*, 2016). The current understanding of how sperm competition might impact cephalopod mating systems is still in its infancy. However all of the above mechanisms could potentially influence relative fertilisation success of male cephalopods. The following will summarise the current knowledge of sperm competition in cephalopods based on observations of sperm loading, sperm removal, female choice of sperm and relative paternity patterns.

2.4.2: Sperm Competition in Sepioidea

Sperm competition behaviours have been relatively well documented within sepiid taxa during observations in both the laboratory and field, and relatively recent laboratory investigations have additionally revealed insights to processes of postcopulatory mate choice within members of the Idiodepiidae and Sepiolidae (Sato *et al.*, 2013; Squires *et al.*, 2015; Sato *et al.*, 2016). Sepiid males perform sperm removal (Hanlon *et al.*, 1999; Wada *et al.*, 2005b; Wada *et al.*, 2006; Wada *et al.*, 2010), some degree of sperm loading (Hall & Hanlon, 2002; Wada *et al.*, 2006) and non-random patterns of fertilisation have been observed within females' egg masses (Naud *et al.*, 2005). As mentioned in the section on precopulatory behaviour, sepiid males compete for consort status with females whom they guard from rival males and occasionally pass more than one spermatophore (Corner & Moore, 1981; Adamo *et al.*, 2000; Hall & Hanlon, 2002). Copulating multiple times with the same female and mate guarding suggests that relative sperm contributions are likely important for fertilisation success within these mating systems. Additionally, Hanlon *et al.* (1999) observed three stages of copulation in *S. officinalis*. The first stage, which is the longest, is spent using the siphon to flush water over females' buccal areas, likely attempting to remove sperm from either

from seminal receptacles or from spermatangia left on females' exterior. The second stage, which lasted an average of 14 seconds, was for transferring new spermatophores, and the third stage was spent placing and/or manipulating the new spermatophores.

This behaviour has also been observed in *S. apama* (Hall & Hanlon, 2002; Naud *et al.*, 2004). However, Naud *et al.* (2005) found that water flushing did not reduce the counts of spermatangia found on females' buccal areas in *S. apama*, suggesting that males of at least this species possibly aim to remove sperm specifically from within seminal receptacles. Male *Sepia lycidas* use arm III to scrape old sperm masses from females' buccal areas, and spend more time doing this if they are not the last male to have copulated with the female (Wada *et al.*, 2010). This same study identified that larger males of this species will also spend more time removing sperm than smaller males. These authors suggest that smaller males might choose to pass spermatophores sooner if copulation might be likely to get interrupted by a larger male. *S. japonica* has also been observed to briefly remove previous males' spermatangia using arm IV (Wada *et al.*, 2006). However, these authors suggest that male *Sepiella* spp. might invest more time towards sperm loading than removal compared to Sepia spp. In this study, *S. japonica* males were observed to display intense mate guarding and in most cases would transfer more than one spermatophore to guarded females.

Currently, sepiid fertilisation patterns have been investigated only within wild populations of *S. apama.* Naud *et al.* (2004) found that males of all sizes and mating strategies had equal fertilisation success among eggs sampled from spawning areas. However, paternity comparisons within individual females' egg clutches were biased to spermatangia left on females' mantles and buccal areas in (Naud *et al.*, 2005). This suggests that it might be advantageous for males to copulate with females shortly before egg deposition and to place sperm externally on females rather that in the seminal receptacle. This pattern is supported in a study by Hanlon *et al.* (2005), in which a female-mimicking sneaker male that achieved a copulation with a female directly prior to egg deposition, was observed to fertilise that egg. If there is a last-male paternity bias to egg fertilisation in *S. apama* this would fit well with male sperm removal behaviour, and the fact that consort males attempt to guard females from rival males while escorting them to egg-laying sites (Hall & Hanlon, 2002).

It is also noteworthy that Naud and Havenhand (2006) discovered that sperm, stored within intact spermatophores in females' seminal receptacles, have longevities

up to two months. As *Sepia* spp. are intermittent spawners (Rocha *et al.*, 2001), this suggests that females might be able to use stored sperm for future egg fertilisations, and might possibly do so outside of spawning aggregations. Future studies investigating which males' sperm are stored in seminal receptacles vs. placed externally as spermatangia might yield further information about sperm competition in this species. Also, the fact that female sepiids are often selective of mates combined with the suggestion of a last-male paternity bias, presents a question of whether females might assess potential male partners differently based on the types of males they have recently copulated with (see trade-up behaviour in Pitcher *et al.*, 2003). Future studies observing female receptivity to sequential males, either in the field or laboratory, might elucidate whether female trade-up behaviour occurs in sepiid mating systems.

Similar studies, focused on members of two additional sepioid families (Idiosepiidae and Sepiolidae) have suggested that both sperm competition and CFC might be prevalent within the mating systems of these taxa. Laboratory studies with the Japanese pygmy squid (*Idiosepius paradoxus*) revealed that both larger males and males who copulated for longer with females, externally transferred more spermatophores to the base of females' arms during mating (Sato *et al.*, 2016). However, females of this species were observed to use their buccal masses to remove spermatophores from larger males, favouring the retention of spermatophores by males who copulated with them for longer durations (Sato *et al.*, 2016). Additionally, these females were more likely to be selective of transferred spermatophores during subsequent copulations, suggesting possible female postcopulatory trade-up behaviour in this species (Sato *et al.*, 2013). In the sepiolid, *E. tasmanica*, paternity analyses among genotyped candidate parents revealed biased fertilisation patterns to the most recent males to copulate with females (Squires *et al.*, 2015). Such findings emphasise that mating chronology may be of critical importance to paternal success within some sepioid taxa, and although not yet empirically demonstrated in other sepioid examples, a last-male paternity bias would be consistent with observations of male sperm-removal (Hanlon *et al.*, 1999; Wada *et al.*, 2005b; Wada *et al.*, 2006; Wada *et al.*, 2010), mate guarding (Corner & Moore, 1981; Adamo *et al.*, 2000; Hall & Hanlon, 2002), female trade-up behaviour (Sato *et al.*, 2013) and limited longevity of male sperm in female sperm storage structures (Naud & Havenhand, 2006).

2.4.3: Sperm Competition in Loliginidae

Sperm competition in loliginid squid has been investigated relatively more thoroughly than in sepioids or octopods. The current literature suggests that males employ sperm loading (Hanlon *et al.*, 1997; Hanlon *et al.*, 2002; Jantzen & Havenhand, 2003), but that also sperm placement (Iwata *et al.*, 2005), the interval between copulation and egg deposition (Buresch *et al.*, 2009; Hirohashi *et al.*, 2016), and possibly CFC of stored sperm (Shaw & Sauer, 2004; Buresch *et al.*, 2009) can all influence fertilisation patterns. Similar to sepiids, loliginid males compete for consort status with females that they copulate with repetitively and guard from rival males (Hanlon *et al.*, 1997; Hanlon *et al.*, 2002; Jantzen & Havenhand, 2003). This suggests that sperm loading may be important for male fertilisation success. This pattern has been confirmed in laboratory paternity experiments with *Heterololigo bleekeri* (Iwata *et al.*, 2005) and *D. pealei* (Buresch *et al.*, 2009) where higher copulatory rates resulted in higher male fertilisation success. These studies also found that paternities were biased to males that copulated with females in a parallel position that enabled internal placement of spermatophores (Iwata *et al.*, 2005; Buresch *et al.*, 2009), however that sperm from sneaker males, placed in the seminal receptacles, had greater longevity (Hirohashi *et al.*, 2016). Recent genotyping of egg strings obtained from wild spawning assemblages of the chokka squid, *L. reynaudii* have confirmed that paternity was typically biased to the male observed guarding the female at the time of collection (Naud *et al.*, 2016). Paternity bias to higher copulatory rates, the parallel mating strategy and mate guarding males suggest that consort males will generally achieve higher fertilisation success within the mating systems of these species, and this further explains both why males compete vigorously for this mating strategy (Hanlon *et al.*, 1997; Hanlon *et al.*, 2002; Jantzen & Havenhand, 2003) and why sneaker males have to metabolically invest so heavily into producing competitive sperm (Hirohashi *et al.*, 2016).

It is also strongly suggested that female loliginid squid have the capacity to influence the paternities of their egg capsules post-copulation. A female *D. pealei* has been observed to eject spermatophores from her mantle after a forced copulation (Buresch *et al.*, 2009), and these authors also identified that the interval between copulation and egg deposition greatly affects egg capsule paternity. When females of this species laid egg capsules within 40 minutes of copulation, the egg capsules were fertilised mostly by older sperm from previous male partners. However after 140

minutes, egg capsules paternities were biased to the most recent male to have copulated with the female. Additionally, Naud *et al.* (2016) observed a distinct switch in embryo paternity along *L. reynaudii* egg strings, suggesting that females of this species might have been using different males' sperm for egg fertilisation in non-random patterns. If females can reject spermatophores and presumably can choose when to lay egg capsules (Buresch *et al.*, 2009), then these observations combined with females' capacity to be selective of stored sperm use during external fertilisation (Shaw & Sauer, 2004; Naud *et al.*, 2016), suggest that female loliginids might be capable of controlling which males' sperm fertilise their egg capsules.

2.4.4: Sperm Competition in Octopodidae

The mechanisms of sperm competition are much less understood within octopus mating systems. It is probable that males of several species perform sperm loading and sperm removal. However this has only been formally addressed within one laboratory study, and currently no controlled paternity experiments have allowed fertilisation success to be compared among different males. Copulation durations are generally much longer in octopuses than in decapods (Hanlon & Messenger, 1998). Copulations have been observed to last more than an hour in most studied taxa, with the longest copulation being reported as 360 minutes in laboratory observations of *O. tetricus* (Joll, 1976). Field observations of *A. aculeatus* also report males to guard and copulate repeatedly with certain females (Huffard *et al.*, 2008a), and laboratory studies with *H. maculosa* have observed male of this species to mate for longer with both unfamiliar females and females that had recently mated with another competing male (Morse *et al.*, 2015). Prolonged copulation durations and multiple copulations with the same females, suggest that sperm loading might be an important factor for male fertilisation success. However, it is currently not known whether longer copulation times allow males to pass more spermatophores to females, and/or also might allow males to remove more sperm from previous males.

One study, assessing sperm removal in an unidentified pygmy octopus, found that this species had three phases of copulation, similar to sepiids (Cigliano, 1995). This author suggested that males might use their ligulae to remove competing sperm from females' oviducts during an initial phase of copulation, prior to transferring new spermatophores. Males were also observed to spend more time with the ligula inserted

in the female's mantle cavity prior to spermatophore transfer if the female had recently copulated with a different male. However males spent less time doing this if they were the last males to copulate with the same female. Males were most likely able to assess females' recent mating history based on the presence or absence of sperm in either the distal portion of females' oviducts of the oviducal glands (Cigliano, 1995). However it is impressive that males were able to determine if that sperm was their own, as the mechanism enabling them to do this is currently unknown and evidence for mate recognition among octopuses is very limited (Boal, 2006; but c.f. Caldwell *et al.*, 2015; Morse *et al.*, 2015).

Three molecular studies have so far confirmed multiple paternities within egg clutches of *O. tetricus* (Morse, 2008)*, Graneledone borealis* (Voight & Feldheim, 2009) and *O. vulgaris* (Quinteiro *et al.*, 2011). It has so far been postulated that female octopuses might benefit from polyandry due to increased genetic diversity of their offspring (Quinteiro *et al.*, 2011). However, so far no controlled paternity comparisons with known candidate fathers have been able to determine whether certain males displaying different morphologies, behaviours or mating strategies are able to gain increased fertilisation success within females' broods. As copulation durations are markedly longer in octopuses than decapods (Hanlon & Messenger, 1998), it would be interesting for future investigations to compare copulation duration to fertilisation success. Additionally, separate studies with two octopus species have reported that females might be able to control the duration of copulations, as observed by copulations consistently being ended by females (*O. digueti*, Voight, 1991; and *H. lunulata*, Cheng & Caldwell, 2000). If extended copulations lead to increased male fertilisation success, and females can choose to copulate for longer or shorter durations with different males, then this could potentially be a form of intra-copulatory mate choice in some species.

Although not yet empirically demonstrated, the reproductive system of female octopuses suggests that CFC may also occur in this family. Female octopuses also possess paired, muscular and innervated oviducal glands (Froesch & Marthy, 1975), from which they could potentially use to selectively pump sperm to the egg during fertilisation. Additionally, chemoattractant peptides have been found in egg capsules of *O. vulgaris*, that can influence the chemotaxis of male sperm (De Lisa *et al.*, 2013). This suggests that both mechanical and chemical processes might potentially be used by some female octopuses in manipulating the storage or fertilisation success different

males' sperm in their oviducal glands. However, at the time of writing this topic has not yet been investigated.

2.5: CONCLUSIONS AND SUGGESTED AREAS FOR FUTURE RESEARCH

Currently, the mechanisms of sexual selection are more thoroughly understood within some decapod mating systems than in those of octopuses. The coastal spawning aggregations of *S. apama* and loliginid squid have enabled much more detailed investigations within natural settings to have taken place for these taxa. Within sepiid mating systems, females appear highly selective of male partners. It is presently unknown what cues females might use to discriminate between potential male partners, whether certain males get preferential spermatophore placement in females' seminal receptacles or buccal areas, whether the suggestion of a last-male paternity bias is accurate and whether this consistently leads to increased female choosiness with successive males. It is suggested here that further studies of sepiid taxa, either in the field or in large aquaria with male-biased OSR, might provide this information if they can asses the context of different spermatophore placements, compare egg paternities to the order of copulation with genotyped males and compare female-male rejection rates between the first and subsequent males that attempt to copulate with females within egg-laying intervals.

Within loliginid mating systems, females of most studied species appear receptive to copulations with every attempting male (c.f. *S. lessoniana,* Wada *et al.*, 2005a). However it is strongly suggested that females might be selective of which sperm they use to fertilise egg capsules (Naud *et al.*, 2016). As copulations are usually very quick in these taxa (1 - 300 s, Hanlon & Messenger, 1998), it might be more parsimonious for females to avoid potential male aggression and the time or energy spent on rejecting males, by being receptive to every copulation and then to control egg capsule paternities post-copulation. Continued observations in the field might be able to further identify the context of both spermatophore rejections and varying intervals between copulation and egg deposition. If females eject spermatophores more or less often with and/or can adjust the timing of egg capsule deposition after copulating with different males that have varying displays, mating strategies or morphologies, then females might use these mechanisms as a form of CFC to bias paternity to genetically fitter and/or more compatible males (Eberhard, 1996; Tregenza & Wedell, 2000).

There is still much that can be learned about the processes of mate choice and sperm competition among the octopuses. Further observational studies and/or laboratory choice trials in species where visual courtship displays and female-male rejection are common might unveil whether cues such as ligula morphology or visual displays influence precopulatory mate choice in these taxa. Additionally, paternity comparisons with genotyped candidate fathers could reveal whether certain types of males gain higher fertilisation success within octopus mating systems, and also whether female brood paternities might be biased towards longer copulation durations, indicating sperm loading, or towards recent males, suggesting the influence of sperm-removal. If sperm loading is identified as an important factor in male fertilisation success, then it will be worthwhile investigating differential copulation durations in species where copulations are frequently terminated by females. This might determine whether females can influence their brood paternities by adjusting copulation times with males that display different morphology or behaviour.

As females of at least two octopus species are suggested to be capable of conspecific sex recognition based on odour cues (Walderon *et al.*, 2011; Morse *et al.*, 2017), it is worthwhile continuing to investigate the role of chemoreception within octopus mating systems. Two interesting follow-up questions that could be investigated within laboratory odour response experiments are a) Whether males respond differently to odours from sexually mature vs. immature females; and b) Whether either sex responds differently to odours from novel versus familiar conspecifics. Answering these questions could help to define the role of chemosensory in octopus social recognition and mate choice behaviours. Additionally, as mentioned in the section on visual signalling, visual displays have been reported as part of precopulatory behaviour of all sepiids, loliginids and octopuses studied in the field (Corner & Moore, 1981; Hanlon *et al.*, 1994; Hall & Hanlon, 2002; Huffard, 2007; Huffard & Godfrey-Smith, 2010; Schnell *et al.*, 2015). However, in order to make sense of these behaviours it is necessary to interpret how these displays are perceived by receiving conspecifics. As most cephalopods are polarisation-sensitive (Moody & Parriss, 1961), yet colour-blind (Mäthger *et al.*, 2009), the further integration of imaging polarimetry into field studies and laboratory mate choice trials is suggested to reveal valuable information about the way cephalopods might communicate within spawning assemblages or in a context of sex identification and/or courtship.

A common theme amongst all studied cephalopod mating systems is the extremely high level of both male and female promiscuity (Hanlon & Messenger, 1998). Male promiscuity is common within animal mating systems, and can develop easily as an evolutionarily stable strategy because promiscuity directly increases male reproductive success (Bateson, 1983). Female promiscuity is less common among species where females do not receive material resources or parental care from the males they mate with, because females have a finite number of eggs they can lay in a lifetime and therefore their reproductive success is typically not limited by the numbers of males they can copulate with (Kodric-Brown & Brown, 1987). Additionally, copulating with lots of different males can be potentially quite costly to females due to the increased risk of potential harm during copulations (Adamo *et al.*, 2000; Hoving *et al.*, 2010b), decreased foraging time (Huffard *et al.*, 2008a), increased risk of disease transfer (Thrall *et al.*, 2000) and increased energy expenditure (Franklin *et al.*, 2012). Therefore it appears mandatory in cephalopod mating systems for promiscuous females to achieve some type of selective advantage over non-promiscuous females in order for this phenomenon to be an evolutionarily stable strategy (Maynard Smith, 1982).

So far, polyandry in cephalopods has been suggested to benefit females by either helping to overcome potential sperm-limitation (van Camp *et al.*, 2004), increasing the genetic diversity of females' offspring (Quinteiro *et al.*, 2011), and/or optimising offspring quality (Squires *et al.*, 2012; Naud *et al.*, 2016). Sperm limitation might be an important factor to female reproductive success in species that have high egg-laying capacities and that might have infrequent encounters with opposite sex conspecifics (e.g. *Architeuthis* spp., Hoving *et al.*, 2004). However sperm limitation can probably not explain polyandrous behaviour in female cephalopods that have smaller fecundities and that would have the capacity to fertilise all their offspring to one or a few mate guarding males (e.g. *Sepia* spp., Mangold, 1987). Offspring diversity probably does increase the fitness of promiscuous females, however this mechanism alone being the drive for cephalopod polyandry is not consistent with observations of female-male rejections in many taxa, or with observed paternities consistently biased towards particular males (Iwata *et al.*, 2005; Naud *et al.*, 2005; Morse, 2008; Buresch *et al.*, 2009; Squires *et al.*, 2015; Naud *et al.*, 2016) rather than shared more equally between candidate fathers, as would be expected in a bet-hedging strategy.

The optimisation of offspring quality appears to be a robust hypothesis for the evolution of polyandry in cephalopod mating systems (Squires *et al.*, 2012; Naud *et al.*, 2016). However, the exact processes for how female promiscuity might lead to enhanced offspring quality still remain unclear. Postcopulatory fertilisation bias to either reproductively successful males or genetically compatible males are two possible mechanisms by there could be selective advantages for polyandry (Zeh & Zeh, 1996; Yasui, 1997; Zeh & Zeh, 1997) and neither has yet been investigated within a cephalopod mating system. Postcopulatory mechanisms might be especially applicable if females either cannot accurately assess male fitness or relatedness during precopulatory choice, and/or have limited control of which males they copulate with. In these contexts, polyandrous females could theoretically benefit from accepting sperm from multiple males if differential sperm fertilisation ability, or CFC consistently bias brood paternities to either the fittest or least related males. In the former scenario, if females' offspring are disproportionately sired to males that are innately capable of obtaining a higher fertilisation success, then promiscuous females are also likely to have sons with higher fertilisation success and therefore more grandchildren than non-promiscuous females (Yasui, 1997). This mechanism could potentially be investigated within laboratory paternity comparisons over several generations, and might be supported if copulatory rates and/or fertilisation success are correlated between fathers and their sons.

In the case of postcopulatory mechanisms biasing paternity to genetically compatible males, it is possible that female promiscuity is a form of ensuring inbreeding avoidance (see Tregenza & Wedell, 2002). Currently no molecular studies have assessed the relatedness of individuals within decapod spawning aggregations or interacting octopus during a breeding season. It is possible, that the frequency of close relatives might be quite high among potential mates, especially in species with limited dispersal such as *H. maculosa* or *Metasepia* spp. (Tranter & Augustine, 1973; Roper & Hochberg, 1988). Genomic studies within wild cephalopod populations, and paternity comparisons with known relatedness between mothers and candidate fathers could explain whether inbreeding avoidance might be one of the evolutionary drives for promiscuous behaviour in cephalopods.

Finally, it is worth noting again that the bulk of current knowledge for cephalopod sexual selection is still confined to the three families: Sepiidae, Loliginidae

and Octopodidae. The extreme depths and pelagic environments that other cephalopod taxa inhabit make it virtually impossible to observe them in their natural habitats. However, at least nautilids appear amenable to aquarium settings (Mikami & Okutani, 1977; Arnold, 2010), and hopefully methods will become available in the future for maintaining other deep-sea or pelagic cephalopod species successfully in the laboratory. Investigating precopulatory behaviour and fertilisation patterns of additional cephalopod taxa, either through laboratory rearing or ROV voyages, can likely provide valuable context to the current understanding of sexual selection and behavioural ecology in this unique class of animals.

CHAPTER 3: Nocturnal Mating Behaviour and Dynamic Male Investment of Copulation Time in the Southern Blue-Ringed Octopus, *Hapalochlaena maculosa* (Cephalopoda: Octopodidae)

Citation:

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3.1: ABSTRACT

The southern blue-ringed octopus**,** *Hapalochlaena maculosa* (Hoyle, 1883) is a nocturnal species that exhibits a mating system in which females hold sperm from multiple males over a one to two month breeding window before laying a single egg clutch. Contrary to most studied animal mating systems where anisogamy exists, gamete package production is limited for both males and females of this species (~50 spermatophores/eggs). This presents an animal model for studying aspects of sperm competition and dynamic mate choice behaviours. The present study reports on the mating behaviour of *H. maculosa* observed under laboratory conditions using infrared closed-circuit television video footage. Rates of male copulation attempts increased with male size, while female receptivity to mating attempts increased with female size, resulting in larger animals of both sexes gaining more copulations and spending more time per day in copulation. There was some evidence of female preference of larger males, but no male preference of females based on measured morphological traits. Both sexes terminated copulations in equal frequencies but male-terminated copulations were significantly shorter in duration. Males were more likely to terminate copulation early with females they had previously mated with, however were less likely to do so if the female had recently mated with a different male. Among male-terminated copulations, males mated for longer with females that had previously mated with other males in the trial. Male-male mounts were as common as male-female mounts, suggesting that male *H. maculosa* are not able to discriminate the sex of conspecifics. These findings suggest male strategic allocation of spermatophores based female mating history is an important factor influencing mating behaviours of this species.

ADDITIONAL KEYWORDS: mate choice – *Octopus* – operational sex ratio – sperm competition.

3.2: INTRODUCTION

Sexual selection is a form of intra-specific competition in which differential reproductive success within one or both sexes in a species can lead to the evolution of phenotypic traits and/or behaviours that aid individuals to increase their own level of reproductive success (Darwin, 1906; Bateson, 1983). To date, the processes of sexual selection have been predominantly studied within vertebrate and insect mating systems (West-Eberhard, 1983; Andersson & Simmons, 2006). Within these animal models,

anisogamy, which is the differential investment between males and females towards their gametes, leads to the reproductive success of most females of these taxa to be limited by the resources they have access to, and male reproductive success to be primarily limited by the numbers of females they can successfully mate with (Kodric-Brown & Brown, 1987). Therefore, where anisogamy exists, sexual selection typically imposes females to selectively mate with higher quality and/or genetically compatible males, and males to evolve traits or behaviours that enable them to achieve more copulations with a higher number of females, and to attain greater fertilisation success with the females they mate with (Darwin, 1906; Bateson, 1983; Kodric-Brown & Brown, 1987).

Cephalopods (Mollusca: Cephalopoda) present a different style of mating system from many other taxa, especially vertebrates, because male mate choice might be a critical factor influencing reproductive behaviours and spawning patterns within this class of animals. The fact that spermatozoa are encased in a finite number of discrete spermatophores, one or more of which are transferred to the female during copulation, imposes a disparity in which male spermatophores may be as limited or more limited than female eggs (Mann, 1984; Wodinsky, 2008). This disparity might be especially prominent among the octopods (Cephalopoda: Octopoda). For example, at any given time males of the giant Pacific octopus, *Enteroctopus dofleini*, (Hochberg, 1998) carry up to approximately ten spermatophores (Mann *et al.*, 1970), each of which can take over an hour to be placed during mating (Anderson *et al.*, 2003). By contrast, females of this species spawn up to 100,000 eggs, and are not limited in the number of males with which they can mate (Hartwick, 1983). The constraint of having a male reproductive capacity that could potentially be limiting might lead to a high investment by male cephalopods towards their gametes, and therefore a system in which male investment towards mate selection might influence reproductive success of individuals within a species. Accordingly, precopulatory mate choice by males has been observed in the algae octopus, *Abdopus aculeatus*, (d'Orbigny, 1834) where males preferentially guard and have longer bouts of male-male aggression over larger females that are likely to have higher egg-laying capacities (Huffard *et al.*, 2008a, 2010). Simlarly, in the California two-spot octopus, *Octopus bimaculoides,* (Pickford & McConnaughey, 1949) there is increased male-male aggression over immature females, which are likely to hold fewer sperm from competing males (Mohanty *et al.*, 2014).

Female octopods store sperm internally in the oviducal glands until they are ready for egg deposition, which is the time when fertilisation occurs (Mangold, 1987; Hanlon & Messenger, 1998). This system can lead to multiple paternity (Morse, 2008; Voight & Feldheim, 2009; Quinteiro *et al.*, 2011). Sperm competition, in the forms of sperm removal, sperm-loading and mate guarding have been documented amongst male cephalopods (Hanlon *et al.*, 1999; Iwata *et al.*, 2005; Wada *et al.*, 2005b; Wada *et al.*, 2006; Huffard *et al.*, 2008a). However, studies investigating the differential time investment that males allocate towards copulating with different females, based on either female novelty or recent mating history of females, are limited (c.f. Cigliano, 1995; Wada *et al.*, 2010). Males of both an unidentified pygmy octopod and the kisslip cuttlefish, *Sepia lycidas*, (Gray, 1849) have been observed to spend longer copulations, either performing sperm removal, or transferring more spermatophores with females that had recently mated with a competing male (Cigliano, 1995; Wada *et al.*, 2010).

As the availability of spermatophores are limited for most male cephalopods (Mann, 1984; Wodinsky, 2008), it is predicted that the strategic allocation of spermatophores and/or time by males might be commonplace amongst the Cephalopoda. Relevant models of sperm competition, where sperm supply is limited, imply that male cephalopods could potentially achieve optimal fertilisation success by investing less time copulating with females that they have already mated with (Parker, 1970), with females that are holding less sperm from competing males and therefore pose less risk of sperm competition (Ball & Parker, 2007), and/or when additional factors such as male mating order might give males an inherent advantage towards successful fertilisation (Parker, 1990). Likewise, male cephalopods should be expected to invest more time and/or spermatophores with novel females and females posing a high-risk of sperm competition (Parker, 1970, 1990; Parker *et al.*, 1997; Ball & Parker, 2007).

The southern blue-ringed octopus (*Hapalochlaena maculosa*) presents a model for addressing hypotheses concerning cephalopod mate choice for several reasons: 1) Copulations in this species are protracted compared to copulation times reported across other cephalopod taxa (see review in Hanlon & Messenger, 1998), suggesting that sperm-loading, sperm removal or male monopolization of females might be important in this species; 2) Copulations can either be terminated by the male or female, suggesting that either sex can regulate their time and/or potential gamete investment during

copulation; and 3) Sexually mature, virgin males bear approximately fifty spermatophores at any given time, and sexually mature females have approximately the same number of eggs (Tranter & Augustine, 1973), suggesting that male strategic allocation of their spermatophores might be critical to male reproductive success. Additionally, this species is small and easy to maintain in captivity, and adults are often found in very close proximity of each other in the wild (P. Morse, unpubl. data) making it feasible to recreate realistic population densities in laboratory settings. Finally, this species also has synchronous seven month life-cycles with a terminal breeding season (Tranter & Augustine, 1973), making it easy to obtain sufficient numbers of sexually mature adults for simultaneous study.

One of the limitations to studying cephalopod mating systems is that it is difficult to make long-term observations of most species in a natural setting. While field studies have been possible for some large decapods that spawn in aggregations (Corner & Moore, 1981; Hanlon *et al.*, 1997; Hall & Hanlon, 2002; Jantzen & Havenhand, 2003), and a diurnal octopus of moderate size, (Huffard, 2007; Huffard *et al.*, 2008a, 2010), small or highly cryptic cephalopods may be more efficiently studied in a semi-natural setting. As with many cephalopod taxa (Boyle, 1987), *H. maculosa* often live in subtidal and usually turbid water, and are nocturnal, making it currently impractical to gain long-term observations of natural mating behaviour for this species in the wild. Therefore, this study aimed to describe key aspects of both male and female mate choice behaviours in *H. maculosa* by reporting on focal animal observations made under laboratory-simulated natural conditions using infrared closed-circuit television (CCTV) and an experimentally manipulated operational sex ratio (OSR). Specifically, this study aimed to address the following questions relevant to the mating behaviour of *H. maculosa* within simulated natural conditions:

Approach Behaviour:

a) Does either sex make more approaches to conspecifics within trials, and is this affected by the OSR?

Copulatory Success:

b) Can any measurable morphological or behavioural trait be linked to higher copulatory rates or time spent copulating by either males or females, and is this affected by the OSR?

Copulation Terminations:

c) Are males more likely to terminate copulations early with females based on the novelty of the female, or her recent mating history in a manner consistent with predictions of sperm competition and/or strategic allocation of finite spermatophores?

Male-Male Mount Comparisons:

d) Do males attempt to mount other males, and if so how does the frequency, success and duration of male-male mounts compare to male-female mounts?

3.3: METHODS

3.3.1: Animal Acquisition and Maintenance

Wild adult *H. maculosa* (males: *N* = 12; females: *N* = 12) were sourced from false-shelter traps and from the by-catch of commercial fishermen between the Mandurah and Cockburn Sound coastlines in Western Australia (32°17'59" S, 115°39'4" E ± 40 km) from November 2013 to June 2014. A variety of false-shelter traps were used to obtain animals, and ranged from 20 mm lengths of plastic pipe (19 – 25 mm diameters) and concrete traps adapted from Schafer (2001). The cavity and entrance sizes were modified versions of the concrete traps used in (Schafer, 2001), which corresponds with the size of shells and structures that *H. maculosa* are observed to inhabit in the wild (P. Morse personal observations). Two sizes of concrete trap were used in this study to limit the size bias in collections. Small concrete traps had 50 x 30 mm cavities with 10 x 20 mm entrance holes. Large concrete traps had cavities sized 70 x 45 mm with 15 x 25 mm entrances. Animals were successfully found using all trap types, and *H. maculosa* also readily used the same trap types as shelters within the lab. Animals sourced from by-catch were obtained through commercial fishermen that fished for the gloomy octopus, *Octopus tetricus*, (Gould, 1852) under the license of the Fremantle Octopus Company. Commercial fishermen used a combination of larger false-shelter traps (approximately 20 cm in diameter), and Trigger Traps designed by Octopus Technologies PTY LTD. All animals were taken from between 3 – 28 m of water depth.

Animals were sourced under Western Australia DPaW permit: SF00963. The use and treatment of the animals were approved by the James Cook University Animal Ethics Committee (Approval Number: A1850). All animals were housed within individual 1 L plastic containers connected to a closed flow-through system with a

1,000L sump at Fremantle Octopus Company facilities in O'Conner, Western Australia (WA). Seawater was obtained from Cockburn Sound, WA where most of the animals were sourced, and water parameters we continuously maintained at 22 ^{o}C and between 34 – 35 ppt salinity. Male and female containers were separated by an opaque divider, and activated carbon was used to neutralise odours in seawater before entering animals' individual containers to limit animals' awareness of any pre-existing OSR prior to trials (see Kvarnemo & Ahnesjo, 1996). Each animal was given an appropriately sized shell for use as a den, and animals were fed *ad libitum* with sections of prawn and occasional live crabs. ReefOne™ biOrb fluorescent LED lights were used to simulate daylight for 14 hours per day, which corresponded to local daylight hours when trials began. All individuals were of adult size on capture and so were likely to have mated in the wild prior to experiments. Therefore all animals were maintained under these laboratory conditions for a minimum of two weeks prior to trials to help minimise any bias of different mating histories prior to capture.

Focal animal observations during copulatory behaviour trials were made in a larger experimental tank that was set up to simulate the substrate as similarly as possible to where *H. maculosa* were sourced. The bottom of the tank was 1 m^2 and had a water depth of 50 cm. The bottom of the tank was lined with sandy rubble. Twelve shells of various shapes and sizes, all large enough for *H. maculosa* to hide in, were scattered haphazardly across the tank floor. An aerator was used to keep water oxygenated during focal animal trials. Animals were fed *ad libitum* with sections of prawn throughout trials, and excess waste was removed from the experimental tank daily using a net.

3.3.2: Animal Measurements

Morphological traits were measured on all animals one day prior to entering trials. Wet weights were recorded using a digital scale. Mantle length (ML) and interocular width were recorded to the nearest mm using gloves and a ruler, while keeping the animal out of the water for a maximum of two mins. Male ligula lengths (Robson, 1929) were very small and had little variability (Mean = 2.08 mm ± 0.23 S.E.), so were not included within analyses. Length measurements were confirmed by additionally photographing the animal over a grid of 1 cm squares. Individual colouration patterns, markings and arm injuries were noted to aid in identifying individuals during trials (Adamo *et al.*, 2000; Huffard *et al.*, 2008a).

3.3.3: Copulatory Behaviour Trials

Copulatory behaviour trials consisted of focal animal observations recorded for six animals at a time within the experimental tank. In total, 24 animals were used to make up four distinct trials, each having one of three levels of OSR. A male-biased OSR trial was comprised of four males and two females; a female-biased OSR trial contained two males and four females; and two equal OSR trials both contained three animals of each sex. Each of the 24 animals was randomly selected from the available animals housed in the laboratory at the time of the trial, and each animal only entered one trial. All animals had a mantle length of at least 20 mm as this was the minimum size of animal observed to mate during pilot studies. Male sizes ranged from 2 – 9 g wet weight, (26 – 38 mm ML; 7 – 14 mm IO). Female sizes ranged from 1 – 12 g wet weight (20 – 42 mm ML; 5 – 17 mm IO). The six animals entering each trial were put into plastic containers with holes in them that were suspended within the experimental tank for 12 hours preceding the start of each trial in order to acclimate them to the new tank.

Each trial was planned to run for five days. However, in the first equal OSR trial one of the males became almost completely inactive on the fourth day after copulating for 197 minutes with a female much larger than himself. Therefore this trial was terminated after 3.28 days, and a new equal OSR trial was created using six new animals, and was allowed to run for another two days giving a total of 5.28 days of data for equal OSR trials. An overhead CCTV camera (Anran: High Resolution SONY CCD 700TVL Waterproof 78IR Zoom: 2.8 – 12 mm) was used to record all trials continuously to an external hard drive. Because this species is nocturnal (P. Morse personal observations) infrared video was used to monitor interactions taking place during the ten night-time hours each day. Visual checks were made daily to identify animals based on their individual markings recorded during animal measurements, and their locations within the tank in order to ensure that IDs were correct during video playback.

3.3.4: Focal Animal Video Observations

During video playback, behaviours were scored for each of the 24 individuals among the total 15.28 days of focal animal observations in the four trials. The following behaviours were scored: number of approaches made by each animal; first animal to make contact after an approach; retreating individual after any interaction; male mount attempts with females; female receptivity to male mount attempts; successful male-

female copulations; copulation durations; individuals terminating each copulation; and the identification of individuals in all interactions. Definitions of female receptivity and female-terminated copulations are outlined for the greater blue-ringed octopus, *Hapalochlaena lunulata*, (Quoy & Gaimard, 1832) by Cheng and Caldwell (2000), and were used for categorising the behaviours within this study. In short, females were considered receptive to male copulation attempts if there was no grappling phase or obvious attempt to retreat between male contact and a successful male mount. It was not always possible to observe hectocotylus insertion due to the small size of the animals and the fixed camera angle. Therefore, male-female copulations and male-male mounts were considered successful if the mount lasted for a minimum of 30 s. Similarly, it was not possible to enumerate spermatophore release, and so this was not addressed within analyses. Copulations were considered terminated by the female when the female was observed using her arms to push the male off of her. Male-terminated copulations were categorised by the male passively unmounting the female without female instigation. It was not possible to identify which sex terminated two of the copulations during video playback, and these copulations were omitted from analyses that used copulation termination as a factor.

3.3.5: Behavioural Analyses

A total of 29 male-female copulations and 557 approach/contact/retreat interactions were observed among the four trials. Where appropriate, measures were scaled to daily rates to accommodate the different lengths of observation time for animals in separate trials. Copulation durations were transformed to a normal distribution using a log-scale transformation prior to analyses. Some animals did not copulate during trials, and so time spent copulating per day for individual animals was transformed to a log + 1 scale to normalise this distribution that contained values of zero. Frequencies of copulations terminated by either sex were compared between each level of OSR using Fisher's Exact Test, and all other behavioural comparisons were made using general linear modelling (GLM).

Approach frequencies between each pair of animals within trials were compared using Negative Binomial Models with response values offset by the log-value of days that animals were observed for, as this test is robust against data that has a high residual deviance (Jones *et al.*, 2013). Copulatory rates and male-female copulation attempts,

being frequency data, were analysed using GLMs fitted to a Poisson distribution. Proportional data were analysed using Logistical Regression. Comparisons made using daily time spent copulating per animal as the response variable were analysed using a Fixed-Effects ANOVA as each animal only had one data point within these analyses. Most animals copulated more than once, so all comparisons of copulation durations among the 29 observed copulations were analysed using a Mixed-Effects Model with the identification of individual males and females both set as random effects to account for individual variation between animals. Comparisons of male-male mount attempts to male-female mount attempts within different levels of OSR were also performed using a Mixed-Effects Model with male ID as a random effect to account for males that made attempts to mount both males and females. Additionally, as in this analysis male-male mount attempts were greatly influenced by trial OSR, individual rated of daily mount attempts towards males and females were divided by the relevant number of other males and females in the trial respectively to make the rates comparable between trials containing different OSRs. Finally, these OSR-corrected daily rates were fitted to a 'square root + 1' transformation to normalise the distribution of these frequency data containing zeros.

Although all animals had a minimum mantle length of 20 mm, two of the females that had wet weights of less than five grams were unreceptive to all male copulation attempts and did not copulate during trials. One of these females was from the female-biased OSR trial and the other was from the second equal OSR trial. These two females might have been sexually immature and so their rejections of attempts by males to copulate were omitted from comparisons of female receptivity to male size. All males gained copulations within trials, and males made copulation attempts with all females. All statistical analyses were carried out using S+ software under license to James Cook University.

3.4: RESULTS

3.4.1: Approach Behaviour

With very few exceptions, focal animals spent all daylight hours hiding within shells or under gravel. Daytime behaviours consisted only of occasionally changing shelter locations between shells or gravel, or approach/copulatory behaviours during the first two hours after animals were placed in a trial. All other approach, feeding and

copulatory behaviours occurred during simulated night-time hours when interactions were recorded using infrared CCTV. All animals were relatively active during night-time hours. Males made significantly more approaches to conspecifics within trials than females (Negative Binomial Model: $X^2 = 11.284_{117}$, $P < 0.001$; Fig. 3.1). Male approach frequencies were significantly affected by the OSR of the trial, in that individual males made more approaches to conspecifics in trials that contained fewer females (Negative Binomial Model: $X^2 = 4.159_{58}$, $P = 0.041$; Fig. 3.1). However, female approach frequencies were unaffected by OSR (Negative Binomial Model: $X^2 = 0.218_{58}$, $P = 0.64$; Fig. 3.1). Individual approach fequencies were independent of animal size among both males (Negative Binomial Model: $X^2 = 1.245_{57}$, $P = 0.265$) and females (Negative Binomial Model: $X^2 = 0.025_{57}$, $P = 0.874$).

Among pairwise approach combinations between all individuals in trials, the sex of the approached animal had no effect on approach frequencies among either male (Negative Binomial Model: $X^2 = 0.423_{58}$, $P = 0.515$) or female (Negative Binomial Model: $X^2 = 0.333_{58}$, $P = 0.564$) approaches. This suggests that animals within trials were either unable to discriminate the sex of consepecifics while approaching, or chose to approach both sexes equally.

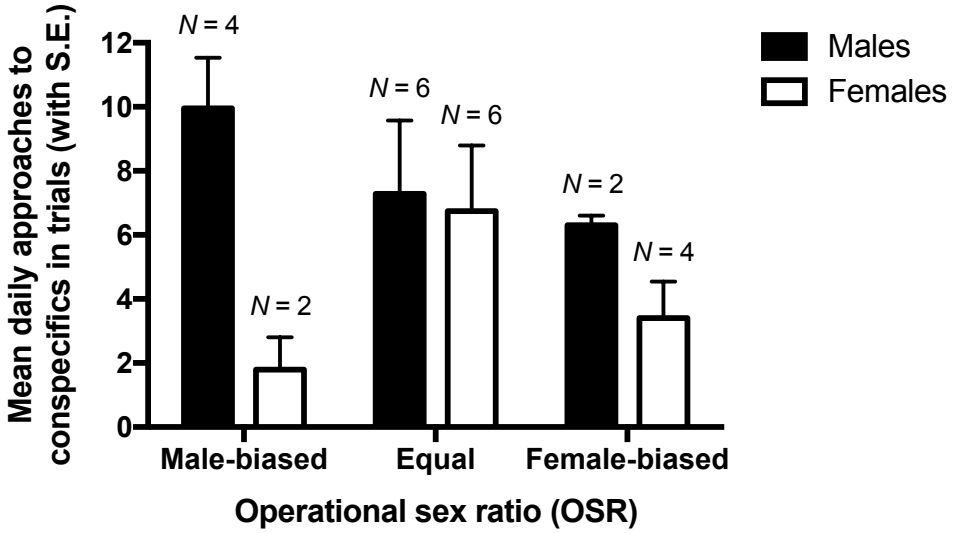

Figure 3.1: Mean male and female approach rates (approaches per day per individual) by OSR treatment. Males made significantly more approaches towards conspecifics within trials than females, and male approaches were significanty affected by the OSR of the trial.

3.4.2: Copulatory Rates

A total of 29 successful male-female copulations were observed among the four trials. Observations were consistent with behaviour recorded by Tranter and Augustine (1973), in that males mounted females by wrapping their arms around their mantle and inserting the hectocotylus through the female's aperture. Although males made more approaches overall (Fig. 3.1), and female rejection of male copulation attempts was common (see below), successful copulations among all trials were initiated equally by approaches from both sexes (Generalised Linear Poisson Model: $X^2 = 0.311_{10}$, $P = 0.577$). Copulation durations ranged from 38 to 348 mins (Mean = 117.66 mins ± 14 S.E.). All males copulated during trials, however females only participated in copulations at a minimum of five grams wet weight (Fig. 3.2A).

Copulatory rates (mean per 24 h) within trials increased with body mass for both males (Generalised Linear Poisson Model: $X^2 = 5.216_9$, $P = 0.005$) and females (Generalised Linear Poisson Model: $X^2 = 12.791_9$, $P < 0.001$). Similarly, mean daily time spent in copulation increased with size for both males (Fixed-Effect ANOVA with a Log + 1 Transformation: $F = 9.121_{1\ 8}$, $P = 0.017$; Fig. 3.2A), and females (Fixed-Effect ANOVA with a Log + 1 Transformation: $F = 9.645_{1\ 8}$, $P = 0.015$; Fig. 3.2A). Additional measures of size: mantle length (Fixed-Effect ANOVA with a Log + 1 Transformation: $F = 8.458_{1\ 10}$, $P = 0.016$) and interocular width (Fixed-Effect ANOVA with a Log + 1 Transformation: $F = 8.116_{1\ 10}$, $P = 0.017$) had significantly positive relationships with time spent copulating by females. However, these morphological traits were highly correlated with body mass among females (Mantle Length/Wet Weight: r = 0.908; Interocular Width/Wet Weight: r = 0.886). Therefore wet weight was used to represent body size in following analyses. These traits measured on males were less correlated to body mass (Mantle Length/Wet Weight: r = 0.254; Interocular Width/Wet Weight: r = 0.847) and did not have significant relationships with average time spent copulating by males (Mantle Length: Fixed-Effect ANOVA with a Log + 1 Transformation: $F = 271_{1\ 10}$, $P = 0.254$; Interocular Width: Fixed-Effect ANOVA with a Log + 1 Transformation: $F = 3.825_{1\ 10}$, $P = 0.079$).

Trial OSR had no significant effect on average time spent copulating per day among either males (Male-Biased OSR: Mean = 46.4 min ± 23.2 S.E.; Equal OSR: Mean = 132.48 mins ± 54.08 S.E.; Female-Biased OSR: Mean = 60 mins ± 42.43 S.E.; Fixed-Effect ANOVA with a Log + 1 Transformation: $F = 0.023_{1\ 8}$, $P = 0.884$), or females (Male-Biased OSR: Mean = 85.7 mins ± 60.6 S.E.; Equal OSR: Mean = 132.48 mins ± 54.08 S.E.; Female-

Biased OSR: Mean = 30 mins ± 15 S.E.; Fixed-Effect ANOVA with a Log + 1 Transformation: $F = 3.754_{1\ 8}$, $P = 0.089$). However average female copulatory rates in trials were significantly affected by OSR (Generalised Linear Poisson Model: $X^2 = 13.79_{10}$, $P < 0.001$), with females gaining more copulations in trials that contained more males (Fig. 3.2B). Contrastingly, male copulatory rates were not significantly affected by OSR (Generalised Linear Poisson Model: $X^2 = 0.692_{10}$, $P = 0.406$; Fig. 3.2B).

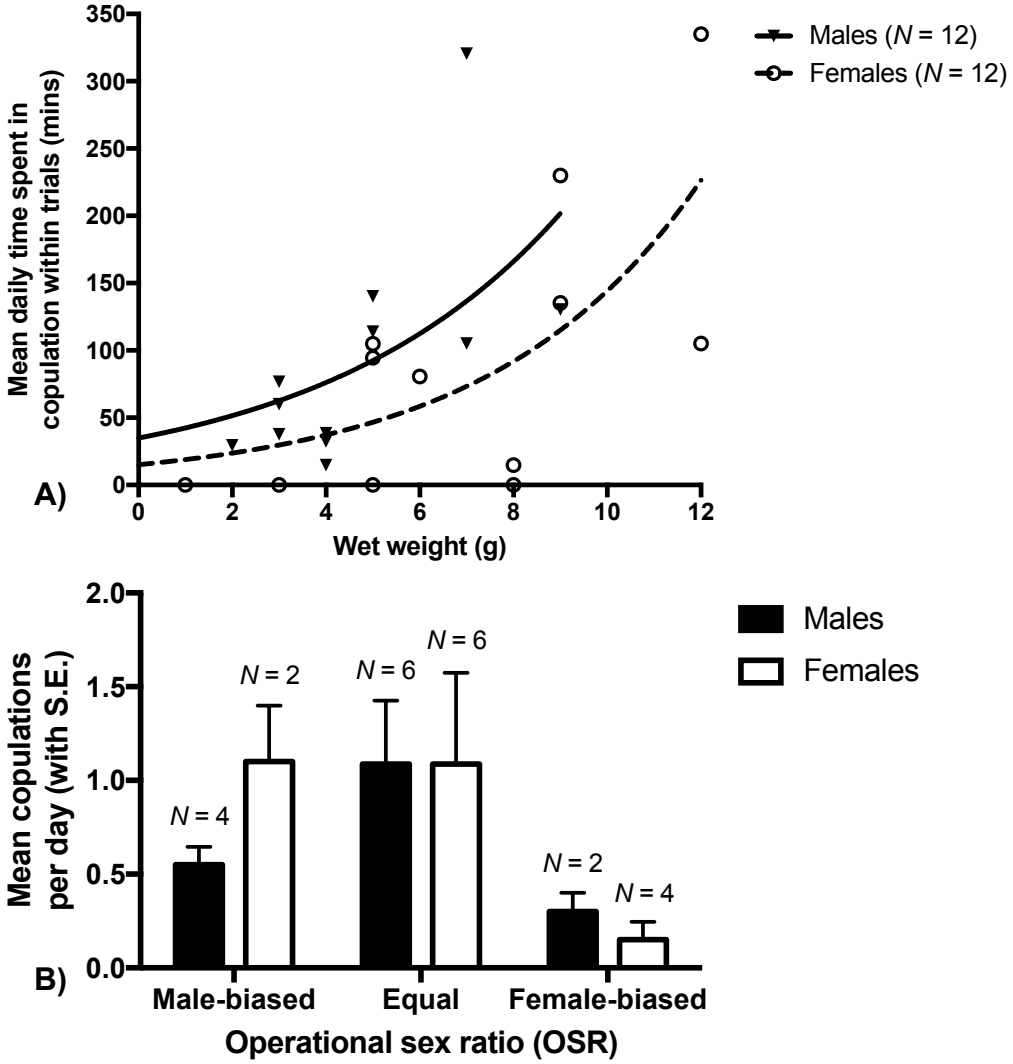

Figure 3.2: A) Time spent copulating per day increased with size for both males and females. Copulation times were unaffected by trial OSR. The solid and broken lines represent linear regressions to log + 1 transformed data: $y = e^{((0.274x + 2.932)-1)}$ for males ($P = 0.017$); and $y = e^{((0.449x - 0.007)-1)}$ for females ($P = 0.028$) respectively; B) Male copulatory rates were not significantly affected by trial OSR, however females had significantly more copulations within trials containing more males.

3.4.3: Copulation Terminations

Among the 29 observed copulations, two copulations were terminated either inside or behind a shell and so the terminating member could be identified for a total of 27 copulations during trials. Among these observations, 15 copulations were terminated by females and 12 by males. Copulations were terminated equally by both sexes and there was no effect of OSR on these frequencies (Fisher's Exact Test: d.f. = 2, $P = 0.699$). Among focal animals, there was no effect of female wet weight on the likelihood of a female to terminate copulations (Logistic Regression: X2 = 0.8745, $P = 0.35$). Larger males did terminate significantly more copulations than smaller males (Logistic Regression: X2 = 4.9059, $P = 0.027$). However, this pattern was driven by larger males having more copulations than smaller males (Fig. 3.2), and males being more likely to terminate copulations during subsequent matings (see Male Mating Behaviour below). Among observed copulations, there was no effect of size difference between males and females influencing the sex that terminated copulation (Logistic Regression with a Binary Response: X2 = 0.83425, $P = 0.361$). After accounting for variability among individuals as a random effect, copulations terminated by males were significantly shorter than copulations terminated by females (Linear Mixed-Effects Model with male and female identifications as random effects and a log transformation of Copulation Time: F = 6.3011 6, $P = 0.046$; Fig. 3.3).

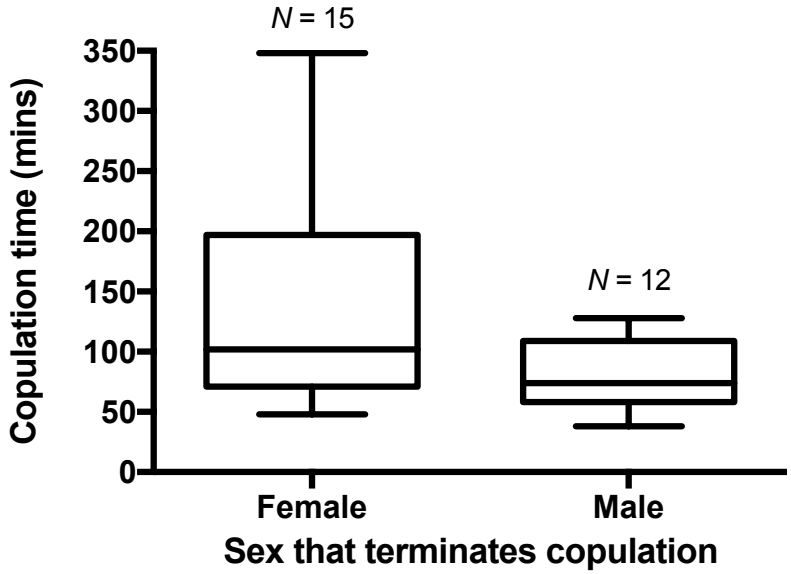

Figure 3.3: Male terminated copulations were significantly shorter than female terminated copulations.

3.4.4: Female Mating Behaviour

Female copulation times decreased significantly during subsequent copulations within trials (Linear Mixed-Effects Model with male and female IDs as random factors and a log transformation of Copulation Time: $F = 9.519_{1\ 10}$, $P = 0.012$). Females were often unreceptive to male mounting attempts by pulling their arms over their mantle apertures, grappling with the male and/or attempting to retreat when the male tried to mount. This mode of mate rejection occurred on 25 occasions among trials, and on nine of these occasions the male managed to mount and copulate with the female anyway. Female receptivity to males was similar between all levels of OSR (Logistical Regression: $X^2 = 0.903_{10}$, $P = 0.342$). Excluding copulation attempts with females smaller than five grams, male size had no effect on female receptivity (Logistical Regression: $X^2 = 2.655_{9}$, $P = 0.103$). Female receptivity to copulations did significantly increase with female size (Logistical Regression: $X^2 = 9.155_{9}$, $P = 0.002$; Fig. 3.4A). Additionally, there was some evidence for females to mate for longer with larger males (Fig. 3.4B). After accounting for individual variation as a random effect, there was a significant interaction between the sex that terminated copulation and male size impacting on copulation time (Linear Mixed-Effects Model with male and female IDs as random effects and a log transformation of Copulation Time: $F = 9.712_{1\ 7}$, $P = 0.017$).

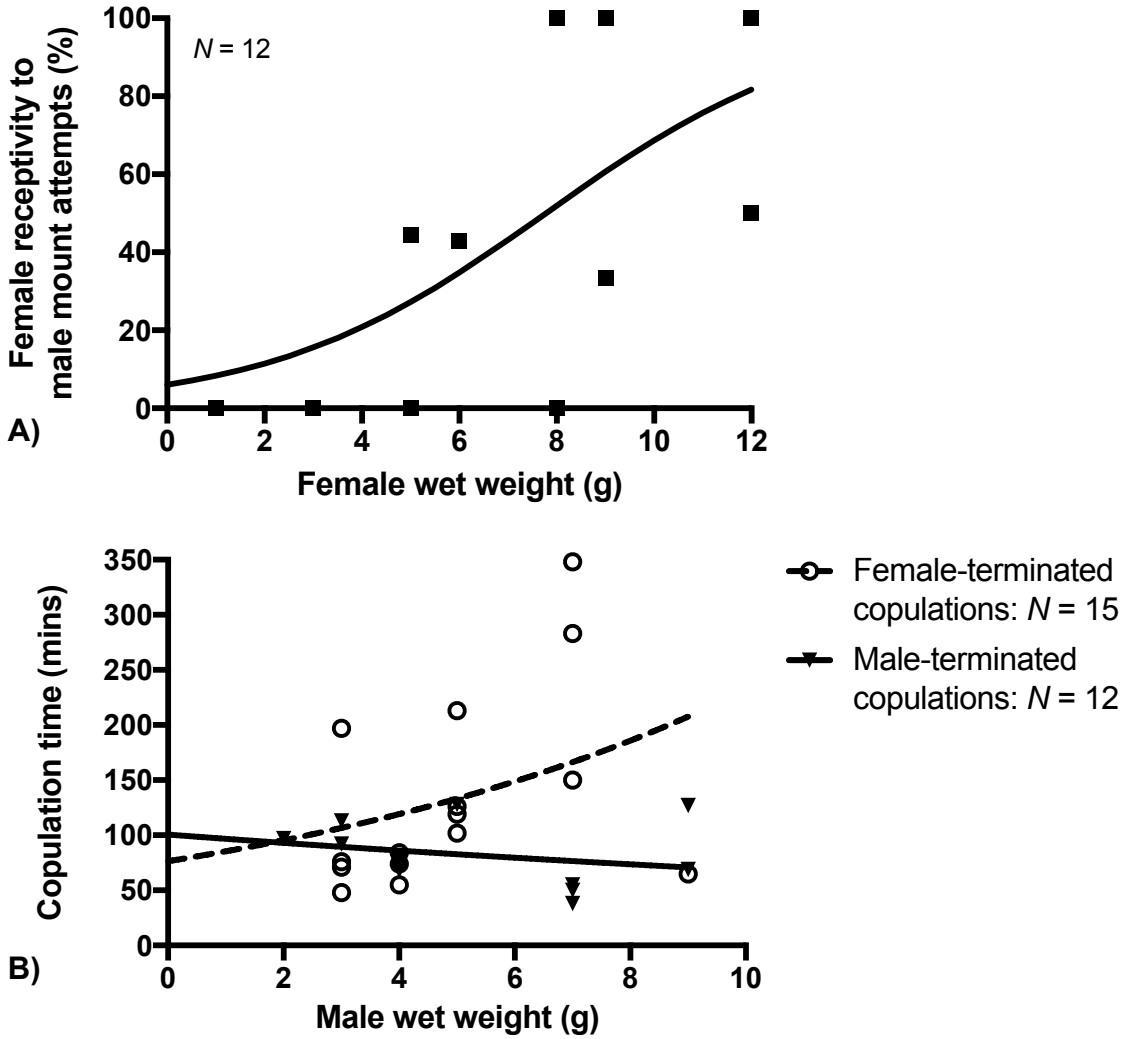

Figure 3.4: A) Larger females were receptive to male copulation attempts significantly more often than smaller females Females did not begin being receptive to copulations until they reached a minimum of five grams wet weight. The solid line represents the logistic regression: $y = 1 / (1+e^{-(3.354x - 2.75)})$; $P = 0.002$; B) There was a significant interaction between the sex that terminates copulation and male size impacting on copulation time. Among female-terminated copulations, copulations tended to be longer with larger males, while the lengths of male-terminated copulations were independent of male size. The solid and broken lines represent Linear Mixed-Effect Models fitted to log transformed data: $y = e^{(-0.096x + 4.907)}$ for male-terminated copulations ($P = 0.017$); and $y = e^{(0.172x + 3.889)}$ for female-terminated copulations ($P = 0.047$) respectively.

3.4.5: Male Mating Behaviour

Male-male physical aggression over females and mate guarding were not observed during this study. Instead all male-male interactions were confined to contact-retreat behaviours or mount attempts. Male-female copulation times decreased significantly with all subsequent copulations during trials (Linear Mixed-Effects Model with male and female identifications as random factors and a log transformation of Copulation Time: $F = 8.084_{1\ 10}$, $P = 0.018$). Larger males attempted to copulate with females more frequently than smaller males within trials (Generalised Linear Poisson

Model: $X^2 = 7.463_9$, $P = 0.006$; Fig. 3.5A). However, female size had no effect on the number of copulation attempts she received (Generalised Linear Poisson Model: $X^2 = 1.357_9$, $P = 0.244$).

Among the 27 observed terminations of copulation, males terminated copulations based on different criteria depending on if it was his first or subsequent copulation within a trial. During first copulations of all males in a trial, they always waited for the female to terminate the copulation if the female had not yet copulated with another male during the trial. In contrast, males always terminated their first copulations early if they were mating with a female that had mated with a different male previously in the same trial (Logistical Regression: $X^2 = 13.863_8$, $P < 0.001$; Fig. 3.5B). Among all subsequent male copulations, female novelty significantly influenced the likelihood of a male to terminate the copulation early (Logistical Regression: $X^2 = 8.614_{15}$, $P = 0.003$; Fig. 3.5C). Males were significantly more likely to terminate copulations with females that they had already mated with during the trial, and copulation times between repeating pairs of males and females were significantly shorter than copulations between novel pairs (Linear Mixed-Effects Model with male and female IDs as random effects and a log transformation of Copulation Time: $F = 7.079_{1\ 10}$, $P = 0.024$; Fig. 3.5D). Among the eleven copulations between repeating pairs of males and females, males were significantly more likely to terminate the copulation if they were the last male to have mated with the female but were more likely to wait for the female to terminate if the female had last mated with a different male (Logistical Regression: $X^2 = 4.18_9$, $P = 0.041$). Additionally, the lengths of male-terminated copulations varied significantly according to recent female mating history. Male-terminated copulations were significantly longer with females that had previously mated with more competing males during the same trial (Linear Mixed-Effects Model with male and female IDs as random effects and a log transformation of Copulation Time: $F = 9.334_{1\ 6}$, $P = 0.022$).

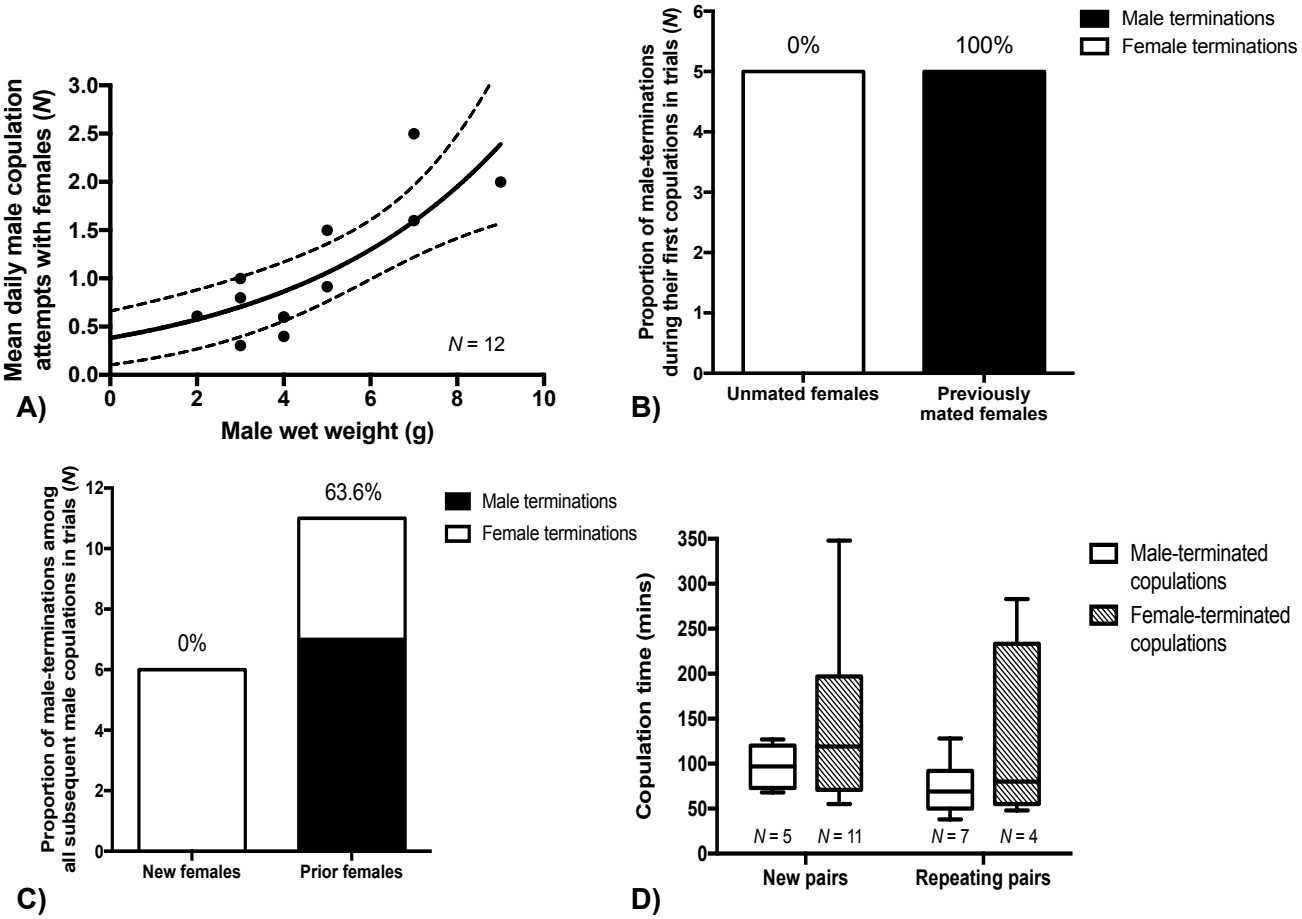

Figure 3.5: A) All males attempted to mate with females, and among all trials larger males attempted to copulate with females more frequently than smaller males. The solid line represents the Poisson regression: $y = e^{(0.318x - 1.088)}$; $P = 0.006$. The broken lines represent the 95% confidence limits of the equation; B) Among male's first copulations of the trial, the male was always the terminating member when the female had previously mated with another male in the trial. When it was the first copulation of the trial for both the male and the female, the male always waited for the female to terminate the copulation; C) Among all male subsequent copulations, males were significantly more likely to terminate the copulation if they had previously mated with the female, and would always wait for the female to terminate the copulation if he had not yet mated with her; D) After accounting for individual variation among individuals as a random effect, copulation times during subsequent copulations between males and females that had already previously mated together were significantly shorter than copulations between new pairs of males and females. Sample sizes were too small to detect differences in copulation times within only male or female-terminated copulations.

3.4.6: Male-Male Mount Comparisons

Male-male mounts were frequent among trials. After making daily rates of male attempts to mount comparable between trials by dividing daily rates of male-male and male-female mount attempts by the number of other males and females in trials respectively, the overall frequencies of male-male and male-female mount attempts were similar (Linear Mixed-Effects Model with male ID as a random effect and a 'square root +1' transformation of OSR adjusted daily mount attempts: $F = 2.89_{1\ 10}$, $P = 0.12$).

However, there was a significant interaction between trial OSR and the sex that males most frequently tried to mount (Linear Mixed-Effects Model with male ID as a random effect and a 'square root +1' transformation of OSR adjusted daily mount attempts: $F = 8.099_{1\ 10}$, $P = 0.017$). This interaction was driven by there being more than twice as many male-male mount attempts than expected within the male-biased OSR trial (Fig. 3.6). In contrast to male-female mounts, males were never receptive to being mounted by another male, and this was usually followed by grappling and/or retreat behaviour from one of the males. This led to the success rate for male mount attempts to be significantly less for male-male mounts than male-female mounts (Logistical Regression: $X^2 = 26.057_{18}$, $P < 0.001$; Fig. 3.7A). Although successful male-male mount durations ranged from 2 to 162 mins, average male-male mount times were significantly shorter than male-female mounts (Linear Mixed-Effects Model with male and female IDs as random effects and a log transformation of Mount Time: $F = 48.258_{1\ 17}$, $P < 0.001$; Fig. 3.7B).

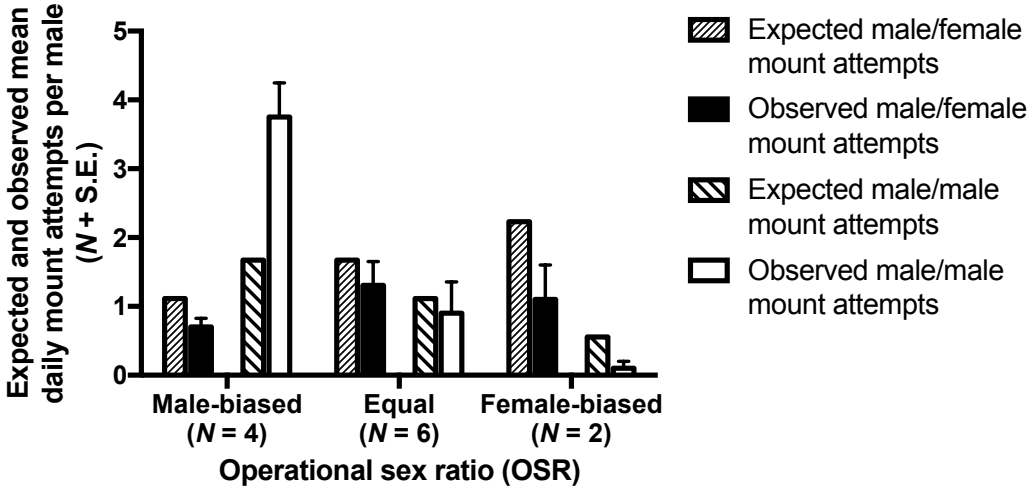

Figure 3.6: Male-female and male-male mount attempt rates were similar among all trials. However there were more than twice as many male-male mount attempts than expected in the male-biased OSR trial. Expected attempt rates are shown for illustrative purposes only and were calculated using the average daily total mount attempts per male and multiplying by the ratio of other males and females in trials to predict expected male-male and male-female mount rates respectively. In the male-biased OSR trial, expected daily rates = 2.787*(2/5) for male-female mounts and 2.787*(3/5) for male-male mounts; Equal OSR Trial expected daily rates = 2.787*(3/5) for male-female mounts and 2.787*(2/5) for male-male mounts; and in the female-biased OSR trial, expected daily rates = 2.787*(4/5) for male-female mounts and 2.787*(1/5) for male-male mounts.

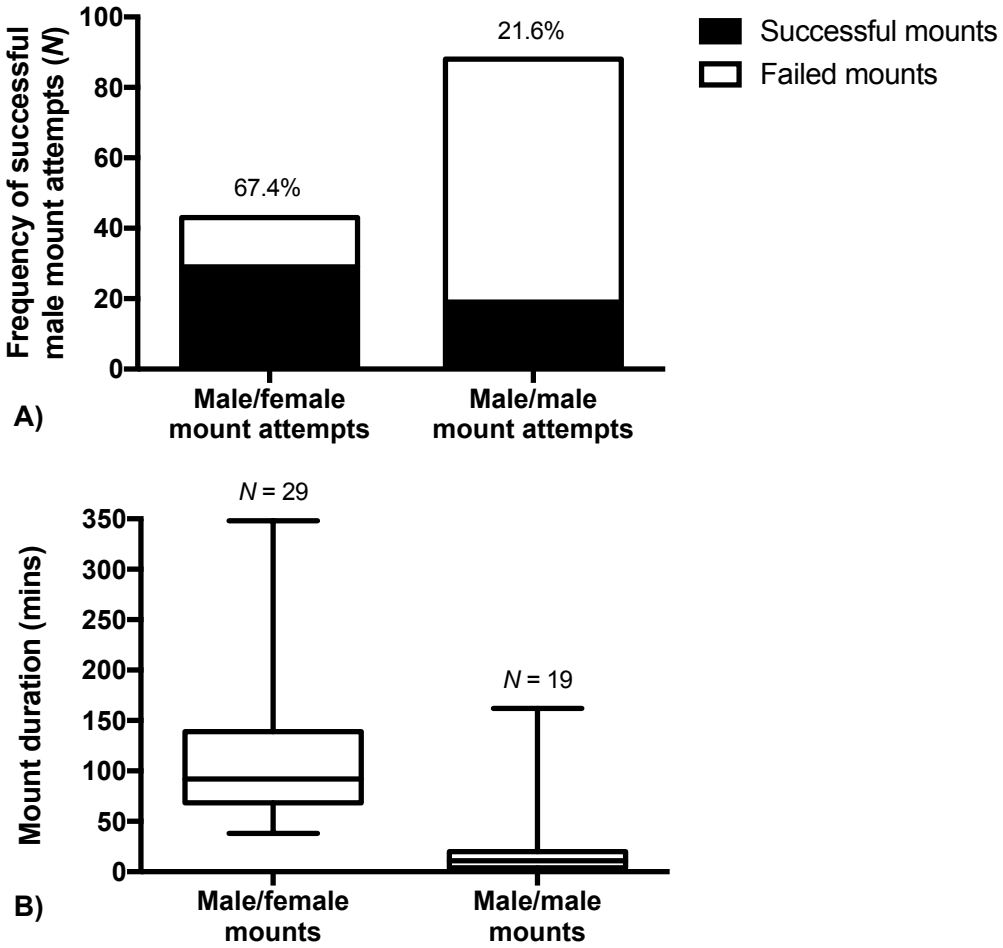

Figure 3.7: A) Male mount attempts were significantly more likely to be successful with females than with other males; B) Successful male-male mount durations were significantly shorter than successful male-female mounts.

3.5: DISCUSSION

Laboratory observations of focal animals in this study indicated that, when sexually mature, both male and female *H. maculosa* approach and contact nearby conspecifics, which often leads to an attempt to mount by males, and copulation. Males made more approaches than females, particularly when the availability of females was low, suggesting that males expend more effort than females to initiate copulation. This result is consistent with patterns observed across most animal groups where anisogamy exists (Kodric-Brown & Brown, 1987). Both males and females were approached in similar frequencies, and male-male mount attempts were very common, suggesting that *H. maculosa* could not discriminate the sex of approached conspecifics within trials. This observation supports findings by Cheng and Caldwell (2000), where male *H. lunulata* was found to attempt mounting other males as readily as female conspecifics.

The apparent lack of sex recognition in *Hapalochlaena* spp. contrasts with field observations of *A. aculeatus*, which recognised the sex of conspecifics from a distance (Huffard *et al.*, 2008a), and laboratory experiments with *O. bimaculoides*, which discriminate conspecific sex based on odour cues (Walderon *et al.*, 2011). Yet even in *A. aculeatus*, male-male mating attempts did occur in low frequencies and in all cases led to physical aggression (Huffard *et al.*, 2008a). The potential use of odour cues were not addressed in this study. However, based on the frequency of male-male approaches and mount attempts, it seems unlikely that male *H. maculosa* use odour cues to identify females to mate with. It is possible that *Hapalochlaena* spp., which have an even shorter breeding window than most *Octopus* taxa (Tranter & Augustine, 1973; Overath & Boletzky, 1974), have not developed the ability to discriminate the sex of conspecifics. This might be due to the risk of missing an opportunity to mate potentially outweighing the cost of intra-sexual aggression. No male-male aggression has been reported within *H. lunulata* (Cheng & Caldwell, 2000), and the only aggression resulting from same-sex mounts in the present study was confined to brief grappling behaviour and never led to noticeable male injury.

Sample sizes within each trial were very low, and may have affected this study's ability to detect differences in mating behaviour between different levels of OSR. For example, there was a consistent trend for females to have longer copulation times in trials containing fewer males. It is possible that a greater sample size might have been able to identify this as a significant pattern. Within the limitations of this study, only three significant behavioural changes were evident between trials containing different OSR: 1) males made more approaches to other individuals when fewer females were available, 2) females had fewer copulations when fewer males were present, and 3) male-male mount attempts were more common than expected in the male-biased OSR trial. The lower female copulatory rates in the female-biased OSR trial coincide with the finding that males approach conspecifics more often in this species, and therefore copulation opportunities for females were reduced in this trial. Similarly, this pattern led to the increase in observed male-male mount attempts during the male-biased OSR trial. Male-biased OSRs have been reported to influence the frequencies of both male sexual displays and male-male competition within other mating systems (Kvarnemo & Ahnesjo, 1996; Jirotkul, 1999; Huffard, 2005). Therefore, it is suggested that the limitation of available females in the male-biased OSR within the present study led to

heightened male activity, resulting from increased male motivation to copulate. Consequently, male-male interactions were more common than by chance in the male-biased OSR trial, and this resulted in a greater number of male-male mount attempts per individual than expected.

Body mass was observed to be the strongest factor influencing the difference in copulatory rates among animals in trials. Larger males invested a greater effort than smaller males towards initiating copulation with females by making more copulation attempts, and larger females were more likely than small females to be receptive to these attempts. There was no evidence for precopulatory female preference to mate with larger males. Female receptivity to male copulation attempts was affected by her own size and not by that of the male. This observation is consistent with studies of both decapods and octopods, where females were not observed to discriminate amongst males based on their size (Corner & Moore, 1981; Adamo *et al.*, 2000; Hall & Hanlon, 2002; Huffard *et al.*, 2008a, 2010; c.f. Wada *et al.*, 2005a). However, within most of these mating systems larger males still obtained greater copulatory success with females by being more successful in male-male aggressive interactions and in more successfully guarding females.

There was however support for female intra-copulatory preference of males based on size. Among female-terminated copulations there was a tendency for females to mate longer with larger males. Male size may be an indication of sexual maturity for females (Kokko *et al.*, 2003), or it is possible that females might benefit from mating with larger males by having larger offspring with higher fecundity (Kirkpatrick, 1982). Females may be able to bias their offspring paternity towards these males by electing to mate with them for longer, as reported within several insect mating systems (Thornhill & Alcock, 1983). Subsequent studies on paternal size and offspring growth rates and/or gamete counts, as well as assessing paternity patterns amongst genotyped candidate fathers might further elucidate these patterns.

No male preference for females based on size or any other measured physical trait was observed in this study. Males did however adjust their durations of copulation according to both female novelty and recent female mating history. During the males' first copulations in trials they mated for longer and never terminated copulation with a female when she had not yet mated with another male during the trial, thus was less likely to be holding competing sperm. It is not known how many spermatophores were

passed during these observations. However, if it is assumed that longer copulation times enable males to transfer more spermatophores to females, as has been reported for some insects (Sakaluk & Eggert, 1996), then this behaviour was consistent with the risk model outlined by Ball and Parker (2007). These researchers suggested that when sperm supply is limited and female mating status is known, it is advantageous for males to allocate more sperm to virgin females in order to ensure paternity amongst future competing sperm given to that female. Although it is unknown whether any of the females had mated in the wild prior to capture, none of them had copulated for a minimum of two weeks prior to entering trials. Therefore it is noteworthy that males treated these females differently to females that had recently mated during trials. Among subsequent male copulations, a male was only likely to terminate copulation early with a female if he was the last male to have mated with her. Additionally, amongst male-terminated copulations, copulation times significantly increased with the numbers of other males that the female had mated with. These patterns are consistent with predicted behaviours based on models of sperm competition (Parker, 1970; Parker *et al.*, 1997), and with behaviours recorded in both *S. lycidas* (Wada *et al.*, 2010) and an unidentified pygmy octopus (Cigliano, 1995) where males of these species were reported to adjust copulation times, presumably spent performing sperm removal and transferring multiple spermatophores, with females based on whether they were the last male to mate with her.

It is not known whether male *H. maculosa* spent time during copulation removing sperm deposited by previous males, or transferring more sperm of their own to females. However, in a mating system where males have a limiting supply of gametes to use over a limited breeding window, both time and spermatophores are likely to be resources that males allocate strategically (Simmons, 1995; Engqvist & Sauer, 2002; McCartney *et al.*, 2010). The present observations support the possibility that male *H. maculosa* adapt the time spent with a female dynamically based on the likelihood of competing sperm in her oviducts to maximise his chance to still be able to mate with additional females, thereby increasing their overall genetic contribution to the following generation. Future studies are required to examine the mechanisms by which male *H. maculosa* might assess female novelty and mating history. As distance sex recognition was not supported for *H. maculosa* in this study, visual recognition of previous mates also seems unlikely in this species. It is possible that following contact or insertion of the hectocotylus,

chemoreceptors as described by Budelmann (1996), might play a role in recognition of the female and/or competing sperm.

It is necessary to acknowledge that the use of two shorter trials for the equal OSR treatment could have affected some of the results. As male copulatory-termination behaviour in particular was heavily dependent on both his own and the female's recent mating history, it is possible that the shorter trials may have missed important patterns that could have been identified if all trials ran for the full five days. However, there were five and ten copulations observed within the two equal OSR trials, which fit within the range of three and twelve copulations observed within the female-biased and male-biased OSR trials respectively. Consequently, males in all trials would have had opportunities to respond to similar conditions of male and female mating history. Additionally, there were no observations of animals behaving outwardly differently with recognised trial members later in trials than when the trials commenced. Therefore it is assumed that any differences in animal behaviour caused by splitting the equal OSR trial into two smaller trials would have been negligible.

Finally, it is also noteworthy that some male-male mounts lasted as long as they did. Same-sex mount durations in the present study were similar to those reported by Cheng and Caldwell (2000) for *H. lunulata*, where the majority of male-male mounts lasted for 5 – 6 mins, but in one case lasted for 44.5 h. These authors reported that spermatophores were not released during same-sex mounts, and this could not be observed during the present study. However, if male *H. maculosa* are apparently selective with their copulation times, and presumably spermatophore investment with females, it remains a mystery why some males engaged in prolonged same-sex mounts to this extent.

3.6: CONCLUSION

This study supports the growing literature that the mating systems of octopods are both unique and involve complexities that are yet to be divulged with further observations and experimentation. Specifically, these results support findings by Cigliano (1995) and Wada *et al.* (2010) in that some male cephalopods appear to strategically regulate their copulation time based on the mating history of the female, presumably to maximise their reproductive output by balancing both their chance of paternity and their ability to successfully copulate with other females. Sperm

competition appears prevalent among cephalopod mating systems (Cigliano, 1995; Hanlon *et al.*, 1999; Naud *et al.*, 2004; Shaw & Sauer, 2004; Wada *et al.*, 2005b; Wada *et al.*, 2006; Buresch *et al.*, 2009; Wada *et al.*, 2010; Iwata *et al.*, 2011; Sato *et al.*, 2013). Future studies using molecular markers might identify correlations between paternity and behavioural patterns including copulation duration and chronology. This work will provide the necessary next-steps in understanding the role of sperm competition in the evolution and maintenance of cephalopod mating behaviours.

3.7: ACKNKOWLEDGEMENTS

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CHAPTER 4: Mating Behaviour and Postcopulatory Fertilisation Patterns in the Southern Blue-Ringed Octopus, *Hapalochlaena maculosa* (Cephalopoda: Octopodidae)

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4.1: ABSTRACT

Female octopuses are known to store sperm from multiple males they encounter throughout a breeding season, before laying a single egg-clutch containing mixed-paternity. Although octopuses display a broad range of precopulatory behaviours, and both sperm competition and cryptic female choice have been hypothesised to occur, the current understanding of how these processes influence resulting paternity remains very limited. This study aimed to identify behavioural factors associated with paternity patterns, and the capacity of females to bias paternity to specific males post-copulation in the southern blue-ringed octopus, *Hapalochlaena maculosa* (Hoyle, 1883). Genetic markers and controlled, sequential, laboratory-pairings of genotyped individuals were used to examine paternity patterns and compare them to relative signatures of male sperm remaining in female oviducal glands after egg-laying. Multiple paternity was discovered in all twelve laboratory-reared broods. There was no indication that the relative time spent in copulation affected the resulting paternity. Males that waited for females to terminate the copulation had greater paternity when they were the first candidate male, but this was not the case among second candidate males. The relative quantities of candidate male alleles detected in female oviducal glands after egg-laying were consistent with relative paternity of the candidate males in all but three cases. In one of these cases, sibship analysis revealed that the male who obtained less paternity than expected was in fact the female's full-sibling brother. Although this study finds no evidence for female postcopulatory selection of male sperm, anecdotal evidence suggests that female *H. maculosa* might benefit from polyandry if chemical processes can favour brood fertilisation to unrelated males. It is recommended that future studies, investigating paternity bias among genotyped males of varying, but known relatedness to the female might help to validate this pattern.

Additional Keywords: *cryptic female choice – inbreeding avoidance – paternity – polyandry – SNP*

4.2: INTRODUCTION

Promiscuity, or extra-pair copulations, is a common occurrence in animal mating systems (Bateson, 1983). Promiscuity among males (polygyny), who often have abundant and low cost sperm (Kodric-Brown & Brown, 1987), can develop easily as an evolutionarily stable strategy (Maynard Smith, 1982). This is because copulating with

additional females leads to more offspring, thereby directly increasing male reproductive success (Bateson, 1983). Promiscuity among females (polyandry), who typically have more costly gametes (Kodric-Brown & Brown, 1987), can also evolve easily within mating systems where copulating with additional males can provide the female with increased resources or paternal care (Reynolds & Gross, 1990; Jennions & Petrie, 1997). However, polyandry might be less common among species where females do not receive material resources or parental care from the males they mate with (Jennions & Petrie, 2000). This is because females have a finite number of eggs they can lay in a lifetime (Kodric-Brown & Brown, 1987), and therefore the number of offspring they can produce is not typically limited by the numbers of males they copulate with (Bateson, 1983). Additionally, copulating with multiple males can be potentially quite costly to females due to the increased risk of potential harm during copulations (Adamo *et al.*, 2000; Hoving *et al.*, 2010b), decreased foraging time (Huffard *et al.*, 2008a), increased risk of disease transfer (Thrall *et al.*, 2000) and increased energy expenditure (Franklin *et al.*, 2012). Therefore, it is generally presumed that where polyandry exists and male provision of resources or parental care does not, that promiscuous females might benefit from additional copulations indirectly, by maximising the genetic quality rather than quantity of their offspring (Zeh & Zeh, 1996, 1997; Jennions & Petrie, 2000; Simmons, 2005). When this is the case, a higher offspring quality can lead to increased success in the F2 generation (grandchildren), and this can be the selective advantage necessary for polyandry to become an evolutionarily stable strategy within a species (Kirkpatrick, 1982; Maynard Smith, 1982).

It can be difficult to quantify the indirect advantages that females might obtain from polyandrous mating systems (Slatyer *et al.*, 2012). However, the cephalopods (Mollusca: Cephalopoda) are ideal for investigating this subject, because polyandry is widespread among this class (Hanlon & Messenger, 1998). It has been observed in every cephalopod mating system studied to date, despite very limited evidence for male provision of resources or paternal care (Hanlon & Messenger, 1998; but see spermatophore consumption in the southern bottletail squid, *Sepiadarium austrinum*: Wegener *et al.*, 2013; and possible food sharing behaviour in the larger Pacific striped octopus: Caldwell *et al.*, 2015). Additionally, female cephalopods can store sperm from the multiple males they mate with (Mangold, 1987), and the resulting fertilisation of their eggs could potentially be influenced by a suite of complex interactions documented

within their mating systems (Hanlon & Messenger, 1998). Some of these mating behaviours include phenotypic-conditional male mating strategies (Norman *et al.*, 1999; Hanlon *et al.*, 2002; Huffard *et al.*, 2008a), multiple types of positioning during copulation (Jantzen & Havenhand, 2003; Iwata *et al.*, 2005; Huffard & Godfrey-Smith, 2010) and differential placement of sperm packages (Hanlon *et al.*, 1997; Hanlon *et al.*, 2002; Jantzen & Havenhand, 2003; Naud *et al.*, 2005; Buresch *et al.*, 2009). Where genetic markers have been used, they have confirmed these behaviours lead to multiple paternity (Naud *et al.*, 2004; Shaw & Sauer, 2004; Iwata *et al.*, 2005; Morse, 2008; Buresch *et al.*, 2009; Squires *et al.*, 2014; Squires *et al.*, 2015; Naud *et al.*, 2016). However, the role that these behaviours play in defining paternity patterns and the resulting selection within these mating systems remains largely unknown (c.f. Squires *et al.*, 2015). Finally, it has been found that extra copulations can be metabolically demanding for at least one female cephalopod (dumpling squid, *Euprymna tasmanica* in Franklin *et al.*, 2012), which emphasises that female promiscuity must also yield a fitness benefit in order to compensate for this extra cost.

It has previously been suggested that polyandry may have evolved within some cephalopod mating systems as a form of bet-hedging strategy, whereby females can ensure their fitness by mating with multiple males to obtain a more genetically diverse brood (Quinteiro *et al.*, 2011). It has also been pointed out that proteins in the seminal fluid can provide additional nutrition for females who engage in more copulations, and this might provide promiscuous females with a direct fitness benefit (Fedorka & Mousseau, 2002; Squires *et al.*, 2012). While the above two mechanisms can certainly explain selective advantages to polyandrous females, observations of complex and often biased paternity patterns (Shaw & Sauer, 2004; Iwata *et al.*, 2005; Naud *et al.*, 2005; Morse, 2008; Buresch *et al.*, 2009; Naud *et al.*, 2016; but c.f. Squires *et al.*, 2014), as well as widespread observations that female cephalopods often reject male copulation attempts (Corner & Moore, 1981; Adamo *et al.*, 2000; Cheng & Caldwell, 2000; Hall & Hanlon, 2002; Wada *et al.*, 2005a; Huffard *et al.*, 2008a; Morse, 2008; Morse *et al.*, 2015) suggest that the function(s) of polyandry in cephalopod life histories is/are probably more complex and yet to be wholly understood.

Polyandrous behaviour may also be selected for in cephalopod mating systems if postcopulatory fertilisation processes can increase the likelihood of promiscuous females siring viable and/or sexually prolific offspring (Kirkpatrick, 1982; Yasui, 1997;

Tregenza & Wedell, 2002). Theoretically, if females are able to assess male genotypes and have control over which males' sperm they use to fertilise their eggs, they could increase their fitness by mating with multiple males as they encounter them within a breeding cycle and then preferentially use sperm from the highest quality and/or most genetically compatible male(s) post-copulation (Eberhard, 1996; Zeh & Zeh, 1997; Tregenza & Wedell, 2002). This mechanism is referred to as 'cryptic female choice' and can occur if the female is able to use either physical or chemical processes to influence the probability of a male's sperm successfully fertilising her egg(s) (Eberhard, 1996). This could potentially happen in a variety of ways among the Cephalopoda and would depend on the morphology of any particular species. For example, in sepiids (Sepioidae: Sepiidae) and most teuthoids (Cephalopoda: Teuthoidae) that have external spermatophore placement and fertilisation (Mangold, 1987; but c.f. Hoving & Laptikhovsky, 2007), the females could simply use their arms to select sperm left in their storage organs and mantle exteriors from preferred males at the time of fertilisation (Naud *et al.*, 2005; Naud *et al.*, 2016). Previous studies have also suggested that some female cephalopods could influence fertilisation patterns by ejecting sperm from their storage organs (Buresch *et al.*, 2009; Sato *et al.*, 2016), controlling the timing between copulations and egg-laying (Buresch *et al.*, 2009; Squires *et al.*, 2015), and regulating copulation durations with males to control how much sperm is transferred to them in the first place (Morse, 2008). However, the current understanding of the context for these behaviours and how they may affect paternity patterns is still sparse (c.f. Buresch *et al.*, 2009; Squires *et al.*, 2015; Naud *et al.*, 2016; Sato *et al.*, 2016).

Octopuses (Octopoda: Octopodidae) and sepiolids (Sepioidae: Sepiolidae), that have internal fertilisation (Mangold, 1987), could theoretically use muscles to pump sperm selectively from the oviducal glands, where sperm is stored, during fertilisation (Froesch & Marthy, 1975), or possibly time the release of sperm-attractant peptides to preferentially store spermatozoa and/or activate it during fertilisation (De Lisa *et al.*, 2013). Despite cryptic female choice having long been hypothesised to occur in octopuses, evidence to support this mechanism is currently lacking due to it being more difficult to assess biased sperm use within animals that have internal fertilisation.

Several male behaviours could also impact paternity patterns in cephalopod mating systems (Cigliano, 1995; Hanlon *et al.*, 1997; Hall & Hanlon, 2002; Wada *et al.*, 2005b; Wada *et al.*, 2006). Mate guarding behaviours have been observed across many

cephalopod taxa in both the laboratory (Adamo *et al.*, 2000; Wada *et al.*, 2006) and field (Hall & Hanlon, 2002; Hanlon *et al.*, 2002; Jantzen & Havenhand, 2003; Huffard *et al.*, 2008a). Sperm-loading behaviours have also been suggested to help males increase their chances of successful fertilisation with females in the presence of competing males' sperm (Hanlon *et al.*, 1997; Hall & Hanlon, 2002; Jantzen & Havenhand, 2003; Wada *et al.*, 2006; Huffard *et al.*, 2008a). Additionally, male sperm-removal has been observed in several sepiids (Hanlon *et al.*, 1999; Hall & Hanlon, 2002; Wada *et al.*, 2005b; Wada *et al.*, 2006; Wada *et al.*, 2010), and has been proposed to occur in some octopuses based on mating behaviour (Cigliano, 1995) and hectocotylus morphology (Thompson & Voight, 2003). In species where male mate guarding, dynamic sperm-loading and/or sperm removal occur, these processes would most likely suggest a paternal advantage to the most recent male that females copulate with (Parker, 1970). However, empirical evidence confirming this pattern within the Cephalopoda is limited (c.f. Hanlon *et al.*, 2005; Buresch *et al.*, 2009; Squires *et al.*, 2015).

Male shallow-water octopuses use the hectocotylus to transfer spermatophores to the distal oviduct of females (Hanlon & Messenger, 1998). During mating, the spermatophore erupts and exudes sperm, which travels to the oviducal gland (Mann *et al.*, 1970). In 1975, Froesch & Marty reported the spermathecae, within the oviducal glands, to be the site of sperm storage and eventual fertilisation during spawning in shallow-water octopuses. Since this seminal work was published, many studies have supported these results (Grubert & Wadley, 2000; Di Cosmo *et al.*, 2001; Tosti *et al.*, 2001; Rodríguez-Rúa *et al.*, 2005; Di Cristo & Di Cosmo, 2007; Cuccu *et al.*, 2013; López-Peraza *et al.*, 2013; Avila-Poveda *et al.*, 2016). This system enables the mixing of sperm by multiple males before fertilisation, and not surprisingly, multiple paternity has been demonstrated in the broods of wild-caught octopuses (Morse, 2008; Quinteiro *et al.*, 2011). Although some studies have hypothesised or assumed a positive correlation between copulation time and fertilisation success (Cigliano, 1995; Huffard *et al.*, 2008a; Morse, 2008), the link between shallow-water octopus reproductive behaviour, genetic composition of sperm in the sperm storage site, and fertilisation patterns has not been formally investigated.

The southern blue-ringed octopus (*Hapalochlaena maculosa*) provides a practical cephalopod model for continuing investigations of paternity patterns and the potential effects of postcopulatory fertilisation processes. This is because the species is short-

lived and females lay a single egg-clutch with a small number of eggs (Tranter & Augustine, 1973), making it easy to assess reproductive output and paternity patterns within an entire generation. Copulations in this species are some of the most protracted among studied cephalopods (Morse *et al.*, 2015), suggesting that copulation duration and sperm competition might be important factors for successful male fertilisation. Additionally, copulations can be terminated by either the male or female in this species (Morse *et al.*, 2015). Female-terminated copulations tend to be longer with larger males, and males adjust their copulation time, and presumably spermatophore transfer, with females based on the female's novelty and recent mating history (Morse *et al.*, 2015). Finally, females of this species are selective of the available males they choose to copulate with but are often forced into copulations by the males (Morse *et al.*, 2015). This in conjunction with the presence of paired, muscular and innervated oviducal glands (Tranter & Augustine, 1973; Froesch & Marthy, 1975), where the sperm is stored, suggests that *H. maculosa* might be a prime candidate for assessing potential patterns of cryptic female choice.

The above biological traits of *H. maculosa* suggest that the chronology of male partners, copulation duration and possibly cryptic female choice of sperm might be critical factors for the resulting paternity of egg-clutches. This study used genetic markers and controlled laboratory pairings of genotyped individuals to investigate both paternity patterns of egg-clutches and relative signatures of sperm remaining in female oviducal glands after egg-laying. This approach was used to unravel some of the behaviours that might influence sexual selection in this species, and the possible mechanisms by which females may be able to maximise offspring quality by influencing paternity among multiple males. Specifically, this study aimed to answer the following questions: 1) Was there multiple paternity among the genotyped *H. maculosa* egg-clutches?; 2) Did the chronology of male partners influence paternity?; 3) Was paternity among the studied egg-clutches correlated with other observable behaviours (e.g., copulation duration, female receptivity to the male or terminating member of the copulation)?; 4) Were the patterns of paternity among egg strings and/or the sperm signatures left in female oviducal glands indicative of either sperm mixing or stratification within the sperm storage organ?; and 5) Was there evidence for cryptic female choice?

4.3: METHODS

4.3.1: Animal Acquisition and Maintenance

False-shelter traps were used to source twelve female and 24 male wild *H. maculosa* from Cockburn Sound, Western Australia over September and October of 2015 under Western Australia DPaW permit: SF010531 and Fisheries exemption: 26367. False-shelter traps consisted of 20 cm lengths of PVC pipe at a range of 20 – 25 mm diameters with a cement plug in the centre. Cables ties were used to connect these traps along 20 m lengths of rope that were held down with cement blocks, and these trap lines were marked and checked weekly using GPS. It was ensured that all captured female animals had a minimum of 5 g wet weight, as this was the minimum size at which females of this species consistently accept copulation attempts from males (Morse *et al.*, 2015).

Animals were brought to the Fremantle Octopus facilities in O'Conner, WA, Australia where they were housed in individual 1 L plastic containers connected to a closed flow-through system in a 1,000 L sump. Water parameters were continuously maintained at 22^{o} C and 34 – 35 ppt salinity. Each animal was given an appropriately sized shell to use as a shelter, and all animals were fed *ad libitum* with sections of thawed frozen bait prawn. Animals were given a 14 h daylight cycle using ReefOne biOrb™ intelligent LED aquarium lights. As there was no means to ensure that females had not already mated in the wild, all female animals were maintained in the laboratory for a minimum of 48 h prior to being introduced to candidate males. This was done to ensure that the time between laboratory pairings would have been at least as long as the time since the female might have mated with another male in the wild. All use and treatment of animals was approved by the James Cook University Animal Ethics Committee (Approval No. A1850).

4.3.2: Brood Preparation

The 36 animals were split into twelve groups of three. Each group was comprised of one female with two 'candidate' males. Candidate males in each group were selected to be within one gram wet weight of each other, and where possible were the same weight in order to minimise any effect of differences in male size (Table 4.1). After a minimum 48 h of laboratory acclimation, the females were paired with each of their two candidate males in two separate pairings, 48 h apart. Pairings took place in ReefOne

biOrb™ Life 30 L square aquaria during night-time hours, when the animals are naturally active (Morse *et al.*, 2015). All behavioural interactions were recorded using closed-circuit television (CCTV) with infrared to observe animals without the use of lights or observers close-by, which might have disturbed the animals' behaviours.

The female was given 30 min to acclimate to the observation aquarium prior to the introduction of the candidate male during each pairing. Behaviours were scored by remotely monitoring the camera footage in real time without disturbing the pair. Specifically, it was of interest whether the female was receptive to male's copulation attempt, how long the copulation was and which of the sexes terminated the copulation. Definitions of female receptivity and female vs. male terminated copulations are described in further detail in Morse *et al.* (2015). Females were considered receptive to copulation if there was no obvious struggle or attempt to retreat from the male between first contact and the male's successful insertion of the hectocotylus into the female's mantle aperture. It is worth noting that forced copulations are common among laboratory observations of *H. maculosa* (Morse *et al.*, 2015, 2017). All laboratory pairings in this study resulted in copulation, regardless of the female's receptivity. Copulations were considered to be terminated by the female if the termination visibly ended with grappling or the female pushing the male off her mantle with her arms. Copulations were considered to be terminated by the male if he passively withdrew the hectocotylus and dismounted the female. Each pairing was ended after the copulation finished, and the male and female were separated before the male was able to mount the female a second time. No female rejection of copulation attempts or forced copulations led to physical harm in any of the animals during laboratory pairings in this study.

Tissue samples for genotyping were taken from the tip (2 – 5 mm) of one of each male's arms, and then all males were returned to their capture site in Cockburn Sound. A reliable local anaesthetic has not been developed for cephalopods, and the dosage of general anaesthetics can be difficult to assess (Fiorito *et al.*, 2014; Polese *et al.*, 2014), leading to overdose and additional animal suffering and/or death (Polese *et al.*, 2014). Octopuses have high rates of arm injury in the wild and regenerate arm-tips quickly (Wada, 2016), suggesting that tissue sampling in this study was likely to be less stressful to animals, or potentially less hazardous to their welfare without the use of a general aneasthetic. Great care was taken to ensure that tissue sampling of animals in this study was less than the observed arm injuries obtained naturally by *H. maculosa*. No animals

in this study were observed to favour their wound sites, or behave outwardly different after the collection of arm-tip samples.

Females were maintained in the laboratory to brood. Between 45 – 50 days after egg-laying, each of the egg clutches was counted and the embryos were frozen to ensure death, then separated from egg capsules and preserved in ethanol for genotyping. The minimum number of embryos required to detect at least a 10% difference in paternity between contributing fathers, at a 95% confidence level, was calculated for each clutch, using the website: http://epitools.ausvet.com.au/content.php?page=1Proportion. This calculation required an estimated true proportion value (i.e. predicted paternity bias), and we used the most conservative estimate (P = 0.50) for sample size calculations. The required numbers of embryos were randomly selected from each clutch for genotyping, while additionally ensuring that at least half the embryos from every egg string were sampled. This was done in order to increase chances of detecting changes in paternity patterns among egg strings. Finally, 84% of all embryos were selected for genotyping, and this ranged from 61% to 100% of each clutch depending on clutch size. The precision of paternity estimates in each egg clutch (minimum detectable difference between paternal shares) were also calculated using the above website. At the time of embryo collection all females were euthanised via freezing, and both their left and right oviducal glands were preserved in ethanol as well as tissue from one of their arm-tips. *Hapalochlaena maculosa* is semelparous, and after egg-laying, females of this species stop feeding, become senescent and die shortly after their eggs hatch (Tranter & Augustine, 1973).

4.3.3: Analysis of Paternity Patterns

All tissue samples were sent to the genotyping service provider, Diversity Arrays Technology (DArTseq™), for DNA extraction, library preparation and GBS data generation. A total library of 22,370 high-quality unique SNP markers was returned from DArTseq™. This library was then filtered by average SNP repeatability, call rate and mean allele frequency (MAF) to obtain the 1,000 most informative SNPs to be used in paternity analysis. The final library had a minimum SNP repeatability of 0.9, a minimum call rate of 0.8 and a minimum MAF of 0.42.

All maternal, candidate paternal and offspring genotypes based on these 1,000 SNP loci were assessed for parentage inference using the 'full likelihood' method in the

software program COLONY Ver: 2.0.6.1 (Jones & Wang, 2010). This program designated which, if any, of the candidate males sired each of the offspring samples at a 100% confidence level. Embryos not assigned to one of the candidate males, were assigned to a new paternal identity that could have its genome partially reconstructed based on the genotypes of its offspring and their mother. As all animals were sourced from the same site, this program was also used post hoc to calculate the genetic relationship between candidate parents used in brood preparations. A paternity value (pX) was assigned to each of the candidate males by calculating the proportion of offspring he sired out of the total number of embryos genotyped in the brood of the female he mated with in the laboratory. Relative paternity for genotyped males was calculated as the paternity of a candidate male (pX) divided by the total number of embryos sired by either candidate male (pM1 + pM2) in the egg clutch of the female he mated with.

Chi-squared tests of homogeneity were used in each egg clutch that had more than one egg string to assess whether the frequencies of embryos sired by different fathers were non-randomly distributed among strings. The difference in paternity values was tested between first and second candidate males using a Wilcoxon signed-rank test. The difference in relative paternity between first and second candidate males was tested by comparing the frequencies of first and second male offspring among each of the twelve broods using a chi-squared test of homogeneity. To test the effect of copulation time on paternity, the relative paternities of first candidate males were linearly regressed on the relative times that they spent copulating with the females (timeM1 / (timeM1 + timeM2)). The effects of female receptivity and sex that terminates copulation on paternity patterns, as well as any interaction these factors might have with mating sequence, were tested for significance using linear mixed-effects models (LMEMs). These LMEMs used arcsin-transformed paternity proportions as the response variable, and the ID of the mother as a random grouping variable. Separate Exact Wilcoxon rank-sum tests were also used to test the effect of terminating sex on paternity among first candidate males and among second candidate males. Finally, the potentially confounding effects of interval between last copulation and egg-laying, female wet weight, and the wet weight of both first and second candidate males on relative paternity between candidate males were all tested using separate logistic regressions.

4.3.4: Assessment of Sperm Remaining in Oviducal Glands

The 24 oviducal gland segments were mosaic tissue samples, comprised of maternal tissue and potentially more than one male's sperm. To obtain quantitative proportions of each individual's genetic contribution, DArTseq™ provided multiple reads of each locus for these samples. This enabled comparison of different allele frequencies across loci. Reads of both alleles were summed at each locus within every sample. Next, a panel of informative loci were selected for detecting each candidate males' sperm. This was done by using Microsoft Excel™ to filter for loci where a candidate male was homozygous for one allele, and the female and competing candidate male from his group were both homozygous for the alternate allele. Additionally, loci where the female was homozygous for one allele the two candidate males homozygous for the other were also identified for use in estimating the contribution of maternal tissue within samples. These informative loci were then filtered further, by ensuring that they each had sufficient read counts for either allele in both of the oviducal gland samples from the relevant female. Where possible, loci were selected if they had a minimum of 100 reads. However, DArTseq™ returned slightly fewer reads for two of the oviducal gland samples (from females F6 and F12), and there were also were fewer informative loci available between a full-sibling pair of candidate parents (F9 and M18; see results). For these three females, informative loci were selected for allele frequency detection in the oviducal glands if they had a minimum of 50 reads. Previous studies have shown that 50 reads per loci are sufficient for estimating allele frequencies within pooled-genotyped samples (Bélanger *et al.*, 2016).

The number of informative loci used to detect candidate male sperm left in the oviducal glands of the females they mated with ranged from 44 – 132 loci (Av = 88.458). The total read counts of each allele at these loci were then calculated to give an estimation of the proportion of alleles present in left and right oviducal gland samples that corresponded to each of the candidate males and the relevant female. It was ensured that all alleles were accounted for within each of the oviducal gland samples (proportions of reads ≈ 1.00). To simplify analyses, the relative allelic contributions between candidate males were calculated as "pM1read counts/(pM1read counts + pM2read counts)" for left and right oviducal gland samples. Three linear regressions were used to compare these relative frequencies between left and right oviducal gland segments, in addition to the relative allele frequencies detected in both left and right

oviducal glands to the relative paternities of candidate males. Due to the likely presence of sperm in female oviducal glands from non-genotyped males that females might have mated with prior to capture, not all selected loci in this calculation necessarily represent exclusive candidate male alleles. For this reason, as many loci as possible were used across candidate male genomes to estimate the relative presence of candidate male sperm. However, this also limits these data to be qualitative in their ability to detect patterns, rather than to be used to draw statistical conclusions from empirical evidence.

4.4: RESULTS

4.4.1: Copulatory Behaviour

The 24 observed copulations were consistent with behaviours described in both Tranter and Augustine (1973) and Morse *et al.* (2015), in that there was a brief tactile phase of the arms followed by the male mounting the female's mantle and inserting the hectocotylus into the female's mantle aperture. Males usually initiated the tactile phase among these interactions, however females were the first to make contact during seven of these copulations. The first contact between the sexes took place an average of 10.9 mins (± 4.3 SE) after the male entered the aquaria. This was almost always quickly followed by the male making contact with the female using his ligula (average of 2.4 mins after first contact ± 1.2 SE). The male then immediately mounted the female and initiated copulation in all but one case, in which the male (M14) retreated from the female after making ligula contact. This male was later coerced into reinitiating contact with the female using a toothbrush. Females were receptive to male copulation attempts in this study in all but three cases (Table 4.1). In these three interactions, females initially rejected mating attempts, but these males sill managed to copulate with them during these pairings. Copulation times ranged from 41 – 292 mins, and were ended by the male during most pairings in this study (Table 4.1). There was no significant difference in copulation durations between first and second candidate males (Paired t-test: $t_{11} = 1.176$, $P_{\text{two-tailed}} = 0.264$).

Table 4.1: The details of the 24 copulations between females and candidate males in the laboratory are given below.

Female	Female wet weight (g)	Days in Captivity[a]	Candidate male	Male wet weight (g)	Mating sequence[b]	Female receptivity	Terminating sex[c]	Copulation duration (mins)
F1	14	3	M1	4	1	Receptive	Female	165
			M2	4	2	Receptive	Male	278
F2	7	3	M3	4	1	Receptive	Male	115
			M4	3	2	Receptive	Male	292
F3	10	12	M5	4	1	Receptive	Male	86
			M6	4	2	Receptive	Male	41
F4	10	3	M7	5	1	Receptive	Male	72
			M8	5	2	Receptive	Male	95
F5	10	3	M9	4	1	Receptive	Male	88
			M10	5	2	Receptive	Female	77
F6	9	19	M11	5	1	Receptive	Female	65
			M12	5	2	Receptive	Female	78
F7	7	10	M13	6	1	Receptive	Male	111
			M14	6	2	Receptive	Male	85
F8	6	10	M15	5	1	Receptive	Female	92
			M16	5	2	Receptive	Female	75
F9	5	17	M17	4	1	Receptive	Male	44
			M18	4	2	Unreceptive	Male	64
F10	6	25	M19	6	1	Unreceptive	Female	87
			M20	5	2	Receptive	Male	95
F11	6	2	M21	5	1	Receptive	Male	124
			M22	5	2	Unreceptive	Male	104
F12	7	2	M23	4	1	Receptive	Male	68
			M24	4	2	Receptive	Male	90

[a]Days in captivity refer to the time that the female spent in the laboratory before her first laboratory pairing. [b]Mating sequence refers to the order of the candidate male's copulation with the female in the laboratory, and copulations with the second male always took place 48 hours after the female's first laboratory copulation. [c]Terminating sex refers to the member that ended the copulation.

4.4.2: Sibship of Candidate Parents

Sibship analysis (post-hoc) revealed one half-sibling pair and two full-sibling pairs among candidate parents that were used in brood preparations. Of these three related pairs, only the full siblings, F9 and M18, interacted within the study. M18 was the second candidate male to have been paired with F9 in the laboratory, despite his genotype revealing that they were full-siblings (Table 4.2). The female was unreceptive to her sibling in the paired breeding trial (Table 4.1).

Table 4.2: The details are given below for all sibling pairs detected among candidate parents used in brood preparation.

Pair	Sibship[d]	Confidence[d]	Relationship in experimental design
F1, F6	Half-siblings	96 %	NA
F9, M18	Full-siblings	100 %	M18 was the second candidate male to be paired with F9 in the laboratory.
F10, M22	Full-siblings	98.3 %	NA

[d]Sibship and confidence levels were calculated using the full likelihood method in COLONY Ver: 2.0.6.1 (Jones & Wang, 2010).

4.4.3: Paternity and Mating Chronology

The twelve females laid egg clutches an average of 9.4 days (± 1.3 SE) after their copulations with the second candidate males (Table 4.3). Clutch sizes ranged from 13 – 80 eggs and were comprised of one to six egg strings (Table 4.3). Multiple paternity was detected in all twelve egg clutches, and all but one candidate male sired offspring (Table 4.3). Additionally, offspring sired to non-genotyped males were detected in four of the twelve egg clutches (Table 4.3). The proportion of offspring sired to non-candidate males ranged from 0.082 – 0.791 (Table 4.3). No patterns were detected regarding the positioning of different male's progeny along egg strings. However, paternity was non-randomly distributed among eggs strings in two of the larger egg clutches from females F5 (Chi-squared test of homogeneity: $X^2_{15} = 39.331$, $P < 0.001$) and F12 (Chi-squared test of homogeneity: $X^2_6 = 17.188$, $P = 0.009$).

Table 4.3: The size and paternity of each of the twelve laboratory-reared egg clutches are given below.

Maternal ID	Days between last copulation and egg-laying	Brood size (# eggs)	Egg strings in brood (#)	Embryos genotyped (*N*)	Precision[e]	Candidate males (ID)	Candidate male offspring (*N*)	Non-genotyped males detected[f] (ID)	Non-genotyped male offspring (*N*)
F1	15	50	2	45	0.05	M1	37	-	-
						M2	8	-	-
F2	6	51	4	46	0.05	M3	11	NG1	35
						M4	0	-	-
F3	8	55	2	50	0.05	M5	31	NG2	15
						M6	4	-	-
F4	11	44	1	41	0.04	M7	6	-	-
						M8	35	-	-
F5	4	80	6	49	0.09	M9	22	NG3	14
						M10	9	NG4	4
F6	10	64	3	43	0.09	M11	41	-	-
						M12	2	-	-
F7	4	32	2	32	0.01	M13	1	-	-
						M14	31	-	-
F8	10	36	1	36	0.01	M15	33	-	-
						M16	3	-	-
F9	15	48	1	48	0.01	M17	24	-	-
						M18	24	-	-
F10	10	41	1	37	0.06	M19	25	-	-
						M20	12	-	-
F11	16	13	1	13	0.01	M21	9	-	-
						M22	4	-	-
F12	4	62	4	43	0.09	M23	2	NG5	34
						M24	7	-	-

[e]The precision values indicate the minimum detectable differences between proportional paternity estimates within each egg clutch based on 95% confidence intervals. These values were calculated using the website: http://epitools.ausvet.com.au/content.php?page=1Proportion. [f]Four egg clutches contained embryos sired by non-genotyped fathers, who mated with the females in the wild prior to capture. These non-genotyped males are listed in the second to last column, and labelled as 'NG' followed by an identifying number.

Mean brood paternity for first candidate males was 50.77% (± 9.48 SE), and mean brood paternity for second candidate males was 30.74% (± 9.09 SE). Overall paternal shares were not significantly different between first and second candidate males (Wilcoxon signed-rank test: Z = 1.178, N = 12, $P_{\text{one-tailed}}$ = 0.12; Fig 4.1). However, these values were confounded by the presence of embryos sired to non-genotyped males. Disregarding paternity by non-genotyped males, the distributions of offspring sired to first and second candidate males were significantly heterogeneous among the twelve egg clutches (Chi-squared test of homogeneity: X^2_{11} = 157.945, P < 0.001; Table 4.3).

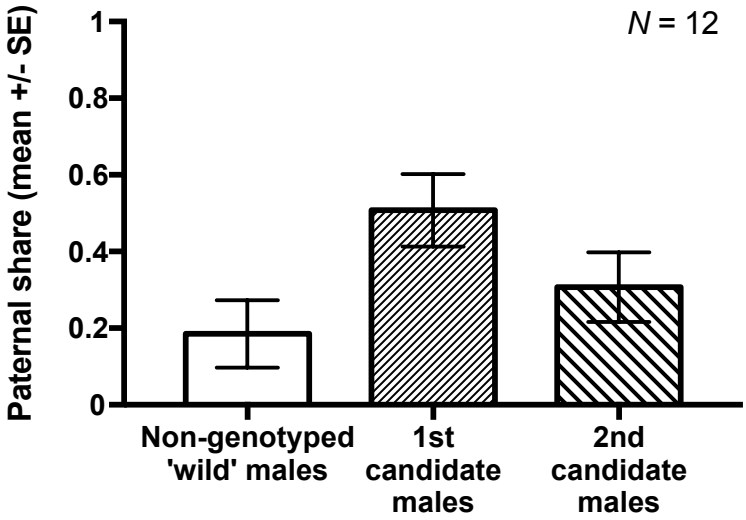

Figure 4.1: There was a general trend for paternity to be biased to the first candidate males to mate with females in the laboratory but this pattern was not significant. Offspring sired to unidentified males were present in four of the broods, yet accounted for ~21% of genotyped embryos. These four females would have mated with the non-genotyped 'wild' males in the field prior to capture.

4.4.4: Paternity and Mating Behaviour

The relative amount of time that candidate males spent copulating with the female did not correlate with relative paternity (ANOVA of linear regression: $F_{1, 10}$ = 0.031, P = 0.862). The interval between females' last copulation and egg-laying did not correlate with the likelihood of paternity being biased to either the first or the second candidate male (Logistic regression: X^2_{10} = 0.292, P = 0.589). Among the four egg clutches where paternity to wild non-genotyped males was detected, there was no indication that the interval between the female's last copulation and egg-laying affected the frequency of offspring sired to males they mated with prior to capture (Logistic

regression: X^2_2 = 0.136, *P* = 0.712). There were no relationships between the relative paternities of candidate males and the wet weight of the female (Logistic regression: X^2_{10} = 0.121, *P* = 0.728), first male (Logistic regression: X^2_{10} = 0.534, *P* = 0.465) or second male (Logistic regression: X^2_{10} = 1.026, *P* = 0.311).

There was a significant interaction between the sequence of candidate males and the sex that terminated the copulation impacting the paternities of candidate males (LMEM: $F_{1,9}$ = 10.466, *P* = 0.01; Fig 4.2). The first candidate males to mate with females in the laboratory had significantly greater shares of paternity among those whose copulations were terminated by the female (Exact Wilcoxon rank-sum test: W = 41, *N* = 4, *P* = 0.008; Fig 4.2A). Among the second candidate males, there was a non-significant trend for males to have lower paternal success if the copulation was terminated by the female (Fig 4.2B). (Exact Wilcoxon rank-sum test: W = 13, *N* = 3, *P* = 0.282; Fig 4.2B). Female receptivity had no effect on the paternities of candidate males (LMEM: $F_{1,9}$ = 0.059, *P* = 0.814).

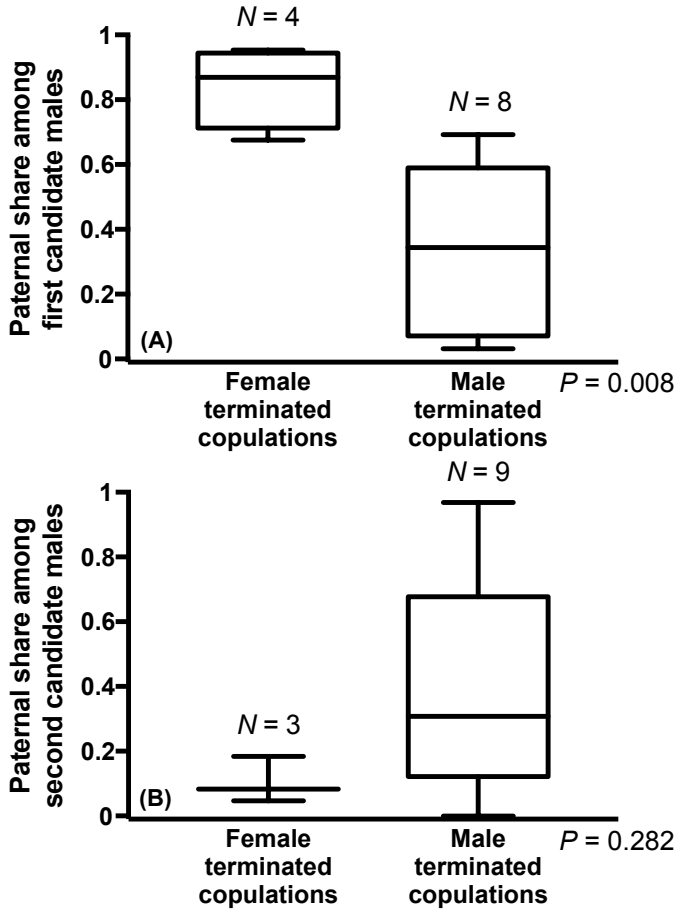

Figure 4.2: First candidate male paternity (A) was significantly greater when copulations were terminated by the female. However, this pattern was not the case among second candidate males (B).

4.4.5: Sperm Remaining in Oviducal Glands

Alleles consistent with all candidate males were detected within the oviducal glands of the females they mated with six to seven weeks after the females laid eggs (Table 4.4). The proportion of reads for candidate male alleles in oviducal gland samples ranged from 0 – 0.223 (Table 4.4). The total proportions of allelic contribution by candidate parents summed to approximately 1.00 in all broods but those of F5 and F12 (Table 4.4). Offspring sired by two non-genotyped males were detected in the brood of F5 and > 79% of offspring in the brood of F12 were sired by a non-genotyped male (Table 4.3). Remaining sperm from these non-genotyped males most likely attributed to the variation in allele frequency counts within the F5 and F12 oviducal gland samples. The slightly larger standard errors found in allele counts within the F6 and F9 samples were due to having fewer informative loci, and therefore read counts, available among these candidate parents (see methods). The relative proportions of detected candidate male alleles were significantly correlated between the left and right oviducal glands (ANOVA of linear regression: $F_{1, 10} = 99.600$, $P < 0.001$; Fig 4.3).

Table 4.4: The proportions of candidate male and maternal alleles found within segments of the left and right oviducal glands of females are listed below.

Female	Contributor to allele counts	Loci used[g] (*N*)	Total reads[h]	Prop. of reads for contributor alleles in left oviducal gland (mean ± SE)	Sum of allele proportions accounted for in left oviducal gland[i] (total ± SE)	Prop. of reads for contributor alleles in right oviducal gland (mean ± SE)	Sum of allele proportions accounted for in right oviducal gland[i] (total ± SE)
	M1	90	13,679	0.202 ± 0.012		0.029 ± 0.012	
F1	M2	132	21,467	0.085 ± 0.008	0.951 ± 0.037	0.025 ± 0.009	0.969 ± 0.042
	maternal	91	13,131	0.664 ± 0.018		0.915 ± 0.020	
	M3	91	14,079	0.068 ± 0.017		0.064 ± 0.018	
F2	M4	77	11,351	0.098 ± 0.021	0.984 ± 0.066	0.100 ± 0.023	1.010 ± 0.068
	maternal	76	11,492	0.818 ± 0.027		0.846 ± 0.026	
	M5	52	8,285	0.001 ± 0.001		0.066 ± 0.006	
F3	M6	51	8,020	0.000 ± 0.000	0.986 ± 0.016	0.017 ± 0.004	0.973 ± 0.025
	maternal	36	5,710	0.984 ± 0.016		0.890 ± 0.015	
	M7	102	16,937	0.059 ± 0.004		0.003 ± 0.001	
F4	M8	101	16,961	0.116 ± 0.006	0.957 ± 0.019	0.007 ± 0.001	0.999 ± 0.004
	maternal	73	11,694	0.781 ± 0.010		0.989 ± 0.002	
	M9	105	17,203	0.044 ± 0.013		0.043 ± 0.014	
F5	M10	91	13,733	0.040 ± 0.015	0.906 ± 0.054	0.037 ± 0.013	0.907 ± 0.053
	maternal	88	12,993	0.822 ± 0.026		0.827 ± 0.026	
	M11	79	6,674	0.087 ± 0.023		0.077 ± 0.020	
F6	M12	75	6,405	0.118 ± 0.028	0.950 ± 0.083	0.121 ± 0.028	0.943 ± 0.082
	maternal	75	5,915	0.745 ± 0.033		0.744 ± 0.033	
	M13	90	14,507	0.053 ± 0.013		0.053 ± 0.013	
F7	M14	114	17,510	0.216 ± 0.018	1.009 ± 0.051	0.223 ± 0.018	1.004 ± 0.052
	maternal	108	17,041	0.740 ± 0.020		0.728 ± 0.020	
	M15	102	17,594	0.070 ± 0.015		0.100 ± 0.014	
F8	M16	115	21,218	0.036 ± 0.011	0.968 ± 0.046	0.035 ± 0.010	0.959 ± 0.043
	maternal	116	19,654	0.862 ± 0.020		0.824 ± 0.019	
	M17	122	18,047	0.022 ± 0.008		0.023 ± 0.009	
F9	M18	55	5,871	0.133 ± 0.030	0.935 ± 0.077	0.142 ± 0.031	0.939 ± 0.081
	maternal	39	3,617	0.780 ± 0.039		0.775 ± 0.041	
	M19	83	14,074	0.031 ± 0.003		0.014 ± 0.002	
F10	M20	94	16,249	0.012 ± 0.002	1.003 ± 0.008	0.003 ± 0.001	1.004 ± 0.005
	maternal	77	11,936	0.960 ± 0.004		0.986 ± 0.002	
	M21	95	15,122	0.041 ± 0.015		0.035 ± 0.014	
F11	M22	112	19,050	0.059 ± 0.016	0.980 ± 0.053	0.062 ± 0.015	0.955 ± 0.053
	maternal	96	16,004	0.880 ± 0.023		0.858 ± 0.025	
	M23	44	3,091	0.166 ± 0.035		0.137 ± 0.034	
F12	M24	51	3,750	0.152 ± 0.032	1.099 ± 0.103	0.150 ± 0.034	1.070 ± 0.106
	maternal	56	3,870	0.781 ± 0.036		0.784 ± 0.038	

[g]Loci were selected to calculate proportions if the candidate male was a homozygote for one allele, while the female and other candidate male were both homozygotes for the alternative allele at the same locus. [h]The total read counts used in calculating allelic contributions are given in the fourth column. [i]The total allelic proportions accounted for by the candidate father and maternal genotypes ≈ 1.00 in all broods except for those of F5 and F12.

Overall, the relative paternity patterns of candidate males were consistent with the relative proportions of their alleles left behind in females' oviducal glands (Fig 4.4). However, this relationship was not significant for either left (ANOVA of linear regression: $F_{1, 10} = 3.975$, $P = 0.074$; Fig 4.4A) or right (ANOVA of linear regression: $F_{1, 10} = 3.663$, $P = 0.085$; Fig 4.4B) oviducal gland samples. Three out of the twelve broods were strong outliers in the above pattern. In the clutch of female F2, no offspring were detected by candidate male M4. However, this male's alleles were still detected in F2's oviducal glands and they were in higher frequencies than alleles from the competing candidate male M3 (Table 4.4). A similar pattern was observed in the clutch of female F6. Candidate male M12 only sired two of the genotyped embryos, but his alleles were in

higher frequencies within the oviducal glands of F6 than his competitor M11 (Table 4.4). Within the clutch of female F9, paternity was exactly equal between both candidate males. However, there was more than five times the proportion of reads for M18 alleles, who was incidentally F9's full-sibling brother, than there was for M17 alleles left in the female's oviducal glands (Table 4.4).

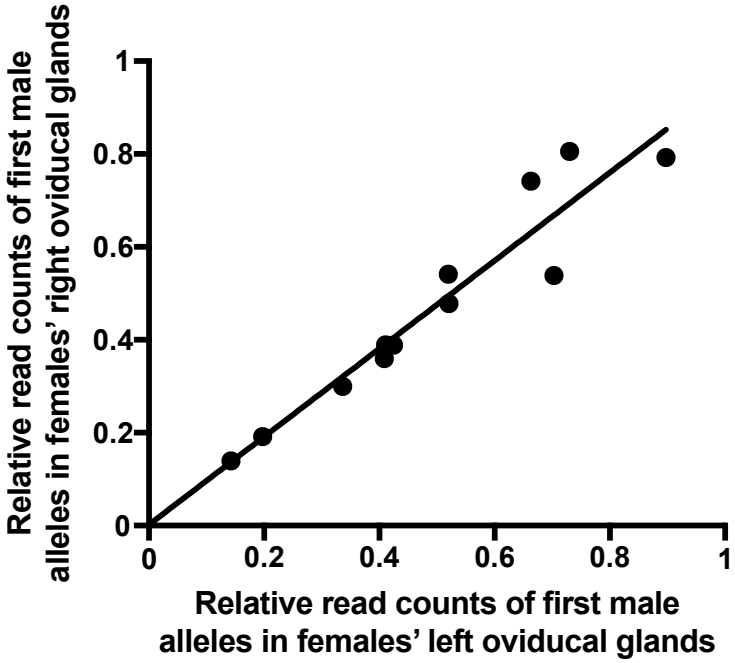

Figure 4.3: The relative proportions of detected alleles between candidate fathers were significantly correlated between the left and right oviducal glands of females (N = 12). Relative read counts were calculated as the proportion of reads in oviducal gland segments that corresponded to alleles exclusive to the first candidate male divided by the total proportion of reads for alleles exclusive to either candidate male. The solid line represents the linear regression: y = 0.948x + 0.002 (P < 0.001).

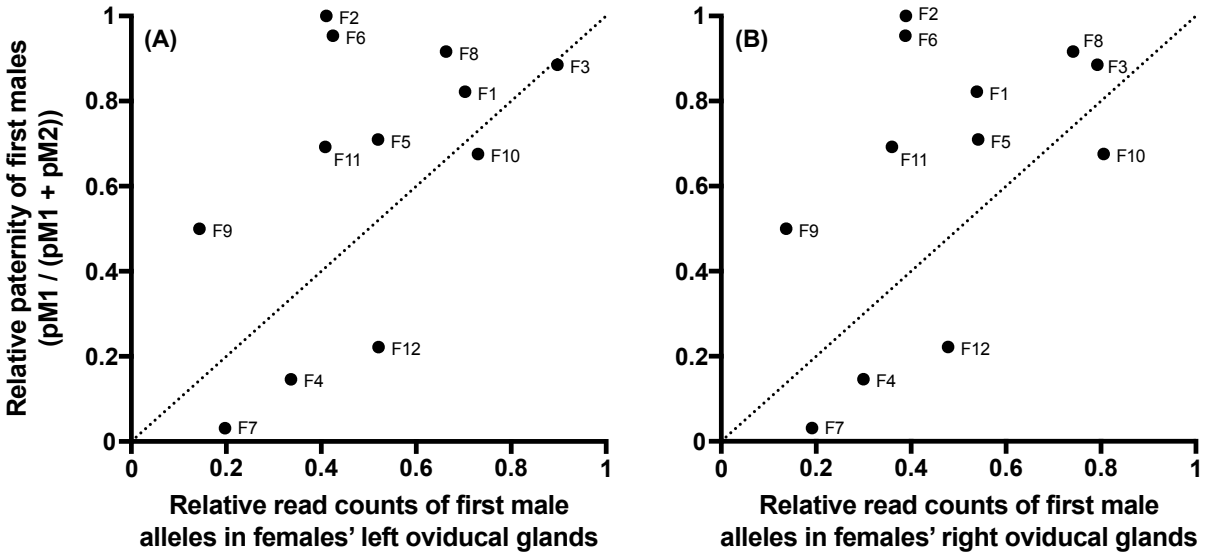

Figure 4.4: There was a non-significant trend for the relative paternity of candidate males to correspond with the relative frequency of read counts for his alleles detected in the left (A; *N* = 12) and right (B; *N* = 12) oviducal glands of the female after egg-laying. Relative read counts were calculated as the proportion of reads in oviducal gland segments that corresponded to alleles exclusive to the first candidate male divided by the total proportion of reads for alleles exclusive to either candidate male. Female IDs are listed next to each data point. The dotted lines represent 1:1 relationships for reference only.

4.5: DISCUSSION

Consistent with all examinations to date of paternity patterns among the Octopodidae family (Morse, 2008; Voight & Feldheim, 2009; Quinteiro *et al.*, 2011), multiple paternity was detected in all twelve genotyped egg-clutches in this study (Table 4.3). Results here indicate that mating chronology appears to have an effect on resulting paternity of embryos. There was a consistent trend for paternity to be biased to the first of the two most recent males that the females in these experiments had mated with, however this relationship was not significant (Fig 4.1). Additionally, in the four broods where non-genotyped males were detected, at least 30% of embryos were sired to these males and in two of these cases non-genotyped males accounted for over 75% of the genotyped offspring (Table 4.3). The females would have mated with these males in the wild at least two days before copulations with the candidate males in the laboratory, and in the case of F3, at least 12 days prior to laboratory pairings (Table 4.1). These results suggest there might be a fertilisation advantage for males that copulate with females earlier in their breeding season. This finding helps to add context to previous findings in Morse *et al.* (2015), who found that males spent relatively more time copulating with females who had no recent mating history and that males frequently attempted to mate with smaller, presumably younger females despite their lack of receptivity. Additionally,

this theory is consistent with observations that some octopuses exhibit higher rates of male-male aggression over small, unpaired females (Huffard *et al.*, 2010) and/or females that are earlier in their maturation cycles (Mohanty *et al.*, 2014). There was no statistical indication that length of sperm storage affected chance of fertilisation, but sample sizes were quite low and lag durations between mating and fertilisation were unknown for copulations prior to capture. Females laid eggs relatively shortly after their final laboratory copulations among the four who sired offspring to non-genotyped males (Table 4.3). It is possible that sperm viability might reduce over time, as has been suggested to occur in *E. tasmanica* (Squires *et al.*, 2015) and the squid, *Doryteuthis pealei* (Buresch *et al.*, 2009). If this is the case, then it is possible that paternity bias to prior non-genotyped males in this study might have been further reduced by the interval between female capture and laboratory pairings.

There was no apparent effect of relative time that the candidate males spent copulating with females on the resulting paternity of their broods. This finding is surprising given that copulation times in *H. maculosa* are highly protracted relative to other cephalopods (Morse *et al.*, 2015), and that previous research has shown females to copulate for longer with larger males and males to regulate copulation time with females based on female novelty and her recent mating history (Morse *et al.*, 2015). Evidence presented here does not indicate that longer copulation times are necessarily associated with increased sperm transfer as previously suggested (Morse *et al.*, 2015). It is possible rather that extended copulation durations might be a form of mate guarding, as it has been suggested for *A. aculeatus* (Huffard *et al.*, 2010). Theoretically, males might spend longer with certain females to defend them from other males and reduce the risk of sperm competition (Parker *et al.*, 1997). This would be consistent with observations that males spend longer copulating with novel females, with whom they would want to ensure paternity (Ball & Parker, 2007; Mohanty *et al.*, 2014; Morse *et al.*, 2015), and also with females that are holding other males' sperm, which might increase the male's perceived risk of competing males in the area (Parker, 1970, 1990; Parker *et al.*, 1997; Morse *et al.*, 2015).

Interestingly, the paternity patterns observed in this study were highly impacted by the sex that terminated copulation (Fig 4.2). Female-terminated copulations, which are typically longer (Morse *et al.*, 2015), resulted in greater paternity among the first candidate males to mate with females. However, it is difficult to reconcile why this same

pattern was not observed among the second candidate males. Only four of the twelve females in this study sired offspring to males they mated with in the wild, and in these cases it is not clear how long they might have been holding sperm from these 'wild' copulations. It is possible that some of the first candidate males perceived these females as having a low-risk of sperm competition. They might have therefore transferred fewer spermatophores and/or invested less time potentially removing other males' sperm before terminating the copulations (Cigliano, 1995; Parker *et al.*, 1997). Contrastingly, the second candidate males were paired with females only 48 h after the first candidate males. The second candidate males tended to have greater paternity if they were the terminating partner. In this context, it is possible that the three females who terminated copulation with the second candidate males did not wait for these males to either finish transferring spermatophores or to possibly perform sperm-removal. However, sample sizes were low and this interpretation requires further verification. It is known that females of this species can regulate copulation duration with males based on their size (Morse *et al.*, 2015). Perhaps female perception of male availability might also influence the context of when they might terminate copulation (Kvarnemo & Ahnesjo, 1996). Additionally, a reduced male perception of male competition, as imposed by this experimental design, might also explain the prevalence of male-terminated copulations in this study (Table 4.1). Male *H. maculosa* have previously been observed to always wait for novel females to terminate the copulation when exposed to potentially competing males (Morse *et al.*, 2015).

The non-random paternity of embryos among egg strings in two of the clutches gentotyped here provides some evidence for separation of sperm within the oviducal glands. However, the sperm signatures found in left and right female oviducal glands were very highly correlated with each other in all twelve females after egg-laying (Fig 4.3). Firstly, this suggests that males transfer sperm equally to both oviducts during copulation. Secondly, and in conjunction with the finding that paternity was apparently random within egg strings, this suggests that mixing of sperm from different males does occur within the oviducal glands. This result is in contrast with observed fertilisation patterns in the chokka squid (*Loligo reynaudii*), in which there can be a distinct switch in embryo paternity along individual egg strings (Naud *et al.*, 2016). There might be some very subtle separation of sperm resulting in the non-random paternity among different egg strings in the present study. However, the lack of complete separation of sperm

casts doubt on the ability of female *H. maculosa* to control brood paternity by selectively pumping sperm from a particular male during fertilisation.

Overall, this study lacks evidence to support the hypothesis of cryptic female choice occurring in *H. maculosa.* The relative sperm signatures of candidate males in female oviducal glands after egg-laying were generally consistent with the relative quantity of embryos sired by each of the candidate males (Fig 4.4). The candidate male that had the most alleles detected in maternal oviducal glands also sired the relative majority of embryos in all but three egg-clutches. This finding strongly suggests that paternity patterns are primarily driven by the relative quantity of sperm the female holds from each male. Therefore, this study does not support cryptic female choice as a means for females to influence brood quality in this species.

It is noteworthy however, that for one of the three clutches where paternity was not explained by sperm signatures remaining in the female's oviducal gland, the female was inadvertently paired with her full-sibling brother. In this group, the alleles detected in the female's oviducal gland were strongly biased to her brother's sperm. However, the brother only sired half of the offspring. This pairing of wild-caught animals was unintentional, and only discovered after genotypes were sequenced. This result was retained in the analysis because it supports the possibility that females might still be able to benefit from polyandry if this enables them to minimise inbreeding (Tregenza & Wedell, 2002). As results presented here indicate that sperm mixing occurs in the oviduct, it is suggested that if paternity is biased against highly related males then this process might occur chemically (Eberhard, 1996). The female might take a passive role in selecting which males' sperm to use in fertilisation, and instead accept sperm from several males and allow chemical processes to favour fertilisation to compatible male genotypes (Zeh & Zeh, 1997). Chemoattractant peptides have been discovered in the eggs of the common octopus (*Octopus vulgaris*), which can affect the chemotaxis of sperm (De Lisa *et al.*, 2013). Females of this species can control the release of these peptides within their reproductive tract, and this may be a mechanism warranting further investigation, by which chemical processes could influence cryptic female choice and/or prezygotic isolation. Ensuring the genomic compatibility of offspring might be particularly important for *H. maculosa*, which is a holobenthic octopus (Tranter & Augustine, 1973) with limited dispersal ability and high levels of relatedness present in areas of suitable habitat (Morse *et al.*, in press). Further studies comparing paternity

between pairs of genotyped individuals with known relatedness are necessary to validate this hypothesis.

4.6: CONCLUSION

In conclusion, this study has found multiple paternity within broods of *H. maculosa*, with paternity patterns generally favouring males that have copulated with the female earlier in her reproductive cycle. Surprisingly, the long copulation times observed in this species did not correlate with resulting paternity, suggesting that extended copulations might instead be a form of male mate guarding. Paternity also appeared to be strongly affected by the sex that terminated the copulation. However, the interacting effects of mating chronology, and possibly male and female perceptions of the operational sex ratio, make the relationship between copulation termination and paternity difficult to define with the present data. Indications of sperm mixing, and patterns of sperm remaining in oviducal glands after egg laying do not support the use of cryptic female choice via selective sperm use in this species. Anecdotal evidence supports the hypothesis that female *H. maculosa* might be able to benefit from a polyandrous mating system if chemical processes can limit brood fertilisation to related males. Future studies investigating paternity bias among genotyped males of varying but known relatedness to the female might reveal the selective advantage responsible for widespread polyandry among the Cephalopoda.

4.7: ACKNOWLEDGEMENTS

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CHAPTER 5: Chemical Cues Correlate with Agonistic Behaviour and Female Mate Choice in the Southern Blue-Ringed Octopus, *Hapalochlaena maculosa* (Cephalopoda: Octopodidae)

Citation:

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5.1: ABSTRACT

Chemoreception cues potentially influence intraspecific interactions of cephalopods, including mate choice. However, at present there is limited empirical evidence demonstrating whether cephalopods can use olfaction to identify the sex or identity of conspecifics. This study examined the responses of the southern blue-ringed octopus, *Hapalochlaena maculosa* (Hoyle, 1883), to conspecific odours during controlled laboratory trials. The ventilation rates in aquaria of 25 wild-sourced animals were measured during four treatments: baseline, sea water, sea water containing male conspecific odour and sea water containing female conspecific odour. When used as 'receivers' in trials, female *H. maculosa* significantly increased their ventilation rates in response to male odours, but not to female odours. However, female response decreased significantly with the receiver's size during female odour treatments. The ventilation rates of male *H. maculosa* were statistically similar in all treatments. However, their ventilation rates showed a significant progressive increase over the observation period during male and female odour treatments. Eighteen of these animals (nine females and nine males) were used in focal-animal trials one week after odour-cue experiments. Of these individuals, females were significantly more receptive to copulation attempts, and spent significantly more time per day in copulation, with males whose odours had elicited a weaker ventilation response in prior trials. These results suggest that female *H. maculosa* can use chemosensory cues to discriminate the sex, and possibly identity, of conspecifics and that this information might influence their mate choice. However, the mechanisms underlying these responses and subsequent copulatory access to females by males remain unknown.

5.2: INTRODUCTION

Social recognition among members of the animal kingdom has been proposed as a necessary prerequisite for the avoidance of unnecessarily competitive or aggressive interactions within species (Colgan, 1983; Wilson, 2000). The coleoid cephalopods have been reported to have relatively complex intraspecific interactions compared with other marine invertebrates (Hanlon *et al.*, 1994; 1997; Hanlon & Messenger, 1998; Norman *et al.*, 1999; Hall & Hanlon, 2002; Huffard *et al.*, 2008; 2010; Godfrey-Smith & Lawrence, 2012; Caldwell *et al.*, 2015). Social recognition has so far been observed in several

cephalopod species, with the ability to identify and signal the sex (Hanlon *et al.*, 1994; Hall & Hanlon, 2002; Huffard *et al.*, 2008), mating status (Cigliano, 1995; Norman *et al.*, 1999; Wada *et al.*, 2010) and dominance (Cigliano, 1993; Boal, 1996; Huffard *et al.*, 2010) of conspecifics. These forms of social recognition are thought to influence both mate choice (Cigliano, 1995; Hall & Hanlon, 2002; Huffard *et al.*, 2008; 2010) and competition for resources (Cigliano, 1993; Huffard *et al.*, 2010; Scheel *et al.*, 2016). While individual recognition based on visual cues has currently been demonstrated for only one cephalopod species (Tricarico *et al.*, 2011), many of them are suspected to use visual context to inform behavioural interactions with conspecifics (Boal, 2006). Explicit chromatophore patterning and signals are used by some species of octopus, cuttlefish and squid to identify the sex, mating strategy and agonistic intent of interacting individuals (Corner & Moore, 1981; Hanlon *et al.*, 1994; Hall & Hanlon, 2002; Huffard, 2007; Huffard & Godfrey-Smith, 2010; Scheel *et al.*, 2016). The size of individuals has also been reported to aid in establishing hierarchies and recognizing dominance of conspecifics in several octopods (Boyle, 1980; Mather, 1980; Cigliano, 1993; Huffard *et al.*, 2010). Additionally, the location of individuals (e.g. den or egg clutch) is thought to help some male octopods and cuttlefish recognize recent mates and to facilitate mate-guarding behaviours (Boal, 1996; Huffard *et al.*, 2008).

While the use of visual cues in intraspecific interactions appears widespread among cephalopods, increasing evidence indicates that chemosensory cues might also be important (Boal, 1997; Boal & Golden, 1999; Walderon *et al.*, 2011). Cuttlefish, squid and octopods can sense chemical stimuli both from a distance using olfactory organs close to the eyes and upon contact with objects using chemoreceptor cells located on the lips and suckers (Budelmann, 1996), and cephalopods are known to use chemosense to aid in locating food items (Wells, 1963; Chase & Wells, 1986). Some cephalopods have also been reported to react to odour from conspecifics, although the reasons for these responses are unclear (Boal & Golden, 1999; Buresch *et al.*, 2003; King *et al.*, 2003; Walderon *et al.*, 2011). For example, *Sepia officinalis* increases its ventilation rate when exposed to sea water containing odour from conspecifics, suggesting that it can detect them by chemical stimuli from a distance (Boal & Golden, 1999). However, *S. officinalis* does not display any change in approach behaviour based solely on odours from conspecifics of different sex or mating history, suggesting that this species might not use odour cues in sex discrimination or mate choice (Boal & Marsh, 1998). The use of non-

tactile chemoreception to detect and interpret information about conspecifics has not yet been investigated for squids, although it has been demonstrated that these animals have the capacity to obtain information from chemical stimuli in the water (Lucero *et al.*, 1992). Tactile chemoreception has been demonstrated in *Doryteuthis pealei* and it has been suggested that a pheromone present in its egg capsules triggers males to engage in male–male agonistic behaviour to compete over females (Buresch *et al.*, 2003; King *et al.*, 2003). The use of odour cues in social recognition also appears possible in octopods. Laboratory trials with *Octopus bimaculoides* revealed that it can detect conspecifics based on odour cues and that ventilation rates of individuals were different depending on the sex of conspecifics that were detected (Walderon *et al.*, 2011). Given that sex discrimination based on chemical stimuli is supported for at least this species, it seems possible that odour cues might play a role in locating or discriminating between potential mates within the mating systems of some cephalopods.

There is some indirect evidence for the use of odour cues in cephalopod mating behaviours. Boal (1997) found that female *S. officinalis* were more likely to choose to mate with newly introduced males that had previously mated with another female than with unmated males. Since females in this experiment could not have seen whether a male had already mated, Boal (1997) hypothesised that females might have used chemical cues to discern recent male mating history in order to mate preferentially with sexually mature, healthy males that had already proved capable of copulation. In an earlier study, Boal (1996) showed that recently-mated male *S. officinalis* were more likely to mate-guard recently-mated females than unmated females, regardless of whether they were the male that had mated with the female. This behaviour suggests that male *S. officinalis* might also depend on chemical cues to identify the recent mating history of females and (presuming the female was not switched) use this information to limit the risk of sperm competition. Additionally, laboratory observations of *S. lycidas*, an unidentified pygmy octopus and *Hapalochlaena maculosa* all showed that males adjusted their copulation times with females based on whether they were the last male to have mated with her (Cigliano, 1995; Wada *et al.*, 2010; Morse *et al.*, 2015). It is possible in the first two of these studies that males assessed the recent female mating history of females by visual means (Cigliano, 1995; Wada *et al.*, 2010). However, the experimental design in the third (nocturnal) study ensured that visual assessment of

female mating history was unlikely, suggesting that odour cues could have been responsible for this behaviour in *H. maculosa* (Morse *et al.*, 2015).

To date, the role of odour cues in mate choice behaviours of octopods has not been formally investigated. *Hapalochlaena maculosa* serves as a good model species for research of this nature for a variety of reasons. As noted above, males of this species might use odour cues to recognize the recent mating histories of females (Morse *et al.*, 2015). Also, females might be selective of potential male partners. Female *H. maculosa* can reject the copulation attempt of one male and within hours be receptive to another male (P. Morse, personal observation). However, patterns of female receptivity appear independent of measured male physical traits including wet weight, mantle length (ML), inter-ocular width and ligula length (Morse *et al.*, 2015). Additionally, *H. maculosa* is a nocturnal octopod that lives in subtidal or turbid environments, where light and therefore visual cues are limited (Tranter & Augustine, 1973). If social recognition is important within the mating system of *H. maculosa,* as it is in some other cephalopods (Hanlon *et al.*, 1994; 1997; Hanlon & Messenger, 1998; Norman *et al.*, 1999; Hall & Hanlon, 2002; Huffard *et al.*, 2008; 2010), then this species would most likely have to rely on forms of sensory input that are useful at a distance, like odour cues, to gain information about conspecifics. Finally, *H. maculosa,* like other octopods, has a ventilation action that is easily observable (Walderon *et al.*, 2011). Mantle ventilation in octopods serves to bring oxygen to the gills, as well as to move chemical signals in the water over olfactory cells (Woodhams & Messenger, 1974).

Therefore, this study was designed to determine whether *H. maculosa* can recognize the scent of conspecifics and to assess whether female ventilatory response to male odours correlates with the performance of males during mate choice trials, thus attempting to clarify the potential role of odour cues within the social behaviour of this species. Specifically this study aimed to answer the following three questions. (1) Does *H. maculosa* change ventilation rate in response to odour from conspecifics? (2) Can either male or female *H. maculosa* discriminate the sex of conspecifics based on odour cues, as determined by differences in ventilation rate? (3) Are female responses to individual male odours correlated with copulation patterns?

5.3: METHODS

5.3.1: Animal Acquisition and Maintenance

Ten female and 15 male *Hapalochlaena maculosa* were obtained from the by-catch of commercial fishermen (under the license of the Fremantle Octopus Company) between Mandurah and Cockburn Sound in Western Australia (WA) from November 2013 to June 2014. Female size ranged from 1 to 12 g, and male size from 1 to 7 g (Appendix 5.1). All animals had a ML of at least 20 mm, the minimum size at which both males and females have been observed to copulate during pilot studies (P. Morse, personal observation). All animals were housed in individual 1-l plastic containers connected to a closed flow-through system with a 1,000-l sump at Fremantle Octopus Company facilities in O'Conner, WA. Sea water was obtained from Cockburn Sound and maintained at 22 °C and salinity of 34 – 35 ppt before and during experiments. Male and female containers were separated by an opaque divider and activated carbon was used to neutralize odours in the sea water entering individual containers to limit each animal's exposure to conspecific odours prior to trials. Each animal was given an appropriately-sized shell for use as a den and was fed *ad libitum* with pieces of thawed, frozen prawns and occasional live crabs. No animals were fed in the 24 h leading up to trials to avoid any effect of recent feeding on ventilation rates. Reef One™ biOrb LED aquarium lights were used to simulate daylight for 14 h per day, which corresponded to local daylight hours when trials began. Animals were obtained under WA Department of Parks and Wildlife permit SF00963. The use and treatment of the animals were approved by the James Cook University Ethics Committee (approval no. A1850).

5.3.2: Odour Preparation

All sea water was obtained from Cockburn Sound the day before each set of odour-response trials. Ten L of sea water were placed in each of three clean plastic buckets in the laboratory. One was the source of the seawater control; a male *H. maculosa* was put in the second bucket and a female in the third to prepare the male and female odour treatments, respectively. A clean aerator was placed in each bucket and the three buckets were left in an air-conditioned part of the laboratory, continuously maintained at 22 °C for 18 h prior to use in odour-response trials. As not all experiments were conducted at the same time and different animals were available at different times, a total of 15 males were used individually in the male odour treatments and ten females

in the female odour treatments. No animals were fed within 24 h leading up to being used as odour sources; however, all animals fed immediately after experiments. All buckets and aerators used in odour preparation were cleaned with fresh water using a high-pressure hose and left to air-dry overnight in a clean section of the laboratory between trials.

5.3.3: Odour-Response Trials

All ten female and eight of the male *H. maculosa* were used as 'receivers' (i.e. animals whose ventilation rates were being recorded) in odour-response trials after two days to one week of acclimation in the laboratory. All observations were made in three Reef One biOrb Life 30-l square aquaria with opaque sides and an opaque barrier that blocked any view from the back of the aquaria. Aquaria were filled with 10 L of clean sea water. All animals were left to acclimate in the observation aquaria for a minimum of 30 min before observation. A CCTV camera in front of the aquaria was used to count ventilations without disturbing the animals.

Odour-response trials entailed counting receiver ventilations for 30 s once each minute for 5 to 10 min in each of a set of three treatments. The 'baseline' treatment was the receiver's normal resting rate without any odour stimulus. The 'sea water' treatment followed immediately after the baseline treatment and was the response after 1 L of sea water was gently poured into the corner of the observation tank in a 30 s action. The seawater treatment was followed immediately by either a 'male odour' or 'female odour' treatment, each applied in the same manner as the seawater treatment, but using sea water with male or female odour, poured from a separate, clean plastic watering can. Ventilation rates were scored for 10 min for all treatments with female receivers. However, due to time constraints during data collection, ventilations were only scored for 5 min during baseline and seawater treatments with male receivers.

No receiver had more than one male or female odour treatment per day. Sometimes a receiver had both a male and a female odour treatment in the same day, but with a minimum of 90 min between trials and only if the ventilation rate had returned to its previous baseline rate. If this was the case, male and female treatments were applied in random order and each included initial baseline and seawater treatments. Observation aquaria were cleaned and filled with new sea water for each new receiver, but not otherwise (to avoid excessive disturbance of the animals).

Each of the ten female receivers was used in at least two male odour treatments. The number of male odour treatments varied between female receivers, due to animal availability and to ensure that each of the females had given a response to every male that would later be used in the same focal animal trial, explained below. Seven of these female receivers were also used in one female odour treatment and an eighth female in two. In total, there were 39 observations of female response to male odours and nine observations of female response to female odours (Appendix 5.1). The eight male receivers each had one male odour treatment and one female odour treatment. However, the baseline ventilation rate of one male preceding a male odour treatment was almost twice all other recorded observations, so this trial was omitted from analyses. This gave a total of seven observations of male response to male odour and eight observations of male response to female odour (Appendix 5.1).

5.3.4: Analyses of Odour-Response Trials

An initial analysis of all raw 30 s observations of receiver ventilation rates used a linear mixed-effects model (LMEM) to determine which factors were correlated with ventilation rate during trials. Ventilation rates (as ventilations per 30 s) were square-root transformed to normalize the distribution (Jones *et al.*, 2013). As each animal was exposed to multiple treatments, 'Receiver ID' was set as a random effect. This enabled an animal's ventilation rate to be compared between different treatments of unequal sample sizes, while eliminating the variance caused by measuring the response of different individuals (Jones *et al.*, 2013). The fixed-effects used in this analysis were: 'treatment' (baseline, sea water, male or female), 'receiver sex' (male or female), 'mass' (receiver wet weight: 1 – 12 g) and 'min' (minute of recorded ventilation: 1 – 10). These fixed effects were represented by the S+ model: square-root ventilation~receiver sex+treatment+mass+min. Preliminary results indicated that all animals reacted strongly to the addition of any water to the observation aquaria for the first 1 – 2 min of observation (Fig. 5.1). This reaction was thought to be associated with the physical disturbance of adding water, so ventilation recorded during the first 2 min of all treatments were omitted from further analyses.

Next, a separate LMEM was applied within each treatment type among both female and male receivers to assess the effects of both time (min) and receiver mass on ventilation rates within individual treatments (square-root ventilation~mass+min).

Finally, additional LMEMs were used to compare the change in ventilation rate of animals between each treatment type for both female and male receivers (square-root ventilation~treatment*mass*min). Where interactions between variables were nonsignificant, the analysis was repeated with the interaction terms removed in order to maximize statistical power (square-root ventilation~ treatment+mass+min).

5.3.5: Focal-Animal Observations

One week after odour-response observations, nine of the 15 males that had had their odours given to females and nine of the ten female receivers were used in focal animal trials addressing mate choice behaviour. These animals were split into three separate trials, each containing six animals (Appendix 5.1). As one of the initial objectives had been to assess differences in behaviour with different operational sex ratios (OSR), the numbers of males and females differed among the three trials. The first trial had four females and two males, the second two females and four males, and the third three females and three males.

The focal-animal trials took place in a 1-m^2 observation tank, with a water depth of 50 cm. The bottom of the tank was lined with sandy rubble, and 12 shells of varying shapes and sizes were haphazardly placed in the tank for animals to shelter in. Water was maintained at 34 – 35 ppt and 22 °C. A Reef One™ biOrb LED aquarium light was used to provide 14 h of daylight per 24 h period and animals were fed *ad libitum* with pieces of prawn throughout the trials. The six animals were allowed to interact freely for the duration of the trial and observed using CCTV with infrared-recording capability. The first two trials each ran for 5 d. However, the third trial (with equal OSR) was terminated after 3.28 d as one of the males had died from excessive copulation (Morse *et al.*, 2015).

Behaviours of each animal were scored during video playback, to quantify the time each pair of animals spent in copulation per day (pair copulation time) and female receptivity to the males' mount attempts (female receptivity). A mount attempt was defined as any attempt by a male to climb onto a female's mantle. Any mount that lasted for more than 30 s was considered a copulation (Morse *et al.*, 2015). Pair copulation time was defined as the average time per day that a male–female pair spent in copulation. Females were considered receptive to male mount attempts if there was no rejection, i.e. a grappling phase or obvious attempt to retreat between male contact and

a successful male mount. Copulations were often successful even when females were not receptive, but in this study female receptivity referred to the reaction of a female to a male, and not to whether males actually succeeded in copulating.

5.3.6: Comparison of Female Response to Male Odours with Mating Behaviour

As each female had previously been exposed to the odour of each male that was included with her in the same focal trial, it was possible to compare each female's response to individual male odours with observed mating interactions. First, a response value (RV) was calculated for each female–male pair. This was defined as the average female ventilation rate from minutes 3 – 10 after exposure to a male's odour, minus her average ventilation rate during minutes 1 – 10 of her immediately preceding baseline treatment. Analyses of the difference in magnitude of behaviours before and after exposure to an experimental stimulus are common in the literature on chemical ecology (e.g. Ferrari *et al.*, 2010; Walderon *et al.*, 2011). In this way, RV represented a relative measure of biological response of females to individual male odours. In some cases this calculation yielded an RV less than zero. However, even negative values were considered meaningful for the purpose of this study.

During focal animal trials, not all of the 25 potential female-male pairs copulated with each other. If the male never attempted to mount the female then this pair was omitted from analyses. However, if a male attempted to copulate with a female, and was unsuccessful due to rejection by the female, the pair copulation time was scored as zero. Values of pair copulation time were normalized using a log (x + 1) transformation (Jones *et al.*, 2013) and linearly regressed on female RV to the corresponding male odours and on female wet weight.

To assess any correlation between female receptivity and female response to male odours, a logistic regression was used to compare both female mass and pair RVs to the proportion of male mount attempts to which the female was receptive during focal-animal trials. As the number of mount attempts was not consistent among all pairs, the 'cbind' function in S+ was used to weight the effect of each proportion by its sample size for this analysis (Jones *et al.*, 2013). For one of the pairs, a copulation began inside a shell and so it was unclear whether the female was receptive to this mount attempt. This pair was consequently omitted from this analysis. Additionally, in order to ensure it was

not a confounding variable, male wet weight was compared with female receptivity using a separate logistic regression, and against pair RV using a linear regression.

5.4: RESULTS

5.4.1: Baseline Ventilation Rates

Mean female and male ventilation rates during baseline observations were 12.5 ± 0.28 SE ventilations/30 s (n = 10 females; 48 trials) and 9.6 ± 0.38 ventilations/30 s (n = 8 males; 15 trials), respectively. Female ventilation rates at baseline were significantly faster than those of males (ANOVA of LMEM: $F_{1, 15}$ = 4.697, P = 0.047). Baseline rates were not significantly affected by animal size for either females (ANOVA of LMEM: $F_{1, 8}$ = 2.693, P = 0.139) or males (ANOVA of LMEM: $F_{1, 5}$ = 0.441, P = 0.536).

5.4.2: Female Response to Treatments

All female receivers reduced their ventilation rates immediately after addition of sea water or odour to their aquaria (Fig. 5.1). After omitting the first 2 min of observation from treatments, female ventilation rate was statistically independent of time for baseline (ANOVA of LMEM: $F_{1, 303}$ = 0.013, P = 0.909), seawater (ANOVA of LMEM: $F_{1, 302}$ = 0.175, P = 0.676) and female-odour treatments (ANOVA of LMEM: $F_{1, 63}$ = 2.436, P = 0.124). However, within male-odour treatments, female receivers increased their ventilation rates significantly with time between minutes 3 to 10 of observation (ANOVA of LMEM: $F_{1, 301}$ = 5.653, P = 0.018; Fig. 5.1).

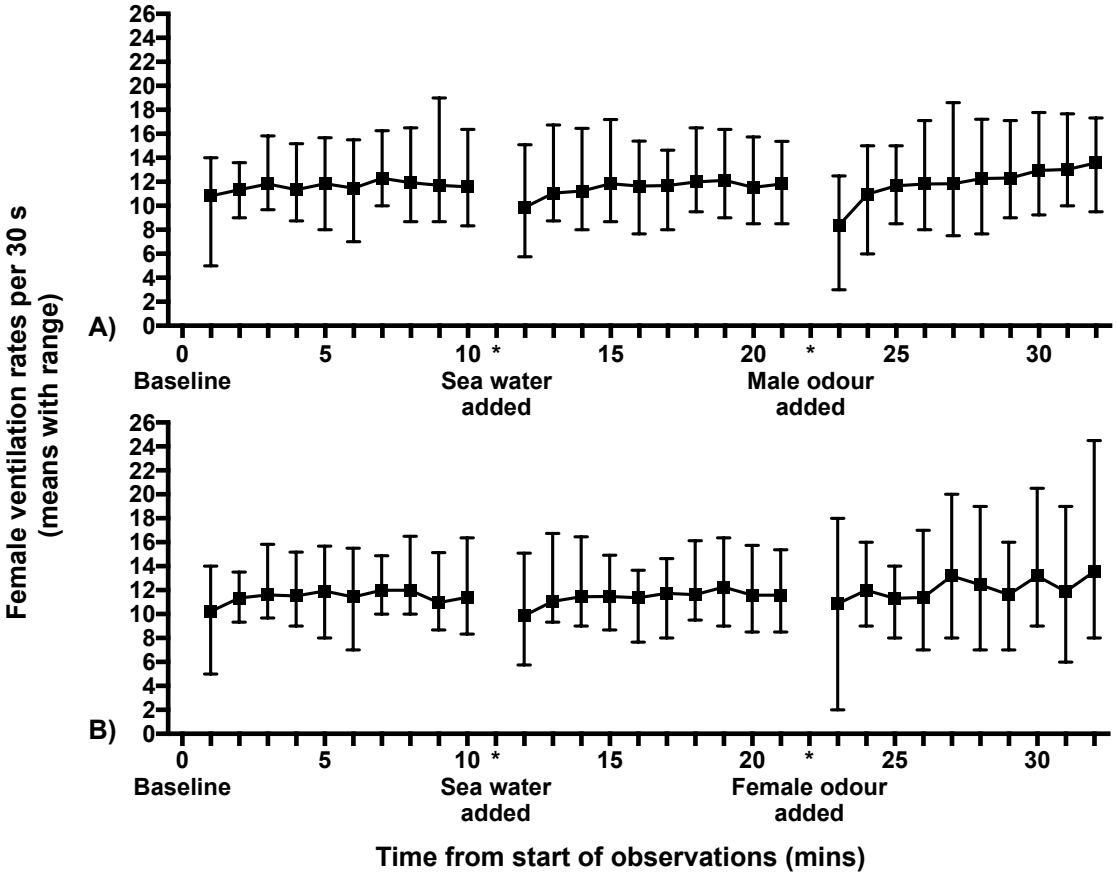

Figure 5.1: Mean ventilation rates per 30 s interval of female *Hapalochlaena maculosa* for each minute of observation. A) Male-odour trials (*N* = 10 animals, 39 trials). B) Female-odour trials (*N* = 8 animals, 9 trials). Asterisks indicate time of additions.

Additionally, female ventilation rates during male odour treatments were significantly faster than female baseline rates (Table 5.1; Fig. 5.2). As there was no statistical difference between female ventilation rates during baseline and seawater treatments (Table 5.1), these could be combined into a 'non-odour' treatment and compared with female ventilation during male-odour treatments for greater statistical power. Female ventilation rates during male-odour treatments were significantly different from the combination of baseline and seawater treatments (ANOVA of LMEM: $F_{1, 926} = 5.682$, $P = 0.017$). Female response to female odour was highly variable and there was no significant difference in female ventilation rates between male and female odour treatments (Table 5.1; Fig. 5.2).

Table 5.1: Results for ANOVAs of linear mixed-effect models comparing ventilation rates of male and female *Hapalochlaena maculosa* between treatments of 'baseline', addition of 'sea water', 'female odour' and 'male odour'. Results for female receivers are given above diagonal, and for male receivers below diagonal. Treatment pairs that yielded differences in receiver ventilation rates at a significance level of 0.05 or less are shown in bold and indicated with an asterisk.

Compared Treatments	Baseline			Sea Water			Female Odour			Male Odour		
	F	df (1, x)	*P*	*F*	df (1, x)	*P*	*F*	df (1, x)	*P*	*F*	df (1, x)	*P*
Baseline	-			0.039	613	0.844	1.452	373	0.229	**4.724**	**613**	**0.030***
Sea Water	2.448	92	0.121	-			1.433	372	0.232	**3.852**	**612**	**0.050***
Female Odour	0.061	95	0.805	3.176	96	0.078	-			0.016	371	0.899
Male Odour	0.082	95	0.775	3.548	96	0.063	0.008	99	0.931	-		

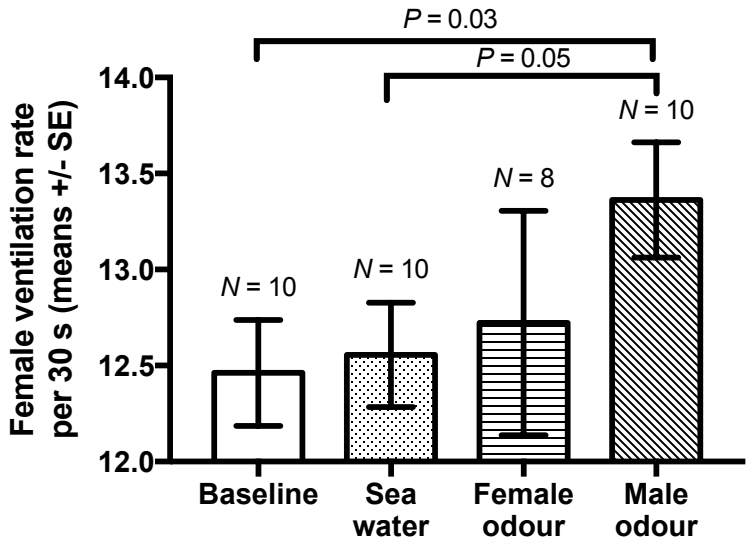

Figure 5.2: Mean ventilation rates of female *Hapalochlaena maculosa* during baseline, and in response to addition of sea water and sea water containing odour from female and male conspecifics. *P* values are indicated for treatment types where receiver ventilations approached significantly different rates. *N*, number of receiver animals used in trials.

Female ventilation rates during female-odour treatments were also statistically similar to female ventilation rates during both baseline and seawater treatments (Table 5.1). However, there was a significant interaction between receiver size and treatment type when comparing female ventilation rates between female-odour treatments and either baseline (ANOVA of LMEM: $F_{1, 373} = 5.933$, $P = 0.015$) or seawater treatments (ANOVA of LMEM: $F_{1, 372} = 4.785$, $P = 0.029$). Female ventilation rates during female-odour treatments decreased significantly with the wet weight of the female receiver (ANOVA of LMEM: $F_{1, 6} = 17.429$, $P = 0.006$; Fig. 5.3). Female ventilation rates were statistically independent of female size for all other treatments (ANOVA of LMEM: $F_{1, 8} = 1.815$, $P = 0.215$).

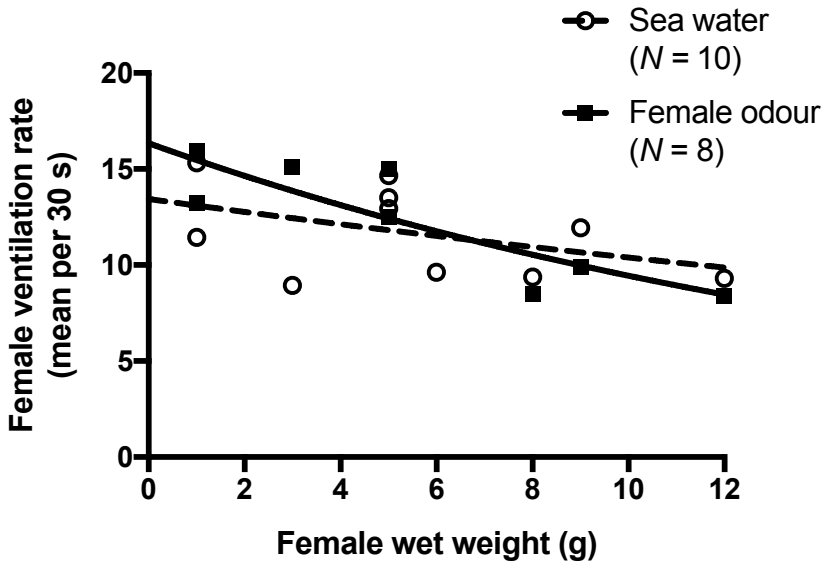

Figure 5.3: Change in ventilation rates of female *Hapalochlaena maculosa* with respect to wet weight, following treatments with seawater controls and female odours. There was a significant interaction between female wet weight and treatment type between these two treatments ($P = 0.029$). Female ventilation rates significantly decreased with receiver size during female odour treatments (solid line shows LMEM fitted to square-root transformed ventilation rates: $y = (-0.093x + 3.696)^2$; $P = 0.006$). However, a female's ventilation rate was statistically independent of her size during seawater treatments (broken line shows LMEM fitted to square-root transformed ventilation rates: $y = (-0.036x + 3.522)^2$; $P = 0.317$).

5.4.3: Male Response to Treatments

Like the females, most male receivers also decreased their ventilation rate for the first 2 min of observation after the disturbance caused by the addition of sea water or odour to their aquaria (Fig. 5.4). After excluding these first 2 min from analyses, male ventilation rates were statistically independent of time in baseline (ANOVA of LMEM: $F_{1, 44} = 2.338$, $P = 0.133$), seawater (ANOVA of LMEM: $F_{1, 45} = 2.823$, $P = 0.1$) and female-

odour treatments (ANOVA of LMEM: $F_{1, 48}$ = 2.794, P = 0.101). However, male receivers increased their ventilation rates significantly with time over observations from minutes 3 to 10 during male-odour treatments (ANOVA of LMEM: $F_{1, 48}$ = 9.524, P = 0.003; Fig. 5.4).

Overall, male ventilation rates were highly variable (Fig. 5.4) and there were no statistically significant differences between any of the treatments (Table 5.1). However, there was an interaction between treatment type and time when comparing male ventilation rates between the seawater treatment and both the male-odour (ANOVA of LMEM: $F_{1, 96}$ = 9.193, P = 0.003) and female-odour treatments (ANOVA of LMEM: $F_{1, 96}$ = 7.297, P = 0.008). The males were observed to increase ventilation rates over the observation periods when exposed to odour from either a male or female conspecific, relative to rates during either a baseline or seawater treatment (Fig. 5.4). Male ventilation rates were statistically independent of the receiver's size for all treatments (ANOVA of LMEM: $F_{1, 5}$ = 0.431, P = 0.541).

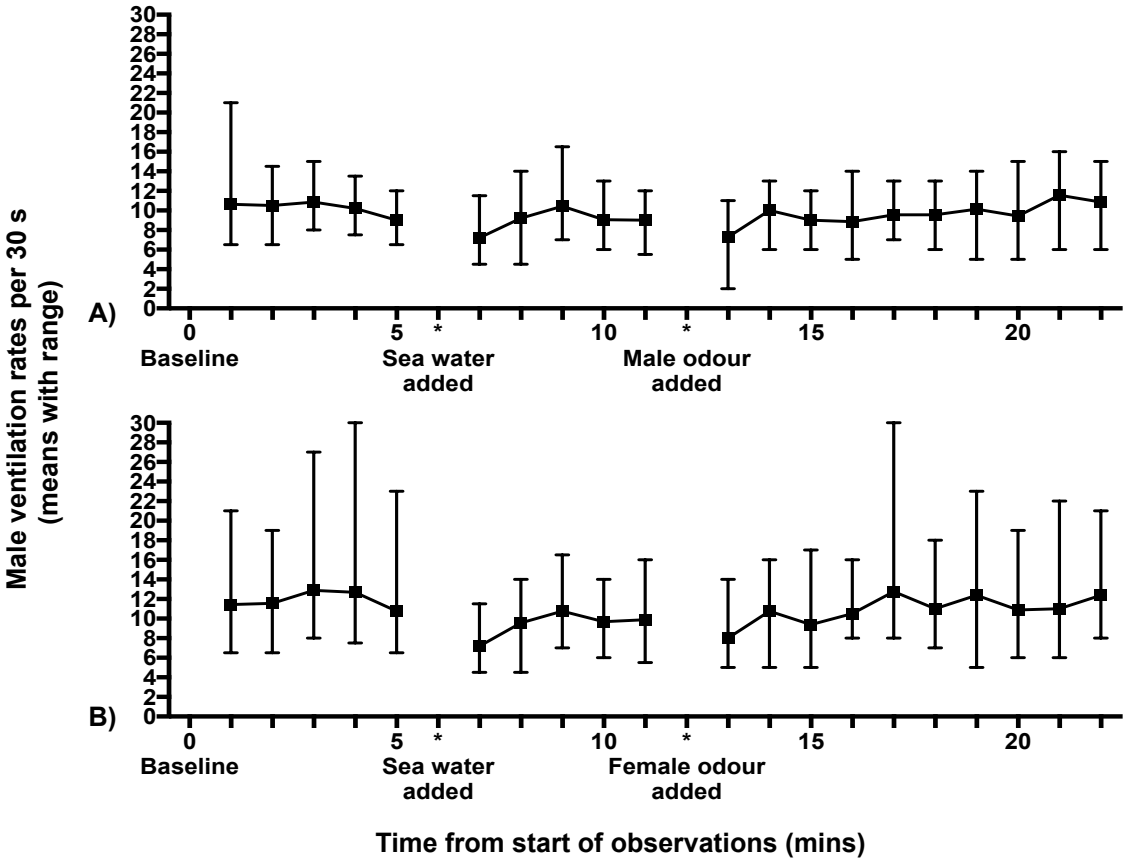

Figure 5.4: Mean ventilation rates per 30 s interval of male *Hapalochlaena maculosa* for each minute of observation. A) Male odour trials (N = 7 animals, 7 trials). B) Female odour trials (N = 8 animals, 8 trials). Asterisks indicate time of additions.

5.4.4: Correlations Between Odour Cues and Female Mate Choice Patterns

Despite the different OSR in the three focal-animal trials, preliminary analyses (not shown) revealed that neither pair copulation time nor male mount success was significantly affected by the trial setup. The results were therefore analysed together. Among the 16 pairs of males and females that had mating interactions during focal-animal trials, there was a significant negative relationship between RV of females to the specific male's odour and the average time per day that the female spent in copulation with that male (ANOVA of linear regression: $F_{1, 13} = 22.754$, $P < 0.001$). However, in this analysis there was also a trend for pair copulation time to increase with the female's wet weight (ANOVA of linear regression: $F_{1, 13} = 2.814$, $P = 0.117$).

Two of the females, which were less than 5 g in wet weight, gave high RV during odour-response trials and later went on to copulate very little during focal animal observations. Excluding these two females from analyses resulted in pair copulation time being slightly less affected by female wet weight (ANOVA of linear regression: $F_{1, 11} = 2.589$, $P = 0.136$). Among the remaining 14 pairs, in which all females weighed at least 5 g, RV was the only measurement that correlated with pair copulation time, supporting the finding that females spent significantly more time per day in copulation with males for which they showed a lower RV during odour trials (ANOVA of linear regression: $F_{1, 11} = 8.028$, $P = 0.016$; Fig. 5).

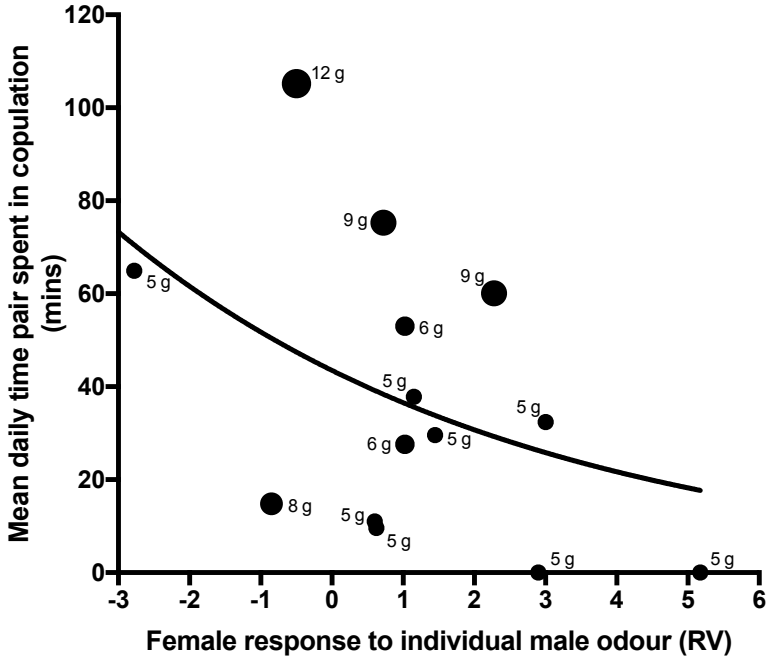

Figure 5.5: Mean time that pairs of males and females (females ≥ 5 g wet weight) spent in copulation given response of the female to odour of corresponding male tested one week previously. RV was calculated as mean female ventilation rate (per 30 s) after exposure to the male's odour minus mean ventilation rate of female during preceding baseline trial. Female size is represented by proportionately sized circles labelled according to wet weight (effect of female size not significant; see text). Solid line shows linear regression fitted to log (x + 1) transformed data: $y = e^{((-0.395x + 1.977)-1)}$; $P = 0.016$ ($N = 14$ pairs).

Among 13 of these same pairs where the female was at least 5 g in wet weight and female receptivity could be observed, there was a significant negative relationship between the proportion of mount attempts by a male that females were receptive to, and the extent to which the same females previously responded to his odour during odour trials (logistic regression: $\chi^2_{11} = 6.384$, $P = 0.012$; Fig. 5.6). In this analysis, female receptivity to male mount attempts was not significantly correlated with the wet weight of the female (logistic regression: $\chi^2_{10} = 1.005$, $P = 0.316$). Male wet weight was also compared with both female receptivity and the RV shown to him by females to test if male size was a confounding variable. Male wet weight was found to be independent of both female receptivity (logistic regression: $\chi^2_{11} = 0.373$, $P = 0.541$) and RV (linear regression: $F_{1, 11} = 0.008$, $P = 0.923$).

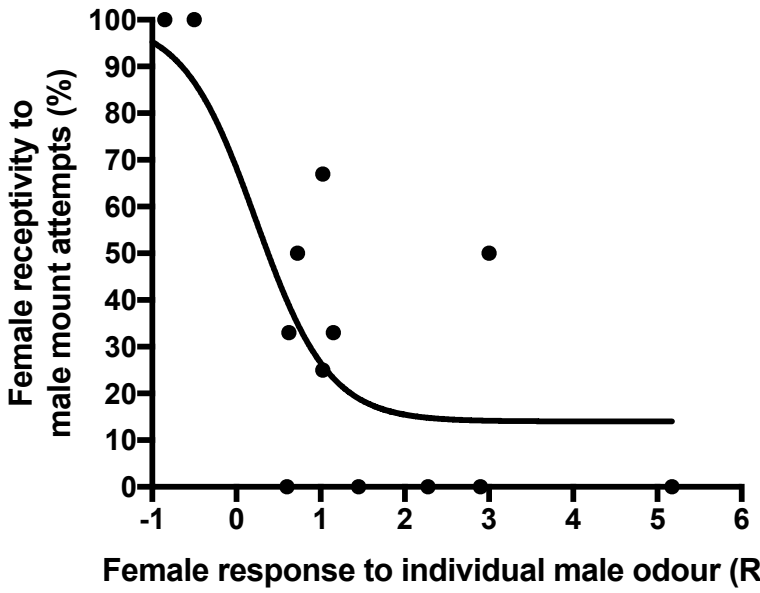

Figure 5.6: Female receptivity to males (measured as proportion of mount attempts by a male to which the female was receptive) as a function of female response to the same male's odour. RV was calculated as mean female ventilation rate (per 30 s) after exposure to male's odour minus mean ventilation rate of female during preceding baseline trial. Females were significantly more likely to try to reject copulation attempts from males to whom they had previously reacted strongly during odour-cue trials. Solid line shows logistic regression: $y = 1 / (1+e^{-(-0.755x - 1.33)})$; P = 0.012 (N = 13 pairs).

5.5: DISCUSSION

Increased ventilation and heart rates in response to social stressors have been documented in a variety of animal taxa (Barreto & Volpato, 2006; von Borell *et al.*, 2007). It is hypothesised that these behavioural mechanisms increase oxygenation of the blood, thus aiding in a 'fight or flight' response (Barreto *et al.*, 2003). In cephalopods, increased ventilation would also aid water movement over olfactory cells, enhancing detection of odour cues in the water (Woodhams & Messenger, 1974). Thus, the *H. maculosa* in this study that increased their ventilation rate after exposure to conspecific odours, might have done so as an alarm response in the same manner that *S. officinalis* increase ventilation rate when presented with odour from a potential predator (Boal & Golden, 1999).

Additionally, the large reduction of ventilation, observed with nearly all receivers in the first 2 min immediately following the addition of sea water or odours (Fig. 5.1 & Fig. 5.4) is consistent with field observations of *Abdopus aculeatus* (Huffard, 2007). This species has been documented to use 'freezing' behaviour to avoid potential predators (Huffard, 2007). It seems likely that many receivers in the present study might have frozen, in a similar manner to *A. aculeatus*, after sudden movement in the water as a

defensive response to reduce visual stimulus and water movement in order to minimize detection by a predator or agonistic conspecific. Therefore, the present observations of both increased ventilation rates and freezing behaviour are consistent with defensive behaviours previously recorded in cephalopods (Boal & Golden, 1999; Huffard, 2007). This being the case, it is likely that changes to ventilation rates observed here were alarm responses, and their interpretation as conspecific recognition should be regarded with this caveat in mind.

Despite the above limitation and the high variability in individual receiver responses, the data presented here show some evidence that female *H. maculosa* are capable of detecting the odours of male conspecifics. Female ventilation rates were significantly faster after exposure to male-conspecific odour than during baseline, and showed a (nonsignificant) trend to be greater than their ventilation rates during seawater trials. Additionally, female receivers showed a progressive increase in their ventilation rate over the 10-min observation period following the introduction of a male's odour, whereas this pattern was not shown in response to other treatments.

The female receivers showed no clear response to odours from other females, as also found in a study of *Octopus bimaculoides* (Walderon *et al.*, 2011). However, female response to female odours did decline significantly with the size of the receiving female. This finding is consistent with the interpretation that higher ventilation rates represent an alarm response, because smaller females might be at greater risk of cannibalism or aggression from conspecific females. Paradoxically, female responses to male and female odours were not statistically different, a pattern consistent with the high levels of unexplained variance within the female-odour trials.

Although ventilation rates of male *H. maculosa* were statistically similar among all treatments, male receivers did show a significant pattern of progressively increasing their ventilation rates over time, after exposure to conspecific odour of either males or females. This suggests that the males might be capable of detecting conspecific odours, but there was no evidence of their ability to discriminate the sex of conspecifics. Regrettably, the sample size for males was low and male ventilation rates were highly variable in all treatments. Therefore the capacity of male *H. maculosa* to detect conspecifics via chemical signals remains unresolved.

It is possible that males of *H. maculosa* do not use odour cues in social recognition. This finding would be consistent with previous observations that males of this species

approach both sexes equally (Morse *et al.*, 2015) and with observations that males of both *H. maculosa* and *H. lunulata* frequently attempt copulations with other males (Cheng & Caldwell, 2000; Morse *et al.*, 2015). However, *H. maculosa* has a limited breeding season (Tranter & Augustine, 1973), is nocturnal and male–male mounts are both time-wasting and can lead to aggressive interactions (Morse *et al.*, 2015). Therefore, it remains a mystery why males do not appear to use odour cues either to locate potential mates or to avoid same-sex mounts, especially given that females in this study seemed to detect conspecific odours.

Female 'masking' of sex-specific chemical cues has previously been documented in an abundant marine snail (Johannesson *et al.*, 2010). This behaviour has been hypothesised to benefit females by enabling them to reduce the predation risks associated with excessive copulations (Johannesson *et al.*, 2010). It is possible that females of the genus *Hapalochlaena* might employ a similar strategy of masking their scent from conspecific males in order to avoid unwanted copulations, but this possibility remains to be investigated. We have no evidence of a mechanism by which copulations might reduce female fitness. Additionally, several observations of octopod mating behaviour report a male tactile phase prior to copulation (Wells & Wells, 1972; Voight, 1991; Morse, 2008). It is possible that males rely on tactile chemoreception for the recognition of conspecifics or their sex. This aspect also remains to be investigated further, but might explain why male–male mounts are typically shorter than male–female mounts in *Hapalochlaena* (Cheng & Caldwell, 2000; Morse *et al.*, 2015).

Interestingly, female response to individual male odours was negatively correlated with both female receptivity and the average time that females spent in copulation with the same males. Females were thus more receptive to copulate, and copulated for longer, with males to whose odour they had previously displayed a lower response. Female rejection of male copulation attempts frequently leads to grappling and often to forced copulations in this species (Morse *et al.*, 2015). Therefore, although sample sizes were small and the evidence limited, these correlations at least suggest that the response to conspecific odours could be linked to defensive or agonistic behaviour, or some other form of stress.

It is also possible that the patterns observed here reflect an ontogenetic shift in the sexual behaviour of females. Smaller females (< 5 g) showed the greatest magnitude of response to male odours, as well as being less receptive to copulation attempts. This

is consistent with previous research on *H. maculosa*, which revealed that females less than 5 g almost always tried to reject male copulation attempts, while males as small as 1 g (but at least 20 mm ML) made frequent attempts to mount conspecifics (Morse *et al.*, 2015). The observed shift in female response to male odours at around 5 g might coincide with the size of most females when they reach sexual maturity. However, it is noteworthy that female response still correlated with mate choice patterns even when females < 5 g were omitted from analyses.

The results reported here are in agreement with the work of Boal (1997), who found that female *Sepia officinalis* consistently spent more time with males that had recently mated with a different female, even though they could not have visually assessed the mating history of the male. If female use of odour cues in *S. officinalis* operates in a similar manner to that in *H. maculosa*, then it is possible that the females were not reacting to the male's recent mating history, but were rather choosing to mate with the males with more attractive chemical cues. This pattern could theoretically evolve through a 'Fisherian' mechanism (Kirkpatrick, 1982); females would benefit from mating with males that emit odours less likely to result in female agonistic behaviour and by also having sons that have similar odours to their fathers. This would increase the reproductive success of the mother (Kirkpatrick, 1982). However, the chemical signal to which females might be responding remains unknown.

Evidence for the detection of conspecifics and the discrimination of their sex via odour cues in *H. maculosa* remains weak. The behaviour of octopods in general can be unpredictable (Mather & Anderson, 1993), so it often a challenge to explain the incredible variance in their observed behaviours. Nevertheless, the data presented here show some significant correlations between odour response and defensive or agonistic behaviour. Although further studies with larger samples and greater statistical power are needed to verify these patterns, our results add to the growing evidence that chemosensory systems play a role in cephalopod cognition and social recognition (e.g. Boal, 1996; 1997; Boal & Golden, 1999; Walderon *et al.*, 2011).

5.6: ACKNOWLEDGEMENTS

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CHAPTER 6: Genome-Wide Comparisons Reveal a Clinal Species Pattern within a Holobenthic Octopod - the Australian Southern Blue-Ringed Octopus, *Hapalochlaena maculosa* (Cephalopoda: Octopodidae)

Manuscript in press:

Morse, P., Kjeldsen, S. R., Meekan, M. G., McCormick, M. I., Finn, J. K., Huffard, C. L. & Zenger, K. R. (in press). Genome-wide comparisons reveal a clinal species pattern within a holobenthic octopod - the Australian southern blue-ringed octopus, *Hapalochlaena maculosa* (Cephalopoda: Octopodidae). *Ecology and Evolution*.

6.1: ABSTRACT

The southern blue-ringed octopus, *Hapalochlaena maculosa* (Hoyle, 1883) lacks a planktonic dispersal phase, yet ranges across Australia's southern coastline. This species' brief and holobenthic life history suggests gene flow might be limited, leaving distant populations prone to strong genetic divergence. This study used 17,523 genome-wide SNP loci to investigate genetic structuring and local adaptation patterns of *H. maculosa* among eight sampling sites along its reported range. Within sites, interrelatedness was very high, consistent with the limited dispersal of this taxon. However, inbreeding coefficients were proportionally lower among sites where sub-structuring was not detected, suggesting *H. maculosa* might possess a mechanism for inbreeding avoidance. Genetic divergence was extremely high among all sites, with the greatest divergence observed between both ends of the distribution, Fremantle, WA and Stanley, TAS. Genetic distances closely followed an isolation by geographic distance pattern. Outlier analyses revealed distinct selection signatures at all sites, with the strongest divergence reported between Fremantle and the other Western Australian sites. Phylogenetic reconstructions using the described sister taxon, *H. fasciata* (Hoyle, 1886), further supported that the genetic divergence between distal sites in this study was equivalent with that of between heterospecifics of this genus. However, it is advocated that taxonomic delineations within this species should be made with caution. These data indicate that *H. maculosa* forms a clinal species pattern across its geographic range, with gene flow present through allele sharing between adjacent populations. Morphological investigations are recommended for a robust resolution of the taxonomic identity and ecotype boundaries of this species.

KEYWORDS: adaptive radiation – cryptic subspecies – ecological genomics – population genetics – SNP

6.2: INTRODUCTION

Dispersal is an important component of animal life histories that influences habitat expansion and the maintenance of population connectivity along the geographic ranges of species (Barton, 1992). Most marine invertebrates and fishes species have a biphasic life history, with a pelagic larval stage that allows them to take advantage of ocean currents for dispersal from natal sites (Gilg & Hilbish, 2003). This phase enables

these organisms to find suitable habitats for settlement and minimises an individual's competition with conspecifics for resources at localised sites (Caley *et al.*, 1996). Furthermore, efficient dispersal mechanisms results in greater genetic connectivity among populations, and this reduces the possibility of inbreeding depression (Charlesworth & Charlesworth, 1987; Gilg & Hilbish, 2003).

Previous molecular studies of the Cephalopoda have revealed that genetic structuring of populations generally mirror life history traits (Shaw *et al.*, 1999; Kassahn *et al.*, 2003; Semmens *et al.*, 2007; Cabranes *et al.*, 2008; Higgins *et al.*, 2013). For example, the squids (Cephalopoda: Teuthida), all of which have planktonic larvae and are nektonic in their adult stage (Boletzky, 1987), are commonly reported to have high levels of gene flow over large spatial scales (Garthwaite *et al.*, 1989; Carvalho *et al.*, 1992; Shaw *et al.*, 1999; Reichow & Smith, 2001). Ecologically relevant differentiation among populations in squid taxa has only been observed over very large distances (ocean basins) or in the presence of a geographic barrier to dispersal (Garthwaite *et al.*, 1989; Carvalho *et al.*, 1992; Shaw *et al.*, 1999). Contrastingly, genetic studies of cuttlefish (Cephalopoda: Sepiidae), which have no planktonic phase (Boletzky, 1987), consistently show genetic structuring at relatively fine scales across species ranges (Pérez-Losada *et al.*, 2002; Kassahn *et al.*, 2003; Zheng *et al.*, 2009). Population structuring in cuttlefish typically follows an 'isolation by distance' (IBD) pattern (Wright, 1943; Pérez-Losada *et al.*, 2002; Kassahn *et al.*, 2003) that reflects the sedentary nature of cuttlefish hatchlings (Boletzky, 1987). Following this pattern, proximal populations within a species might be closely related, but the genetic divergence among populations increases proportionally with the geographic distance between them (Wright, 1943).

Adult incirrate octopuses (Octopoda: Incirrina) are the most sedentary of the cephalopods (Cigliano, 1993; Hanlon & Messenger, 1998). Where studied, their population structure greatly depends on whether the species has a holobenthic or merobenthic life cycle (Cabranes *et al.*, 2008; Juárez *et al.*, 2010; Higgins *et al.*, 2013). For example, a recent study of two sympatric octopuses, one with a planktonic larval phase (merobenthic) and the other without (holobenthic), suggested that this life history trait may drive the type of genetic structuring among populations of these species (Higgins *et al.*, 2013). In the case of the former species, the merobenthic Maori octopus (*Macroctopus maorum* Hutton, 1880), population connectivity was predominantly influenced by ocean currents (Doubleday *et al.*, 2009; Higgins *et al.*,

2013). Contrastingly, genetic structure of the holobenthic pale octopus (*Octopus pallidus* Hoyle, 1885) followed an IBD pattern common to cuttlefish and many terrestrial animals (Wright, 1943; Pérez-Losada *et al.*, 2002; Kassahn *et al.*, 2003; Higgins *et al.*, 2013).

The above studies are useful for advancing hypotheses about the dispersal processes leading to population structure among cephalopod taxa. However, to our knowledge there have been no studies addressing the broad-scale patterns of genomic differentiation or adaptive radiation of a holobenthic cephalopod along its entire species range. Theory would suggest that reduced gene flow would leave populations of holobenthic cephalopods particularly susceptible to genetic divergence due to both increased random drift and differences in selective pressures occurring over varying habitat types (Mayr, 1963; Lenormand, 2002). Such divergence between conspecific populations based on local adaptation over time can lead to the evolution of cryptic subspecies and/or speciation (Doebeli & Dieckmann, 2003; Kirkpatrick & Barton, 2006).

The southern blue-ringed octopus (*Hapalochlaena maculosa*; Fig. 6.1) provides a unique model for addressing biological questions related to mechanisms of population divergence and gene flow. This is due to many unique aspects of this species' distinctive life history. *Hapalochlaena maculosa* is holobenthic, and has a brief seven-month life cycle that terminates in a single breeding season (Tranter & Augustine, 1973). Fecundity in this species is relatively low compared to other cephalopod taxa (Tranter & Augustine, 1973; Boyle, 1987), with females producing up to approximately fifty eggs (Tranter & Augustine, 1973). The mothers invest heavily into their egg-clutch by cleaning and guarding the eggs over a two-month embryonic development phase, until the time of hatching and the mother's eventual senescence and death (Tranter & Augustine, 1973). This extended embryonic phase and maternal care leads to direct development of the offspring (Tranter & Augustine, 1973). Upon hatching, juvenile *H. maculosa* are immediately confined to the benthic environment (Tranter & Augustine, 1973). Juveniles attain sexual maturity after approximately four months of growth, after which they spend most of their time seeking out mates (Tranter & Augustine, 1973).

Throughout its life cycle, *H. maculosa* is capable of swimming only very short distances, via jet propulsion from the siphon (Tranter & Augustine, 1973). Despite its presumably limited dispersal capacity due to the lack of a planktonic phase, *H. maculosa* is widespread along the entire Southern Ocean coastline of the Australian continent (Jereb *et al.*, 2014). Additionally, on the subtropical west coast of Australia an

undescribed sister species has been reported, the western blue-ringed octopus ('WBRO'; Norman, 2000). This potential sister-taxon (referred to hereafter as 'ecotype') appears similar to *H. maculosa* in its external morphology and holobenthic life history, but has been delineated based on its possession of a functional ink-sac (Norman, 2000). However, the geographic boundary between these distinct ecotypes remains unclear due to a lack of genetic and morphological data along this part of the genus range, and both ecotypes will be considered as part of the '*H. maculosa* group' here for simplicity.

It is hypothesised that *H. maculosa* and the WBRO might interbreed at population boundaries, and that limited gene flow between all adjacent populations might lead to a clinal species pattern (see Slatkin, 1973) along the south-western and southern coasts of Australia. This could potentially result in a gradient-like species complex, until the range reaches the described species distribution of the blue-lined octopus (*H. fasciata*) on the subtropical eastern coast, or environments become too warm on the tropical west coast (Jereb *et al.*, 2014). It is also hypothesised that the inferred limited dispersal of these animals, combined with differences in selective pressures along this taxon's range, such as temperature gradients, depth profiles or predation risks could lead to the presence of additionally unique genetic groups and/or possible subspecies within the *H. maculosa* group. Due to their cryptic nature, there is currently very little known about the behavioural ecology or mating system of *Hapalochlaena* spp. that occur along this range (c.f. Morse *et al.*, 2015, 2017). However, these life history characteristics also have the potential to influence the genetic structure and/or reinforce geographic boundaries between potential subspecies within this group (Wright, 1940).

This study used genome-wide Single Nucleotide Polymorphism (SNP) markers to explore the micro-evolutionary processes shaping the genetic structure of the *H. maculosa* group across its range. In particular, the genetic diversity and connectivity were compared among eight sample sites along the *H. maculosa* group distribution, from Fremantle, WA to Stanley, Tasmania. Additionally, genetic signatures of selection were identified at each sampled location in order to estimate the role(s) of local adaptation in driving of the observed genetic divergence between regions. Finally, this study aimed to resolve the phylogenetic relationships among members of the *H. maculosa* group across their geographic distribution, and to provide insight for the taxonomic identity of the species group.

6.3: METHODS

6.3.1: Sample Collection

A total of 248 samples from the *H. maculosa* group were sourced from eight sampling sites across the south-western and southern coastlines of Australia (Fig. 6.2): Fremantle, WA (FRE, n = 91; Sampling area ≈ 61 km^2); Rockingham, WA (ROC, n = 2 Sampling area ≈ 0.1 km^2); Mandurah, WA (MAN, n = 37 Sampling area ≈ 220 km^2); Misery Beach, WA (MIS, n = 3 Sampling area ≈ 0.1 km^2); Emu Point (Albany), WA (ALB, n = 35 Sampling area ≈ 1 km^2); Gulf St. Vincent, SA (SA, n = 22 Sampling area ≈ 0.02 km^2); Port Phillip Bay, VIC (VIC, n = 22 Sampling area ≈ 0.02 km^2); and Stanley, TAS (TAS, n = 36 Sampling area ≈ 22 km^2). Specimens from the Fremantle, Mandurah, Emu Point and Stanley sites were obtained through the by-catch of commercial fishermen. Samples from the Rockingham and Misery Beach sites were obtained through false-shelter traps comprised of both 200 mm lengths of 20 mm diameter PVC pipes, and concrete cavity traps (modified from Schafer, 2001) with cavity sizes of 50 x 30 mm. Samples from the Gulf St. Vincent and Port Phillip Bay sites, as well as two *H. fasciata* samples used as a known sister-taxon for phylogenetic analyses, were obtained during field surveys by J. Finn. Distal 2 mm arm segments were sampled from all animals and placed in 70% ethanol until DNA extraction. Due to small sample sizes in the Rockingham and Misery Beach sites, these were only included in phylogenetic analyses and were omitted from all other genetic evaluations. The use and treatment of the animals were approved by the James Cook University Animal Ethics Committee (Approval Number: A1850). Animals were sourced under Western Australia DPaW permit: SF00963, Western Australia Fisheries exemption: 2393 and the Department of Environment and Primary Industries fisheries research permit: RP699.

6.3.2: DNA Extraction and Genotype by Sequencing

DNA was extracted from all tissue samples using a modified CTAB/Chloroform – Isoamyl method (Adamkewicz & Harasewych, 1996), and further purified using Sephadex™ G-50 spin columns to ensure removal of any small molecule contaminants prior to sequencing (as per Lal *et al.*, 2016; 2017). Quality of DNA and visual indicators of contaminants were resolved using a 0.8% agarose gel. All samples were quantified and standardised to a 50 ng / µL concentration using Biotium ACCUBLUE™ High Sensitivity dsDNA quantification kit. Finally all samples were sent to the genotyping

service provider, Diversity Arrays Technology PL, Canberra ACT, Australia, for full restriction enzyme digestion, library preparation, genotype by sequencing data generation and QA/QC of sequences via DArTseq™ 1.0 technology (Sansaloni *et al.*, 2010; Kilian *et al.*, 2012). DArTseq 1.0 technology generates two independent genetic marker types – Single Nucleotide Polymorphisms (SNPs) and Presence-absence variant (PAV, dominant loci) markers identified from restriction site-associated (RAD) fragments recovered in the sequence data. SNPs were used for both population and phylogenetic analyses, whereas PAVs were only used in phylogenetic reconstructions. Sequence quality control, marker filtering and genotype calling at Diversity for both markers types are described in Lal *et al.* (2016; 2017).

6.3.3: SNP and PAV Quality Control

A total of 33,230 high-quality unique SNPs (single SNP per sequence tag) and 39,033 unique PAV loci were resolved by DArTseq™. SNPs were filtered for call rate (> 70%), and minor allele frequency (MAF; < 5 % in all six sites with n > 20) to ensure high-quality data. Additionally, all SNP loci deviating from Hardy-Weinberg equilibrium (HWE) within sample sites were identified using the software package Arlequin (Excoffier *et al.*, 2005). A total of 474 SNPs significantly deviating from HWE ($p < 0.05$ corrected to an FDR threshold of 0.02) across all six sites with n > 20 were removed the library. SNPs were only removed if they were below MAF or HWE thresholds in all six of the larger sampling sites because wide divergences were expected between the distal populations in this study. Accordingly, rare SNPs were still retained if they were informative in at least one of the sites. Finally, SNPs associated with X or Y-linked chromosomes were screened among the 202 individuals with known sex using the full association test in Plink™ (Purcell *et al.*, 2007), to ensure that only autosomal loci were retained in the dataset. The final SNP library contained 17,523 loci with an average call rate of 0.900 (SE ± 0.001), average read depth of 15.994 (± 0.059) and an average repeatability of 0.986 (± 0.001). PAV markers for phylogenetic analyses were filtered manually to retain the most informative marker set across all individuals and taxa. PAV loci were removed based on a MAF of < 2% among sample sites (n > 20) and technical reproducibility of less than 100%. A total of 22,387 PAV loci were retained for phylogenetic analysis across 250 individuals.

6.3.4: Assessing Genetic Diversity within Sampling Locations

To evaluate genetic diversity within and across sample sites, standard diversity indices including mean observed heterozygosity (H_o), mean non-biased expected heterozygosity (H_e) and Wright's inbreeding coefficients (F_{is}) were calculated through Genetix V4.05.2 (Belkhir *et al.*, 1996). Partial digestion during genotype-by-sequencing has previously been reported to result in null alleles, which can lead to inflated estimations of F_{is} (DaCosta & Sorenson, 2014; Andrews *et al.*, 2016). In order to address this issue, within-site and locus by locus F_{is} estimates were calculated again with 1,000 permutations in Genetix V4.05.2 (Belkhir *et al.*, 1996) using stringently-filtered, site-specific SNP libraries from which all loci were removed that did not robustly conform to HWE within the site being analysed ($p < 0.05$ corrected to an FDR threshold of 0.20). These reduced datasets were more likely to omit informative or possible outlier loci, but minimised the likelihood of containing null alleles that could have affected accurate estimations of F_{is} within individual sites (DaCosta & Sorenson, 2014). All other within-site diversity indices were consistent between the two filtering methods, but F_{is} was reported using both methods for comparison. As inbreeding affects the whole genome, a homogeneity test comparing all locus by locus F_{is} values was further conducted within each site to determine whether any positive observations of F_{is} were resulting from inbreeding behaviour (as per Andrade *et al.*, 2005).

To assess individual genome-wide diversity and inbreeding measures, standardised multi-locus heterozygosity (sMLH) and internal relatedness (IR) were calculated for all individuals using the R package *Rhh* (Alho *et al.*, 2010). The 1 - proportion of shared alleles (A_S) individual distance was calculated for each individual pair using the 'propShared' command in *adegenet* (Jombart, 2008). The percentage of polymorphic loci (PPL), average individual multi-locus heterozygosity (Av. MLH), proportion of rare alleles (A_R; MAF < 0.05) and proportion of private alleles (A_P) were calculated for each of the six sites (with n > 20) using custom scripts in Microsoft Excel™. To assess the effective population sizes (N_{eLD}) and sib-ship structure at sampled locations, a subset of 500 loci were selected from the SNP database to be used in these analyses. These 500 loci were selected for having a minimum MAF of 0.05 within all sites and were then filtered for having the highest call rate, repeatability and read depth among the remaining loci. Filtering SNPs for these analyses helped ensure many of the simplifying assumptions used in the calculations were met (see Waples, 2006; Jones &

Wang, 2010; Waples & Do, 2010; Do *et al.*, 2014). N_{eLD} was calculated with NeEstimator v2.0 (Do *et al.*, 2014) using the linkage disequilibrium option. The proportions of full and half-sibling pairs were calculated using the software program COLONY v2.0.6.1 (Jones & Wang, 2010).

6.3.5: Addressing Broad-Scale Divergence

Genetic differences among the six sites with sample sizes > 20 were evaluated using Weir and Crockerham's unbiased F-statistics (Weir & Cockerham, 1984) using Arlequin (Excoffier *et al.*, 2005). The impact of geographic distance on genetic divergence (Mantel, 1967) was assessed by linearly regressing the pairwise Fst values between each site on their geographic distance using the software: GraphPad Prism™ (v6). Hierarchical analysis of molecular variance (AMOVA) among individuals and sample sites in different groupings were calculated in Arlequin (Excoffier *et al.*, 2005). A Discriminant Analysis of Principal Components (DAPC) using the R package, *adegenet* (Jombart, 2008), was conducted for the genotypes of sampled animals obtained from the six sites where n > 20. An optimal A-Score test was run on this analysis using the same package, and the DAPC was run again using the optimal number of principal components and discriminant functions, and visualised through a DAPC density plot. Individual genomic relationships among all samples were calculated and visualised using the NETVIEW (v0.5.1) pipeline (Steinig *et al.*, 2016) at *k*-NN values between 10 and 60. Nei's standard genetic distances (Nei, 1978) and their significance were calculated among samples from the six larger sites (n > 20) with 1000 permutations using Arlequin (Excoffier *et al.*, 2005). The mean pairwise distances were then used for tree construction based on the Neighbour-Joining (NJ) method in Mega6 (Tamura *et al.*, 2013). The resulting tree was then aesthetically edited in FigTree (v1.4.2) to illustrate the inferred clustering relationships among the six primary sample sites (n > 20) in this study.

6.3.6: Identifying Signatures of Selection

Outlier analyses were used to identify candidate loci under directional selection among the six sites with n > 20, following both a frequency-based approach in Lositan (Antao *et al.*, 2008), and a Bayesian method in BayeScan (Foll, 2012). Both of these programs can run the risk of identifying false positives during outlier discovery (Narum

et al., 2013). To reduce this possibility, and putatively identify loci under directional selection, this study isolated overlapping outlier loci between these two programs (as per Jacobs *et al.*, 2017). Samples from three ecologically and spatially-separated sites within Western Australia (Fremantle, Mandurah and Emu Point) and eastern Australia (Gulf St. Vincent, Port Phillip Bay and Stanley) were compared within each region separately as the eastern and western sites were too divergent to be analysed together (see Villemereuil *et al.*, 2014; Whitlock & Lotterhos, 2015). Directional outlier loci were selected for tree construction within the Western Australia region if both programs jointly identified them as directional outliers at false-discovery rates (FDR) of 0.01. However BayeScan, which is more robust to type I errors but can be more sensitive to high background Fst levels of the two packages (Narum & Hess, 2011; Lal *et al.*, 2016), did not identify outlier loci within the eastern region at low FDR thresholds. Therefore, directional outlier loci were reported for this region and used in subsequent tree construction if they were identified by Lositan at an FDR or 0.01 and in BayeScan up to an FDR of 0.36.

The resulting directional outlier loci for both the western and eastern regions were used in tree construction by calculating the pairwise genetic distances (1 – proportion of shared alleles) using the 'propShared' command in *adegenet* (Jombart, 2008). These pairwise values were then illustrated for both regions using the NJ tree method in Mega6 (Tamura *et al.*, 2013). Due to the relaxed FDR used for identifying directional loci among the eastern sites in BayeScan, this NJ tree was used for explorative purposes only. Any interpretations of selection among the eastern sites derived from this analysis were made with extreme caution. A third NJ tree was also constructed, using the same methodology as above, but using all neutral loci for comparison. Finally, the sequences of all identified directional outlier loci were compared against the NCBI nucleotide database and the *Octopus bimaculoides* genome assembly (Albertin *et al.*, 2015) for biologically relevant matches using Blast2Go™ software.

6.3.7: Phylogenetic Reconstruction and Evolutionary Distances

Phylogenetic relationships among all individuals were reconstructed based on both the SNP and dominant loci (DArTseq PAVs) using maximum likelihood (ML) and Bayesian methods. For both analyses, data from the *H. fasciata* sister taxa were included

as an out-group. The maximum likelihood analysis was conducted using the software RAxML v8.2 (Stamatakis, 2016) incorporating the ASC_GTRGAMMA[X] and ASC_BINCAT[X] site-specific heterogeneity models for SNP and PAV loci respectively (see Leaché *et al.*, 2015). For both ML analyses, the ascertainment bias correction (--asc-corr) was set to 'Lewis' and the rapid bootstrap algorithm with 'autoMRE' (Pattengale *et al.*, 2009) and best ML tree option selected (Stamatakis, 2008). In order to determine if heterozygous site variation biased the phylogenetic reconstruction analysis, the SNP ML analysis was re-run using the repeated random haplotype sampling (RRHS) approach with 5,000 trees according to Lischer *et al.* (2013). Bayesian inference of phylogenetic relationships used only the PAV dataset in MrBayes v3.2.6 package (Ronquist *et al.*, 2012). In order to reach convergence, a subset of the 248 individuals that best reflected the PAV ML tree topology were used for Bayesian analysis. The analysis incorporated two runs of 100,000,000 generations, with each run comprising eight independent chains. A temperature of 0.10 was set for the heated chains, with a sampling frequency of 1,000 and burn-in fraction of 25%. The Dirichlet prior for state frequencies was set at (40, 60), matching the frequencies of "0" and "1" PAV scores present in the dataset. Convergence was also independently assessed using Tracer v1.6 (Rambaut *et al.*, 2014). All resulting phylogenetic consensus trees were visualised and aesthetically edited using the software FigTree v1.4.2 (http://www.molecularevolution.org/software/phylogenetics/figtree). In addition, the levels of phylogenetic distance among all pairs of individuals were calculated using the F84 evolutionary model for SNPs and the modified restriction method for PAVs (DNAdist and Restdist respective programs) in the Phyllip v3.695 analysis package (Felsenstein, 2005).

6.4: RESULTS

6.4.1: Genetic Diversity

Among sample sites, mean observed heterozygosity (H_o) ranged from 0.076 – 0.166, and mean nonbiased expected heterozygosity (H_e) ranged from 0.086 – 0.250 (Table 6.1). All sample sites deviated significantly from Hardy-Weinberg equilibrium ($p < 0.001$). Wrights inbreeding coefficients (F_{is}) ranged from 0.043 – 0.182 after rigorous filtering for null alleles (Table 6.1), but homogeneity tests of these coefficients across all loci revealed that locus by locus F_{is} was significantly heterogeneous within all sites ($p = 0.000$; Appendix 6.1). Standardised multilocus heterozygosity (sMLH) and internal

relatedness (IR) ranged from 0.572 – 1.225, and 0.478 – 0.732 respectively (Table 6.1). Samples from the Stanley site returned the lowest values for H_o, H_e, F_{is} and sMLH, with the correspondingly highest results for IR and A_S (Table 6.1). The Fremantle and Mandurah sites returned the highest values of H_o, H_e, sMLH, PPL and A_R while having the lowest proportions of half-sibling pairs, A_S and IR values (Table 6.1). The Port Phillip Bay and Stanley sites had remarkably high proportions of half-sibling pairs and the two highest scores for A_S, despite having relatively low F_{is} scores (Table 6.1). The effective population sizes based on linkage disequilibrium (N_{eLD}) ranged from 43.0 in the Gulf St. Vincent site to 1,794.1 in the Fremantle site (Table 6.1). However, the Mandurah and Port Phillip Bay sites returned N_{eLD} values of infinity. The N_{eLD} estimates should be regarded with some caution, as the assumption of random sampling may not have been met at all sites (Waples & Do, 2010; Do *et al.*, 2014).

Figure 6.1: An image is shown of the Southern Blue-Ringed Octopus (*Hapalochlaena maculosa*) from Port Phillip Bay, Victoria (Photo taken by Julian Finn, Museums Victoria).

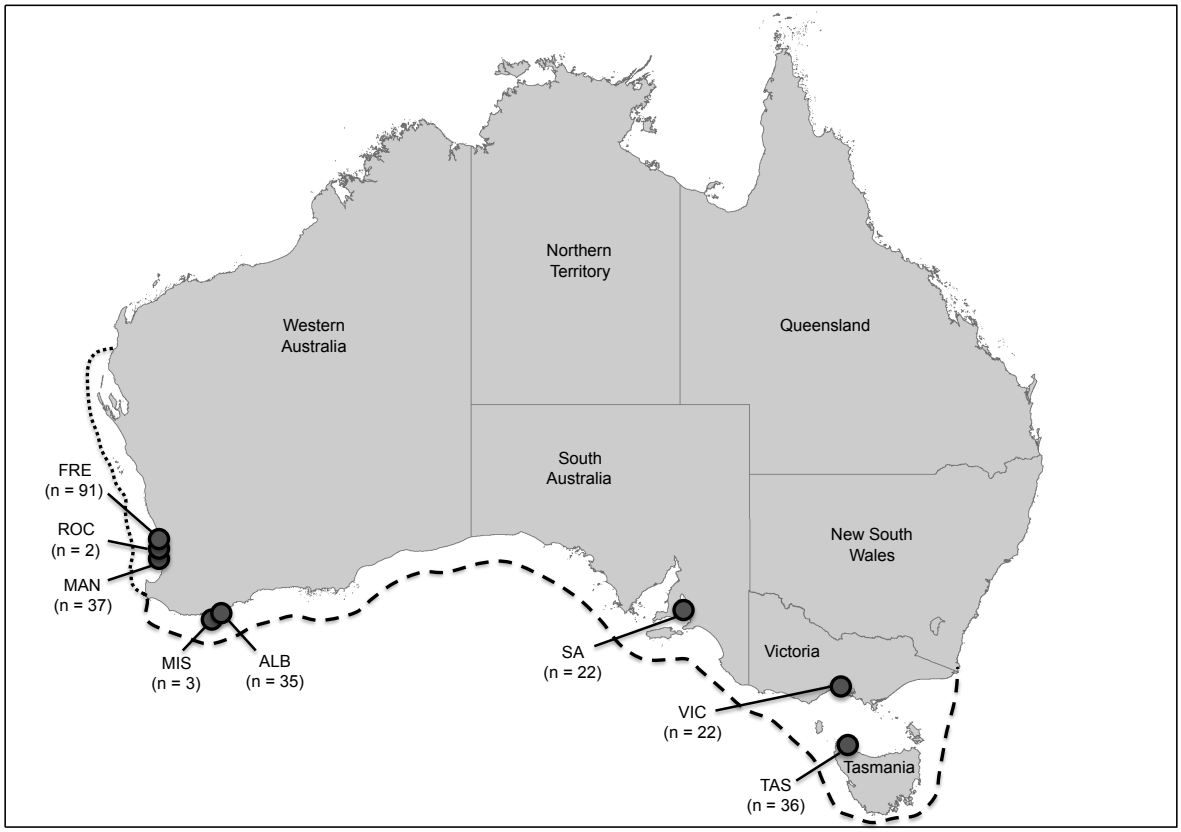

Figure 6.2: Sampling locations for the 248 members of the *H. maculosa* group sourced in this study. Site names and sample sizes are given next to each location. The reported distribution of *H. maculosa* is shown within the dashed line (Jereb *et al.*, 2014). The subtropical region of Western Australia, previously proposed as the distribution for the undescribed WBRO, is represented with the dotted line (Norman, 2000).

Table 6.1: Genetic diversity indices for the six *H. maculosa* sampling sites ($N > 20$) based on 17,523 SNP markers ('site filtered' F_{is} was calculated based on site-specific subsets of loci stringently filtered for HWE; N_{eLD} and Prop. Sibling Pairs were calculated based on a subset of 500 of the most informative loci). PPL stands for the percentage of polymorphic loci within each site. All F_{is} values were estimated from 1,000 permutations at $P < 0.001$. A_S stands for the proportion of shared alleles averaged among individuals for each site. A_R was calculated by the number of alleles having MAF less than or equal 0.05 among polymorphic loci within each site.

Site Name	State	Location	n	N_{eLD} (95% C.I. at $P = 0.05$)	PPL	H_o	H_e	F_{is} (all loci; site filtered)	Av. MLH (± SE)	sMLH (± SE)	IR (± SE)	A_S (SE < 0.001)	A_R (< 0.05 MAF)	A_P	Prop. Sibling Pairs (full siblings; half siblings)
FRE	WA	Fremantle	91	1,794.1 (1,198.7 – 3,519.6)	0.908	0.166	0.250	0.339; 0.108	0.172 (± 0.002)	1.225 (± 0.015)	0.478 (± 0.007)	0.787	0.170	0.053	0; 0.028
MAN	WA	Mandurah	37	Infinite	0.828	0.152	0.228	0.339 0.182	0.158 (± 0.002)	1.116 (± 0.013)	0.472 (± 0.006)	0.803	0.150	0.005	0; 0.024
ALB	WA	Emu Point, Albany	35	283.3 (231.9 – 362.6)	0.610	0.140	0.176	0.210; 0.095	0.146 (± 0.004)	1.037 (± 0.026)	0.490 (±0.012)	0.857	0.113	0.007	0.003; 0.210
SA	SA	Gulf St. Vincent	22	43.0 (40.2 – 46.3)	0.486	0.114	0.153	0.267; 0.175	0.119 (± 0.002)	0.853 (± 0.013)	0.594 (±0.006)	0.876	0.070	0.005	0.009; 0.238
VIC	VIC	Port Phillip Bay	22	Infinite	0.402	0.089	0.112	0.213; 0.153	0.093 (± 0.003)	0.668 (± 0.022)	0.686 (± 0.01)	0.912	0.092	0.001	0; 0.784
TAS	TAS	Stanley	36	468.8 (347.7 – 713.4)	0.363	0.076	0.086	0.120; 0.043	0.080 (± 0.002)	0.572 (± 0.013)	0.732 (± 0.006)	0.935	0.118	0.003	0.033; 0.684

6.4.2: Broad-Scale Divergence

Pairwise Fst values based on Weir & Crockerham's unbiased distances are provided in Table 6.2 for the six sites with sample sizes > 20. Values ranged from 0.159 between the Mandurah and Emu Point sites, which were located ~580 km apart, to 0.507 between the Fremantle and Stanley sites, which were the most geographically separated sites at ~3,530 km apart (Table 6.1). Genetic distances significantly increased with geographic distance (Linear Regression: $F_{1, 13} = 45.97$, $p < 0.001$; Fig. 6.3). The r^2 value of this regression revealed that geographic distance explained 78% of the variation in genetic differences, however comparisons of the three west coast sites were exceptions to this pattern and the Fst value between the Mandurah and Emu Point sites fell well below the regression line (Fig. 6.3).

Table 6.2: Pairwise Fst values are shown for each combination of sampled locations based on Weir & Crockerham's unbiased distances (1984) with 1,000 permutations on the bottom left of the matrix. Nei's Standard Genetic Distances (Nei, 1978) based on 1,000 permutations are given in the top right side of the matrix. All Fst values have a significance of $P < 0.001$, and all Nei's Standard Genetic Distances have a standard error of less than or equal to 0.003.

	FRE	MAN	ALB	SA	VIC	TAS
FRE	*	0.119	0.167	0.253	0.297	0.320
MAN	0.261	*	0.061	0.149	0.193	0.216
ALB	0.341	0.159	*	0.135	0.178	0.202
SA	0.421	0.321	0.339	*	0.056	0.082
VIC	0.469	0.398	0.425	0.227	*	0.041
TAS	0.507	0.459	0.486	0.325	0.230	*

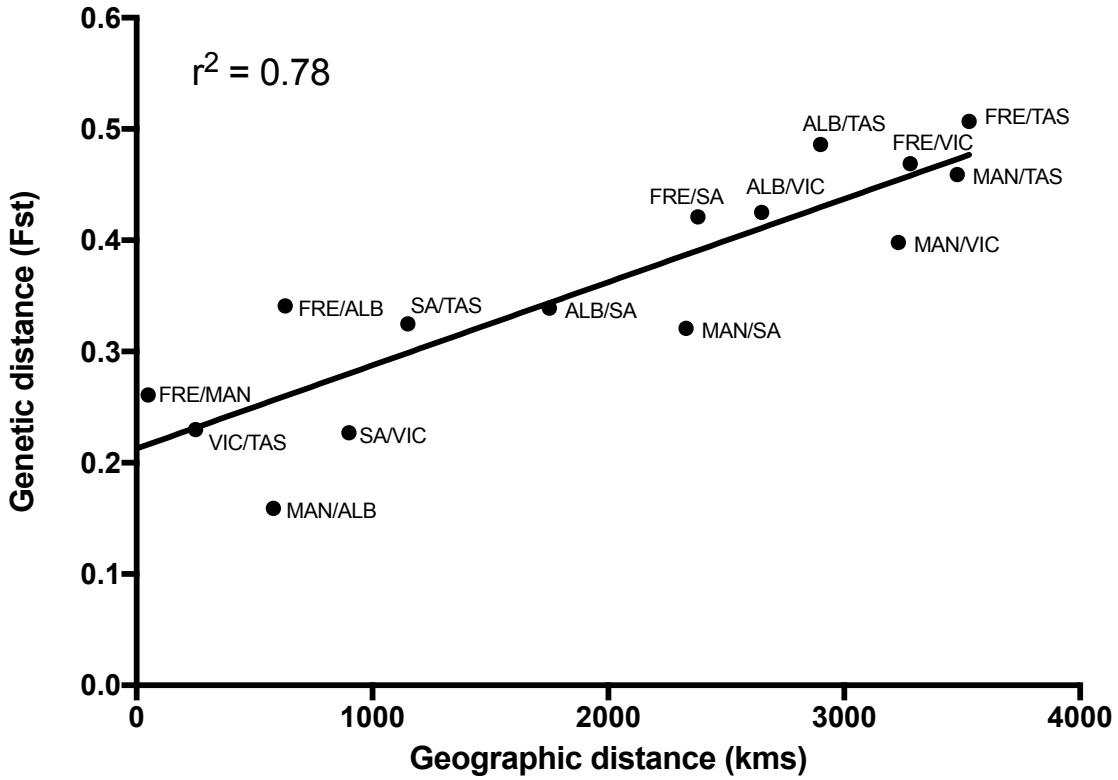

Figure 6.3: There was a significantly positive relationship between Wrights genetic distance (Fst) and geographic distances (kms) between each sampling site. The solid line represents the Linear Regression: y = (7.477e-5)x + 0.213; $P < 0.001$.

Both NETVIEW (*k*-NN = 15) and DAPC analyses revealed that genotyped individuals primarily formed unique clusters based on broad geographic sampling location (Fig. 6.4A & Appendix 6.2). However, two individuals sampled at the Fremantle site fell into the same cluster as other samples from the Mandurah site that was located approximately 50 km away from where they were obtained. Interestingly, NETVIEW analysis (Fig. 6.4A) for Fremantle and Mandurah indicated sub-structuring and higher diversity within these sites, which was also supported by genetic diversity indices (Table 6.1). Based on AMOVA hierarchical analysis, the maximum amount of genetic variance was observed at the individual site level (47.22%; $p < 0.001$). The next highest level of variation was observed when the sampled sites were clustered into the four genetic groups: Fremantle; Mandurah and Emu Point; Gulf St. Vincent; Port Phillip Bay and Stanley. This arrangement accounted for the maximum amount of genetic variation among groupings (35.34%; $p < 0.001$), while 11.66% of variation was among sites within groups ($p < 0.001$) and 53.01% of variation among individuals within sites ($p < 0.001$). This grouping arrangement is inconsistent with both geographic distribution, and the listed distributions of *H. maculosa* and WBRO (Norman, 2000), in that it suggests

the individuals sampled from the Mandurah site are more genetically aligned with individuals from the Emu Point site than they are from the adjacent Fremantle site (Fig. 6.2). The demarcation of these four genetic groups is further supported by the pairwise Fst data (Table 6.2), NETVIEW analysis at *k*-NN = 55 (Fig. 6.4B), and the NJ tree based on Nei's standard genetic distances (Appendix 6.3).

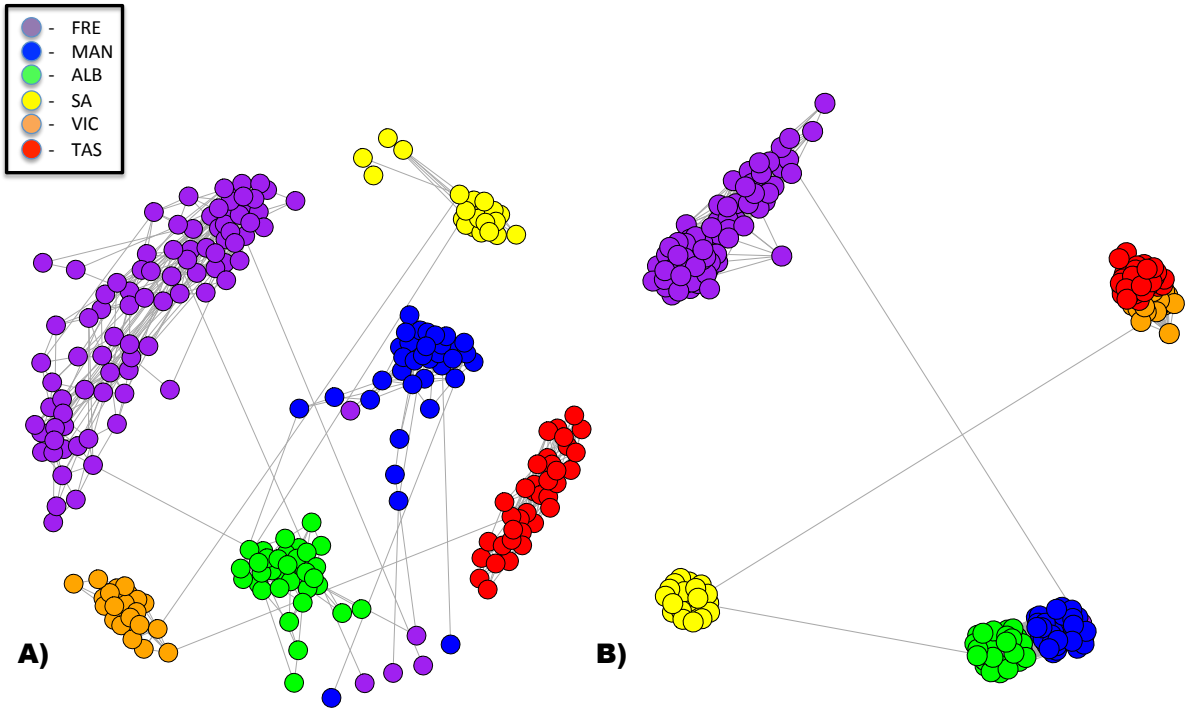

Figure 6.4: The genomic clustering of all sampled individuals using an isolation by state (IBS) constructed using the NETVIEW V5.0 pipeline are visualized at A) *k*-NN = 15; and B) *k*-NN = 55.

6.4.3: Signatures of Selection

A high proportion of directional outlier loci (n = 196, alpha range: 1.267 – 1.938) were jointly identified by both statistical methods at an FDR of 0.01 among the Western Australian sites (Fremantle, Mandurah, Emu Point; Appendix 6.4). A total of 729 directional outlier loci were identified among the eastern Australian sites (Gulf St. Vincent, Port Phillip Bay, Stanley) by Lositan at an FDR of 0.01. However, BayeScan analysis did not identify any outlier loci that overlapped with Lositan results at low FDR thresholds suggesting that outlier loci might be rarer among the eastern range of *H. maculosa*. At FDRs of 0.01 in Lositan and 0.36 in BayeScan, eleven overlapping directional outlier loci were cautiously identified (alpha range: 0.659 – 1.381; Appendix 6.4).

When comparing the 17,316 neutral loci among individuals sampled from all six sites using pairwise values of '1-proportion of shared alleles', six clusters consistent with geographic proximities of sample sites were observed (Fig. 6.5A), with branch lengths between sites consistent with Fst and Nei's genetic distances (Table 6.2). When using the same analysis for the 196 directional loci identified among the Fremantle, Mandurah and Emu Point sites, the Mandurah and Emu Point sites clustered tightly together, and were both separated from the Fremantle site via notably increased branch lengths (Fig. 6.5B). Furthermore, the level of individual diversity among Mandurah and Emu Point was greatly reduced compared to Fremantle, which displayed larger and more variable, individual branch lengths. Interestingly, two individuals from the Fremantle sample site clustered with individuals from the Mandurah site in the neutral loci NJ Tree (Fig. 6.5A). However, both of these individuals migrated back towards the Fremantle cluster in the outlier NJ tree possibly reflecting partial adaptive variation in these individuals (Fig. 6.5B).

Among the Gulf St. Vincent, Port Phillip Bay and Stanley sites, the NJ tree based on the eleven candidate outlier loci revealed slightly longer branch lengths between all three sites compared to the neutral loci tree, but overall topology was similar (Fig. 6.5C). Accordingly, local adaptation was present among the three eastern sites but less pronounced than in the western sites. When annotating outlier loci through Blast2Go™ software, no biologically meaningful matches were identified.

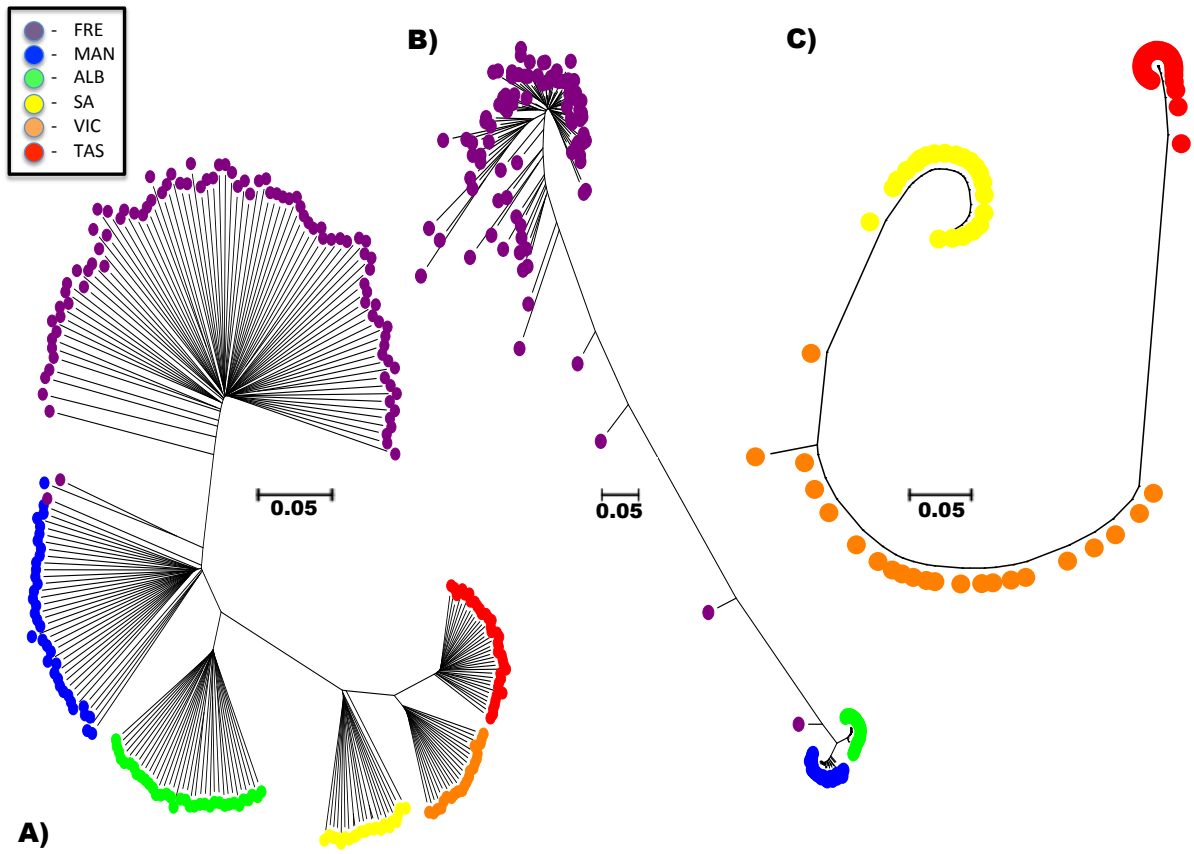

Figure 6.5: The relationships between individuals sampled from different locations are shown using the Neighbour-Joining method based on pairwise "1 – proportion of shared alleles" among A) The 17,316 neutral loci for all sampled individuals; B) The 196 directional outlier loci jointly identified by Lositan and BayeScan analyses among the three west coast sites; and C) The eleven candidate directional outlier loci identified by Lositan at an FDR of 0.01 and BayeScan at an FDR of 0.36. The legend at the top left of the figure displays the colours representing the site where individuals were sampled.

6.4.4: Phylogenetic Reconstruction and Evolutionary Distances

Based on both SNP and PAV phylogenetic inference methods, sampled individuals from the *H. maculosa* group formed distinct clades consistent with geographic proximities of individuals (Fig. 6.6; Appendices 6.5 & 6.6). All clades were well supported in the ML and Bayesian analyses (divergence among all sample sites except Rockingham: bootstraps = 100%, Fig. 6.6 and Appendix 6.5; posterior probabilities = 1, Appendix 6.6), providing strong confidence in the interpretation of phylogenetic reconstructions. There was no difference in tree topology or relative branch lengths when reconstructing trees using the RRHS ML methodology (Lischer *et al.*, 2013), indicating that heterozygous sites were not biasing SNP ML tree reconstruction (data not shown). The placement of the *H. fasciata* out-group clade shifted between SNP and PAV analyses (Fig. 6.6; Appendix 6.5). This might have been

due to the large amount of divergence among all groups, as well as the potentially limited resolving power of branch lengths using PAV loci (see below). Among all analyses, the two individuals from Rockingham were well-dispersed within the Fremantle clade, which suggests that these two sampling locations might be part of a larger single clade. The three samples from Misery Beach formed a separate clade basal to Emu Point indicating that these geographically close populations have most likely diverged in only relatively recent evolutionary history from one another. Individuals within the Fremantle clade displayed longer and more variable branch lengths compared to the other localities, which is consistent with higher diversity indices and a larger N_{eLD} observed in this site (Table 6.1). Upon comparison of relative branch lengths between all clades and the *H. fasciata* out-group, large evolutionary divergence was apparent among all sampled sites for the *H. maculosa* group (Fig. 6.6; Appendices 6.5 & 6.6). Consistent with the hypothesis of an existing sister-taxon in Western Australia (Norman, 2000), there was as much genetic divergence between Fremantle and the three eastern *H. maculosa* sites (range: 0.279 - 0.332) as between *H. fasciata* and all sample sites for the *H. maculosa* group (range: 0.232 - 0.377) based on the SNP ML tree reconstruction and F84 genetic distances (Table 6.3). However, genetic divergences between Fremantle and the Mandurah and Emu Point sites, as well as between Mandurah and Emu Point compared to the eastern sampling sites, were substantially less than with the *H. fasciata* out-group (Table 6.3) which suggests a clinal species pattern across this range. The relative divergence among sample sites and the *H. fasciata* out-group was consistent in the PAV tree reconstructions (ML and Bayesian; Appendices 6.5 & 6.6), and modified PAV genetic distance (Table 6.3). However, divergence estimated by the PAV markers was less pronounced overall. This reduction in relative branch length differences was primarily a function of the PAV loci and their loss of informative sites through the dominantly scored “0” or “1” classification (Lischer *et al.*, 2013). Nonetheless, this constraint has not been shown to affect overall tree topology, particularly for closely-related or recently diverged taxa (Althoff *et al.*, 2007; Lischer *et al.*, 2013).

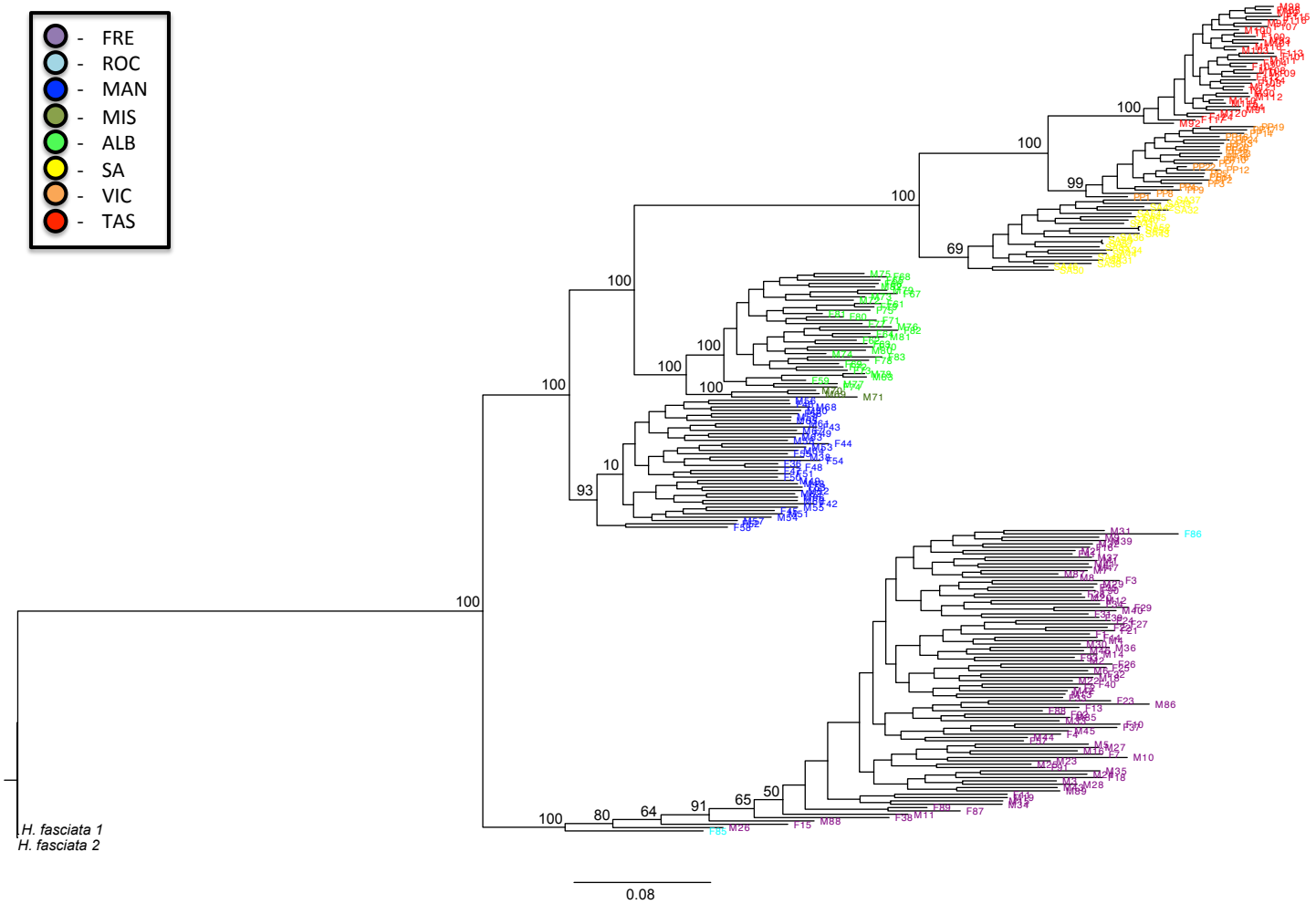

Figure 6.6: A maximum-likelihood tree for all 248 *H. maculosa* group samples from the eight sampling locations based on 100,000 bootstraps and 17,523 SNP loci. Two samples of the sister taxon *H. fasciata* are included as an out-group. The bootstrap values are listed to the top left of major nodes. Sample names are colour-coded to their sampling location, as per the legend in the upper left, with the out-group samples left in black.

Table 6.3: The F84 SNP genetic distances (below diagonal) and the modified PAV genetic distances (above diagonal) are given below between each of the sample sites with $N > 20$ and the sister taxon *H. fasciata*. Genetic distances between *H. maculosa* (also WBRO) sampling sites and *H. fasciata* are given in italics. All F84 SNP genetic distances have a standard error less than or equal to 0.002. All modified PAV genetic distances have a standard error less than 0.001.

	H. fasciata	**FRE**	**MAN**	**ALB**	**SA**	**VIC**	**TAS**
H. fasciata	*	*0.107*	*0.104*	*0.105*	*0.110*	*0.114*	*0.117*
FRE	*0.255*	*	0.053	0.057	0.068	0.074	0.076
MAN	*0.232*	0.176	*	0.042	0.053	0.058	0.060
ALB	*0.251*	0.198	0.094	*	0.048	0.052	0.054
SA	*0.321*	0.279	0.166	0.139	*	0.032	0.035
VIC	*0.359*	0.316	0.196	0.165	0.081	*	0.026
TAS	*0.377*	0.332	0.209	0.179	0.094	0.056	*

6.5: DISCUSSION

Genetic data presented here indicate that individuals sampled from the *H. maculosa* group follow a clinal species pattern across their geographic range, with the geographic extremities displaying levels of genetic divergence consistent with that of sister-taxa (Tables 6.2 & 6.3; Fig. 6.6). Furthermore, genetic divergence even among adjacent sampling sites in this study was remarkably high compared to studies of other cephalopods (Shaw *et al.*, 1999; Reichow & Smith, 2001; Pérez-Losada *et al.*, 2002; Doubleday *et al.*, 2009; Zheng *et al.*, 2009; Keskin & Atar, 2011; Moreira *et al.*, 2011; Higgins *et al.*, 2013). These findings suggest that the high levels of observed genetic divergence among sampling sites are a result of limited gene flow, consistent with a holobenthic life history, leading to a genetic IBD pattern along south-western and southern coasts of the Australian continent. Additionally, differences in strong selective pressures between geographic locations, as detected by outlier analyses, are suggested to increase the genetic dissimilarities of geographically separate populations of the *H. maculosa* group. Together, these data reveal that life history traits and ecological factors are rapidly driving genetic divergence, and possibly speciation within this taxon.

Within sample sites, levels of both observed and expected heterozygosity were quite low compared to other genetic studies in cephalopods (Shaw *et al.*, 1999; Reichow & Smith, 2001; Pérez-Losada *et al.*, 2002; Kassahn *et al.*, 2003; Zheng *et al.*, 2009; Moreira *et al.*, 2011; Higgins *et al.*, 2013). In part, this is due to differences in estimating heterozygosity between SNP and microsatellite markers, which were used in the above studies (see Vignal *et al.*, 2002), as well as the near impossibility of being able to eliminate all null alleles from the SNP library (DaCosta & Sorenson, 2014; Andrews *et al.*, 2016). Nonetheless, the low levels of heterozygosity observed here might also reflect the limited dispersal of this species group (Tranter & Augustine, 1973), leading to aggregations of highly related individuals. Heterozygosity scores were lowest for the Stanley site (H_o = 0.076; H_e = 0.086; Av. MLH = 0.08; sMLH = 0.572), along with the highest observed IR (0.732) and proportions of half-siblings (0.684). These samples were obtained over a one-month period from a commercial fishery that only fished over a ~22 km^2 area of relatively homogenous benthic habitat. However, the Stanley site also had the lowest F_{is} score (0.043 after within-site HWE filtration) and second largest N_{eLD} estimate (468) observed among sample sites in this study. This, in combination with the observation that inbreeding coefficients were significantly heterogeneous at all sites,

suggests that although highly-related individuals are likely to occur within close proximity as they do in Stanley, genetic evidence infers that inbreeding might be extremely rare. Both H_o and H_e were highest at the Fremantle and Mandurah sites, where samples were obtained over ~61 km^2 and ~220 km^2 areas respectively, and these sites also yielded the two lowest levels of half-sibling pairs and IR. The highest values for F_{is} were observed at the Mandurah site. However, due to the low levels of relatedness and large sampling area for this site, the higher F_{is} observed there was likely a result of Wahlund effect (Sinnock, 1975). The influence of Wahlund effect on F_{is} at the Mandurah site is further suggested by the sub-structuring patterns observed by Netview analysis for individuals from both the Mandurah and Fremantle (Fig. 6.4A).

The juxtaposition of high levels of interrelatedness (and sibling pairs) with comparatively low, uncorrelated and significantly heterogeneous inbreeding coefficients throughout the sites sampled in this study suggest that this species group might possess a mechanism for inbreeding avoidance. The low dispersal ability of this taxon, which results in the occurrence of closely-related individuals within small areas, could leave populations of this species group particularly prone to inbreeding depression (Charlesworth & Charlesworth, 1987). Significantly positive inbreeding coefficients have been recorded previously in the golden cuttlefish, *Sepia esculenta* Hoyle, 1885 (Zheng *et al.*, 2009), which also has a limited dispersal capacity. However, it is possible that the relatively lower F_{is} values observed in the current study could be due to the mating system of *H. maculosa* and/or their sister taxa. Females of the WBRO are selective of their mates, males spend different amounts of time copulating with particular females and both sexes copulate with multiple partners within their single breeding season (Morse *et al.*, 2015). It is possible that members of the *H. maculosa* group can avoid inbreeding by either preferentially copulating with non-related partners (Pusey & Wolf, 1996), or by mating with several partners and allowing postcopulatory processes to bias fertilisation to compatible gametes (Zeh & Zeh, 1997; Tregenza & Wedell, 2000). This latter possibility might also help to explain the extreme prevalence of polyandry in both this species group (Tranter & Augustine, 1973; Morse *et al.*, 2015), and possibly the holobenthic cephalopods in general (Hanlon & Messenger, 1998). Further studies investigating the paternity patterns among gentotyped candidate parents with known relatedness would be necessary to verify this hypothesis.

Where estimated, effective population sizes were highly variable among sample sites (Table 6.1). The relatively larger population estimate at Fremantle suggests that this species can be common in some areas, and that individuals might aggregate together due to habitat selection and/or breeding areas to better facilitate its synchronous terminal-breeding season (Tranter & Augustine, 1973). Due to the cryptic nature of the *H. maculosa* group, aggregation behaviour has not been documented in the wild. However, seasonal aggregations to facilitate breeding behaviour have been suggested by observations of predictable abundance and patterns of size structuring in the Cockburn Sound, WA, in addition to synchronous egg-laying events observed in laboratory settings (P. Morse personal observations). It is unknown why Gulf St. Vincent had a lower N_{eLD} compared to other sample sites, but it is possible the limited sample size and observed sampling of related individuals over a smaller area might impacted this calculation.

The observed Fst values among sample sites were very high compared to all comparable studies of population divergence in cephalopods (Shaw *et al.*, 1999; Reichow & Smith, 2001; Pérez-Losada *et al.*, 2002; Doubleday *et al.*, 2009; Zheng *et al.*, 2009; Keskin & Atar, 2011; Moreira *et al.*, 2011; Higgins *et al.*, 2013). Additionally, Fst values increased proportionally with geographic distance, implicating an IBD pattern for gene flow, consistent with *O. pallidus* (Higgins *et al.*, 2013), several species of cuttlefish (Pérez-Losada *et al.*, 2002; Kassahn *et al.*, 2003) and many terrestrial animals (Wright, 1943). This pattern strongly indicates that populations of the *H. maculosa* group are finely structured over distance due to their lack of a planktonic dispersal phase. Such a scenario suggests that the genetic connectivity of this species group might be highly susceptible to geographic barriers such as benthic topography or degradation of suitable habitat (Slatkin, 1973). However, pairwise genetic differences closely fit their expected values predicted by geographic distance, so no obvious genetic bottlenecks or specific barriers to gene flow were identified among sample sites in this study.

The only exceptions to this pattern were that the Fst value between Mandurah and Emu Point sites was much lower than expected based on geographic distance, whereas the Fst value between Fremantle and Mandurah was slightly higher. Interestingly, samples from Fremantle and Emu Point were both obtained in relatively shallow water (4 – 10 m depth), whereas samples from the Mandurah site were obtained from greater depths (17 – 28 m). It is possible that the deeper habitats around

the Mandurah, WA act as a barrier to dispersal and gene flow between the Fremantle and Mandurah sites, delineating the genetic groups between these two sites. Results from the AMOVA, DAPC and NETVIEW analyses all indicated that limited gene flow is present between adjacent sample sites, however support the above results in that animals sampled from the Mandurah site share more genetic similarities with the Emu Point site (Fst = 0.159, and ~580 km away) than individuals in the adjacent Fremantle site (Fst = 0.261, and only ~50 km away; Figs. 6.3 & 6.4: Appendix 6.2). A morphological survey of the ecotypes occurring over this range would be helpful by determining which of these ecotypes might or might not have a functional ink sac. The above genetic data suggests that the delineation between *H. maculosa* and the WBRO might be further north on the western coast than previously reported (Norman, 2000).

The evolutionary divergence of individuals among sites sampled in this study was further supported by phylogenetic analyses using the sister-taxon *H. fasciata*. Consistent with the previous separation of the WBRO from *H. maculosa* (Norman, 2000), phylogenetic reconstructions in this study indicated that the ecotype sampled from Fremantle is more genetically distant from *H. maculosa* ecotypes sampled from eastern sites, than it is from the described sister taxon *H. fasciata* (Fig. 6.6; Table 6.3; Appendices 6.5 & 6.6). Additionally, genetic divergence was sufficiently strong among all six of the primary sample sites in this study to justify investigation into the presence of potentially cryptic subspecies occurring at some or all of these sites. However, these data also indicate that gene flow occurs across the entire sampled region of this study through occasional migrations between adjacent populations. This suggests that the *H. maculosa* species group is in fact a species gradient that follows a clinal pattern across the proposed *H. maculosa* and WBRO distributions. It is recommended that future studies address morphological variation of this group, in order to complement the genetic data provided here and help in further defining the delineations between ecotypes within this potential species complex (e.g. Meudt *et al.*, 2009).

On examination of the 207 directional outlier loci within the *H. maculosa* group genome, it was evident that there were distinct signatures of selection present among the different sites. Although local adaptation was indicated in each of the six larger sites, the greatest divergence in selective pressures was observed between individuals from Fremantle and individuals from both the Mandurah and Emu Point sites. Furthermore, it was suggested that individuals from the Mandurah and Emu Point sites might be under

similar selective pressure, and/or have possibly been separated from the Fremantle site due to a recent genetic bottleneck or range expansion. This pattern adds further support to the delineation between the Fremantle and Mandurah genetic groups, and suggests either environmental pressures (Mayr, 1963) or selective breeding behaviours (Wright, 1940) might be acting to reinforce the divergence of the Mandurah and Emu Point individuals from the Fremantle ecotype. It is noteworthy that the two Fremantle individuals, who had previously clustered with Mandurah within the DAPC, NETVIEW and neutral loci figures, began to re-cluster towards the Fremantle group in the west coast outlier tree. This supports that some migration does occur between the Fremantle and Mandurah sites and that these two individuals, who were obtained near Fremantle, might have been descendants from recent migrants coming from Mandurah.

Selective pressures were subtler among the eastern sample sites (Gulf St. Vincent, Port Phillip Bay and Stanley). No outlier loci were identified using BayeScan analysis for the eastern sites at low FDR thresholds, which was possibly due to less pronounced local adaptation in this region and also the sensitivity of BayeScan to large differences in background Fst among sites (Narum & Hess, 2011; Lal *et al.*, 2016). However, upon examination of the eleven overlapping outlier loci identified at a more relaxed FDR, samples from the eastern sites did show a slight increase in branch lengths when compared to neutral loci (Fig. 6.5). This suggests that different selective pressures might be present among these three sites, although their impact on the *H. maculosa* genome is less defined within this region. None of the outlier loci identified in this study matched any biologically meaningful genes during blast analyses. This is most likely due to the general paucity of genomic sequencing studies in octopods, and the lack of annotation within octopod genomes (c.f. Ogura *et al.*, 2004; Albertin *et al.*, 2015). The increasing availability of genetic markers and techniques may enable future studies to easily link loci under directional selection to biologically meaningful regions of cephalopod genomes.

Together these findings reveal strong divergence among populations of the *H. maculosa* species along its range, most likely due to the limited dispersal capacity associated with this taxon's holobenthic and brief seven-month life history (Tranter & Augustine, 1973). These genetic differences are sufficient to justify the categorisation of two distinct sister-taxa and/or investigation into the possibility of several cryptic subspecies. However, these data also indicate that taxonomic delineations within this

group should be made with caution, as gene flow occurs across the species range through allele sharing between adjacent populations. It is hoped that future annotations of the entire *H. maculosa* genome might enable the identification of what types of directional selection are occurring along the species range, and the role that local adaptation might play in possible speciation within this group. Parallel studies addressing the phylogeny of distinct genetic groups within the greater blue-ringed octopus (*H. lunulata*) might be useful to compare findings in this study to a tropical congeneric possessing a planktonic larval stage (Overath & Boletzky, 1974). Finally, it is also indicated that fine-scale genomic studies in the *H. maculosa* group are warranted. The general processes shaping the broad-scale genomic structure of this species along its geographic range have been identified here. However, there is still much to be learned from this enigmatic taxon via investigating patterns of relatedness, possible subspecies delineations and sex-biased dispersal at much finer geographic scale (100s – 10s km).

6.6: CONCLUSIONS

This study provides the first molecular investigation within the *Hapalochlaena* genus, and the first genetic assessment of a holobenthic cephalopod across its entire range. These findings strongly indicate that *H. maculosa* and the WBRO form a single clinal species, following a genetic IBD pattern, common to terrestrial animal taxa (Wright, 1943) and other marine organisms that lack a planktonic life history phase (Pérez-Losada *et al.*, 2002; Kassahn *et al.*, 2003; Barbosa *et al.*, 2013; Higgins *et al.*, 2013). There was evidence of strong genetic divergence among sampling sites along the *H. maculosa* group distribution, most likely due to the limited dispersal capacity and short seven-month life cycle of the species. Phylogenetic reconstructions including the *H. fasciata* sister-taxon further support that the divergence between *H. maculosa* ecotypes at both ends of their distribution exceeds that observed between some heterospecifics in this genus. However, no two adjacently sampled locations showed comparable divergence to the *H. fasciata* out-group. Therefore, the taxonomic identities and geographic ranges of *H. maculosa* and WBRO require revaluation. Parallel studies with additional sister taxa (e.g. *Hapalochlaena lunulata*) will be useful as a comparison of habitats and life histories, in addition to providing phylogenetic context for the genomic divergence observed here between holobenthic members of the *Hapalochlaena* genus.

Further molecular studies, investigating relatedness and sex-biased dispersal of *H. maculosa* at a more localised scale, will be useful for additional insights into the behaviour of this cryptic taxon.

6.7: ACKNOWLEDGEMENTS

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CHAPTER 7: General Discussion

The current research combined investigations of precopulatory mate choice, postcopulatory fertilization mechanisms, sensory and broad-scale population genetics in order to facilitate a better understanding of the behavioural and molecular ecologies of one of Australia's unique and enigmatic macrofauna, the southern blue-ringed octopus (*Hapalochlaena maculosa*). In the initial component of this study, focal animal observations of precopulatory mate choice behaviours of *H. maculosa* in a laboratory setting indicated no changes in the rates of female receptivity or copulation attempts by males based on any measureable physical trait in the opposite sex (Morse *et al.*, 2015). Rather, both of these behaviours increased in frequency with the size of the animal displaying either receptivity or a copulation attempt (Morse *et al.*, 2015). Like all cephalopods, *H. maculosa* grows continuously through its life history until senescence (Tranter & Augustine, 1973; Boyle, 1987). This might suggest a greater prioritisation of mating by larger individuals, as expressed through decreased female selectivity of males and increased frequency of male attempts to copulate (Morse *et al.*, 2015), consistent with older animals ensuring a successful breeding season before the end of their semelparous, seven-month lifespan. Focal animal observations did reveal that mating preference might occur in this species through a form of intra-copulatory choice. When copulations were not ended by the male partner, females were observed to mate for longer with larger males (Morse *et al.*, 2015). If longer durations of copulation were to correlate with paternity, it could be advantageous for females to prefer larger males should they produce larger, potentially more competitive offspring (Kirkpatrick, 1982), or if larger male size represented a heritable ability to successfully survive the threats of natural selection through to old age (Beck & Powell, 2000). Alternatively, larger males might be more likely to pose a threat to females who terminate copulation too early. Copulations that were ended by females occasionally led to grappling as the male attempted to stay in the mount position (Morse *et al.*, 2015). It is possible that female *H. maculosa* allow larger males to mount them for longer in order to avoid intersexual aggression.

Male mate choice was also observed, in the form of longer mating durations with both novel females and females that had recently mated with more competing males

(Morse *et al.*, 2015). It was originally hypothesised that these differences in copulation times coincided with either male sperm loading or sperm removal behaviours in order to increase chances of paternity. Surprisingly however, paternity assessments in chapter four revealed that copulation duration was not correlated with paternal share. This suggests that longer copulation times were not spent transferring greater quantities of spermatophores, placing doubt on the ability of either male or female *H. maculosa* to regulate copulation duration as a form of intra-copulatory mate choice. The combined observations that males altered copulation times based on the recent female mating histories and that the length of copulation did not impact relative paternal share during controlled laboratory pairings, suggest that extended copulation durations in this species may be a form of mate guarding. In the wild, *H. maculosa* are often found in close proximity to each other during their breeding season (frequently captured in false-shelter traps 50 cm apart: P. Morse, personal observations). It is possible that in the likely presence of sperm competition, a male's chance of successful fertilisation with a female might be greatly improved by limiting the number of competing males to contribute sperm to that female's oviducal gland over the same window of opportunity (Parker, 1970). This hypothesis of preferential mate guarding behaviour would be consistent with the present observations that males favoured longer copulations with females they had not yet mated with, and that male-controlled copulation times were proportional to female recent mating histories (Morse *et al.*, 2015). If males can detect the quantity of sperm that females have recently stored in their oviducal glands, as has been suggested by the focal animal observations (Morse *et al.*, 2015), then this may be an indication of how many competing males are close by. Copulating males might also be able to use this information to determine how much time to spend guarding a female after they transfer their own spermatophore(s). The uncoupling of spermatophore transfer with the length of time spent in the mount position also enables an easier interpretation of the prevalence of male-male mounts observed throughout this study. Despite occasionally lasting for extended periods (up to 162 min: Morse *et al.*, 2015), it is possible that these same-sex mounts did not result in the waste of spermatophores.

Paternity analyses in chapter four did not reveal a paternal advantage to the females' most recent partners, indicating that male *H. maculosa* are unlikely to remove pre-existing sperm from female oviducal glands during copulation. On the contrary, paternity patterns observed in this study revealed a non-significant trend for paternity

to be biased to either the first males to mate with the female in the laboratory or to males the female had mated with prior to capture. This pattern is consistent with observations of male California two-spot octopus (*Octopus bimaculoides*) and algae octopus (*Abdopus aculeatus*) competing more aggressively over smaller and/or younger females (Huffard *et al.*, 2010; Mohanty *et al.*, 2014). If the first males to mate with the female do have a fertilisation advantage within the *H. maculosa* mating system, this could partially explain observations of increased mate guarding among subsequent males (Morse *et al.*, 2015) who might have had to compensate for their chronological disadvantage (Parker, 1990; Parker *et al.*, 1997). Offspring genotyping in this study also revealed non-sequential paternity patterns along egg strings, which suggests that some sperm from different males might get mixed within the female oviducal gland. This implication of sperm mixing, combined with consistent patterns between relative paternity and sperm precedence left in maternal oviducal glands, suggests that female *H. maculosa* are not likely to have the capacity to use cryptic female choice ('CFC': Eberhard, 1996) to selectively fertilise their eggs with one male's sperm over another's. However, the correlation between relative paternity and sperm signatures remaining in female oviducal glands after egg laying was not significant in this study, the sample size was small and there were several strong outliers to this pattern. While the data suggest that CFC via mechanical separation of sperm remains unlikely in *H. maculosa*, the possibility that postcopulatory chemical processes might play a role in biasing paternity to particular males cannot be ruled out (Eberhard, 1996). Additionally, it is emphasised that CFC remains an intriguing and worthwhile topic for investigation among the Cephalopoda, particularly within the sepiid and teuthoid taxa that have external fertilisation (Mangold, 1987; c.f. Hoving & Laptikhovsky, 2007).

At the commencement of this research, it was initially hypothesised that visual signalling might have been an important component of social recognition and corresponding mate choice behaviours in *H. maculosa*. The first pilot studies revealed that *H. maculosa* is active exclusively at night-time and that changes to chromatophore patterning involving the chromatic spectrum of light were uncommon among observed intra-specific interactions (Morse *et al.*, 2015). Furthermore, an assessment using an imaging polarimeter suggested that *H. maculosa* has little capacity to control the polarised properties of light reflected from its skin. These results suggest that it is unlikely for visual signalling to play a meaningful role in the behavioural ecology of *H.*

maculosa, however they also emphasised that alternative sensory modes, such as olfaction, might be particularly important for this nocturnal species. During odour cue trials, this study found that female *H. maculosa* were capable of detecting conspecifics via chemical signals in the water (Morse *et al.*, 2017). Also, differences in female ventilatory response after exposure to odours suggested that they may also be able to use chemical stimuli to discriminate the sex of detected conspecifics (Morse *et al.*, 2017). However, there was a wide variability in female response to odour depending on the identity of the conspecific from which the odour was obtained (Morse *et al.*, 2017). Among male odours, female response was negatively correlated with both her likelihood to be receptive to copulation with the detected male and the average time per day that she spent in copulation with the male during follow-up focal animal observations (Morse *et al.*, 2017). This suggests that the observed female responses to conspecific odours might be related to agonistic behaviour, and that the detection of chemical stimuli in the water might help female *H. maculosa* to avoid potential threats or unwanted interactions with conspecifics. The variability in female response to conspecific odours and its correlation with mate choice behaviours provokes the question of whether chemical cues might aid *H. maculosa* with individual recognition. So far, the ability of cephalopods to recognise individuals has primarily been investigated based on visual cues (Boal, 2006), and has only been empirically demonstrated in one species (common octopus, *Octopus vulgaris*: Tricarico *et al.*, 2011). It might be worthwhile to investigate the response of female *H. maculosa*, as well as additional cephalopod taxa, to the odours of novel and familiar conspecifics in order to help resolve the capacity of these animals to use chemical signals in distinguishing between individuals.

Contrastingly, male *H. maculosa* showed no indication of being able to discriminate the sex of conspecifics via odour cues (Morse *et al.*, 2017). Male *H. maculosa* may be able to detect conspecific odours in the water based on the observation that they gradually increased their ventilation rates over time after exposure to conspecific odours (Morse *et al.*, 2017). However, the reaction of *H. maculosa* to the odour of heterospecific octopuses was not assessed within this study, so it is not possible to determine whether the observed reactions were indicative of *H. maculosa* recognising the odour of a conspecific or whether animals were just increasing ventilation in response to the introduction of a novel odour. Regardless, the apparent

lack of ability by male *H. maculosa* to recognise the sex of conspecifics via chemical stimuli (Morse *et al.*, 2017) was consistent with observations during focal animal studies in chapter three that males approached male and female conspecifics equally, and attempted to mount other males as often as they did with females (Morse *et al.*, 2015). If male *H. maculosa* are able to glean any information about their surrounding environment via olfaction, it remains unclear why they do not appear to be able to use this for either locating females or avoiding infertile same-sex mounts. As was hypothesised in chapter five, it is possible that due to their limited breeding window and potentially fierce competition with other males, male *H. maculosa* cannot afford to miss an opportunity to copulate and therefore mount every conspecific they encounter before taking the time to determine the other individual's sex (Morse *et al.*, 2017).

The apparent lack of chemosensory in male *H. maculosa* (Morse *et al.*, 2017) conflicts with observations that they seem to be able to assess the novelty and recent mating histories of the females they mate with (Morse *et al.*, 2015). It was discovered during focal animal observations in chapter three that males appear to recognise if they have previously mated with a female, and they would also spend less time in copulation with a female if they were the last male to have mated with her (Morse *et al.*, 2015). The lack of sex discrimination via chemical cues in the water means it is unlikely that male *H. maculosa* can recognise individual females via olfaction (Morse *et al.*, 2017). Additionally, given that these interactions are nocturnal, any type of recognition would probably not be based on visual cues (Morse *et al.*, 2015). However, octopuses have chemosensitive cells on the ventral surfaces of their arms that they can use to taste objects in their environment (Budelmann, 1996). Therefore, it remains possible that males can recognise familiar conspecifics via tactile chemoreception, which was not addressed within the scope of this study. Previous descriptions of octopus mating behaviour report a tactile phase prior to copulations (Wells & Wells, 1972; Voight, 1991; Morse, 2008), and this could potentially aid the males in assessing the novelty or identity of the female, which might help him decide whether or not to attempt a copulation, how many spermatophores to transfer and/or how much time to invest in mate guarding. Tactile chemoreception may also enable male *H. maculosa* to identify whether stored sperm in female oviducal glands is either their own or belonging to a competing male. Such an ability might explain how focal males in this study were able to change their lengths of copulation based on female recent mating histories, even when they could not have seen

the female with the other males (Morse *et al.*, 2015). The role that this sensory system plays in octopus mating behaviour or social recognition requires further investigation.

At a population level, the ecology of *H. maculosa* appears to be highly structured by several aspects of its unique life history. Genetic evidence from chapter six in this study confirms that the brief seven-month lifespan of *H. maculosa*, paired with its large investment into relatively few but precocial young, leads to the potential for rapid divergence among different groups throughout its geographic distribution. The lack of a planktonic phase in larval development and short generation time in *H. maculosa* limit its dispersal capacity (Tranter & Augustine, 1973), and this reduced gene flow leaves distant populations vulnerable to other micro-evolutionary processes such as mutation, genetic drift and local adaptation (Slatkin, 1973; Lenormand, 2002; Doebeli & Dieckmann, 2003; Kirkpatrick & Barton, 2006). As hypothesised, all sample sites in this study were genetically distinct from each other. However, no complete barriers to migration were identified in the data and this appears to enable allele sharing between adjacent populations, resulting in gene flow to occur in a cline throughout the entire *H. maculosa* distribution. This clinal pattern of gene flow from Perth, Western Australia to Stanley, Tasmania maintains the species identity of *H. maculosa* in a gradient, while individuals sampled at either end of the distribution displayed sufficient genetic differentiation to theoretically be considered distinct sister-taxa.

A specific demarcation in genetic similarity was observed between Rockingham and Mandurah, WA. Outlier analyses revealed that this was driven by a difference in local adaptation patterns between sample sites north and south of this point on the species distribution. This study was unable to identify what change in environmental pressures might result in this difference of adaptation patterns, but both a review of morphological traits for *H. maculosa* occurring over this part of its distribution and future annotations of the *H. maculosa* genome might lead to an understanding of the types of selective pressures responsible for this divergence. Individuals sampled from both the Mandurah and Fremantle, WA sites were held in the laboratory throughout parts of this research. Although some minor morphological differences were noted among individuals sampled from these sites, no behavioural differences were observed. In addition, members from the two sites engaged in normal copulatory behaviour, which supports the genetic evidence that they are still conspecifics despite appearing to be from currently diverging populations.

Another interesting observation during the genetic component in chapter six of this study was that local diversity indices strongly indicated that the mating system of *H. maculosa* possesses a mechanism to avoid inbreeding. Despite high-levels of interrelatedness and half-sibling pairs within most sample sites, inbreeding coefficients were relatively low among sites and they were not correlated to either measure of relatedness. The proposed presence of an inbreeding avoidance mechanism was further supported by paternity analyses in chapter four. One of the females was accidentally paired with her full-sibling brother during laboratory trials. Although the full-sibling male had more than five times the sperm remaining in the female's oviducal gland after egg-laying than the competing candidate male, he only sired half of her offspring. A fertilisation bias to non-related and more genetically compatible males could potentially be the selective advantage necessary to explain the high-investment that some female cephalopods are observed to direct into their polyandrous mating systems (Eberhard, 1996; Zeh & Zeh, 1996, 1997; Tregenza & Wedell, 2000, 2002). Ensuring the acquisition of sperm from genetically compatible males might be particularly important for holobenthic cephalopods, such as *H. maculosa*, as dispersal from natal sites is more likely to be reduced in these species, resulting in closely-related individuals occupying the same habitat during breeding seasons.

This research has contributed to the existing knowledge of cephalopod ecology by providing insights into the precopulatory mate choice behaviour, postcopulatory fertilisation mechanisms, sensory system and broad-scale genetic structuring of *H. maculosa*. The four studies comprised in this thesis each prompt new hypotheses and/or additional lines of enquiry that could be pursued in further research using the same or similar species model. As mentioned above, the roles of both tactile and distance chemoreception in individual recognition and mate choice justify additional research within cephalopod taxa. Such a study could compare the responses of the focal animals after they have either made contact with or detected the odour of novel and familiar conspecifics. The clinal genetic structuring of *H. maculosa* over its broad geographic distribution could be examined more closely by investigating finer-scale patterns of relatedness within and between neighbouring populations. A fine-scale genetic assessment might reveal additional insights into the behaviour of *H. maculosa* by enabling a better understanding of population sub-structuring, familial proximity and sex-biased dispersal patterns. In conjunction with a census of morphological differences

along the species range, this could help to provide a robust taxonomic review of the *H. maculosa* species identity, which the data suggest as warranted given the vast genetic differences observed among sample sites in this study. Such investigations could help to further define micro-evolutionarily processes driving speciation in *H.* maculosa, as well as potentially in other holobenthic marine taxa.

Additional findings in the present study implicate inbreeding avoidance as a potentially strong selective advantage for both male and female promiscuity within the *H. maculosa* mating system. This hypothesis requires verification by comparing paternity patterns of genotyped candidate males that have varying but known relatedness to a female mate. If an inbreeding avoidance mechanism exists, then it would be expected for paternities to be biased to less related males, and for polyandrous females to have better offspring viability and consequently more grandchildren. At a genomic level, the presence of prezygotic isolation between related individuals can also be assessed by subsequent analyses of allelic segregation between full-sibling genotypes. Furthermore, if inbreeding avoidance is confirmed then it would be of interest to compare these patterns between different octopus taxa having holobethic and merobenthic life histories to assess whether the extent of paternity bias is stronger in species where relatives are expected to live in closer geographic proximities. If inbreeding avoidance and/or bias to genetically compatible gametes is observed as a common feature among cephalopod fertilisation patterns, then this mechanism might help to explain the high investment that females across the cephalopod class are observed to allocate towards polyandry (Hanlon & Messenger, 1998; Franklin *et al.*, 2012) despite very little evidence for obtaining resources or parental care from the males they mate with (c.f. spermatophore consumption in *Sepiadarium austrinum*, Wegener *et al.*, 2013). Overall, the outcomes of the present research are consistent with previous literature on cephalopod taxa in illustrating that this class of marine invertebrates displays profoundly complex behavioural and molecular ecologies (Hanlon *et al.*, 1994; Hanlon & Messenger, 1998; Hall & Hanlon, 2002; Boal, 2006; Mäthger & Hanlon, 2007; Huffard *et al.*, 2008a; Huffard *et al.*, 2008b; Albertin *et al.*, 2015; Caldwell *et al.*, 2015; Naud *et al.*, 2016). Broadening our understanding of the diverse range of reproductive modes and behaviours displayed among this unique class can enable deeper examination of mating system evolution and the development of life-history characteristics among the animal kingdom.

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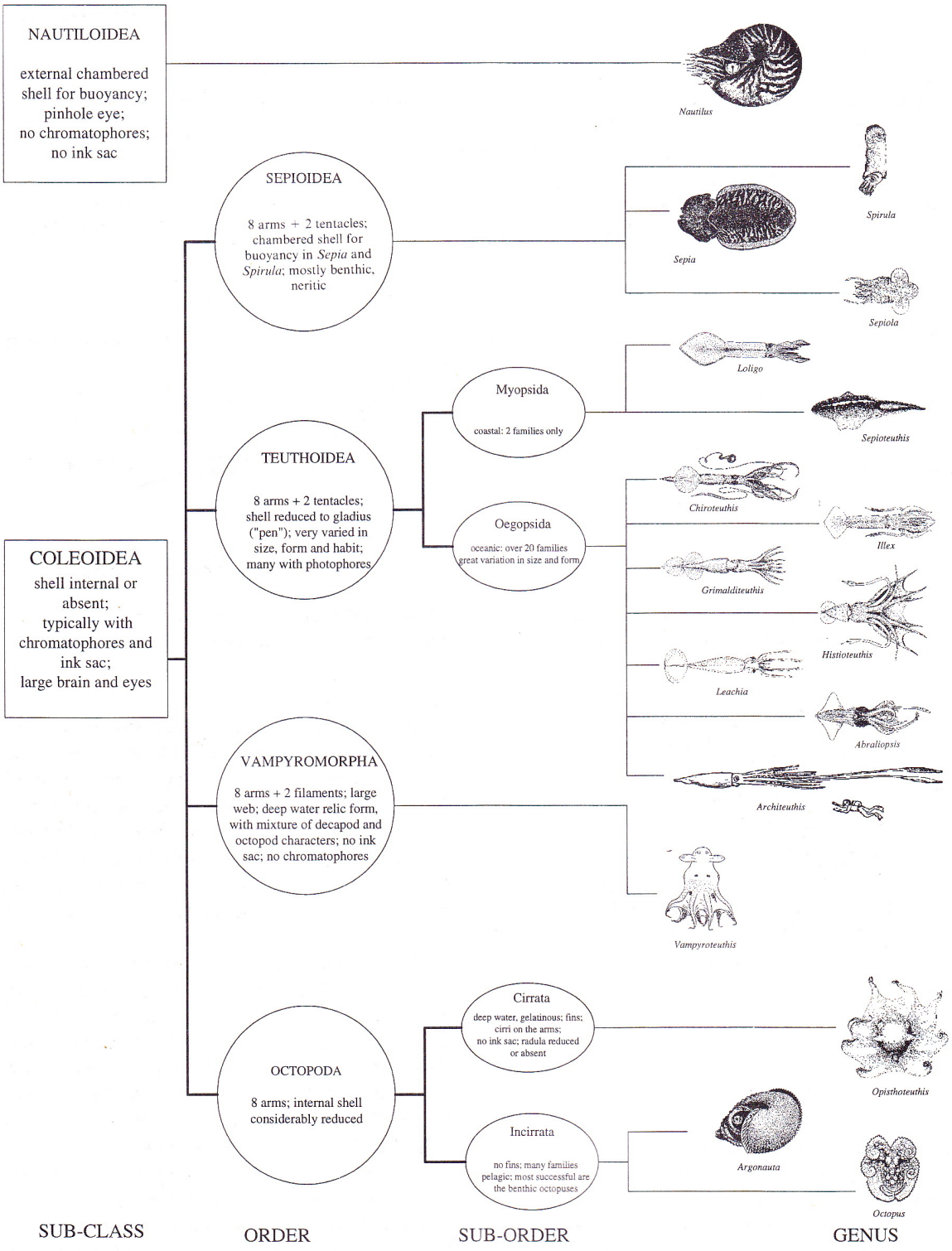


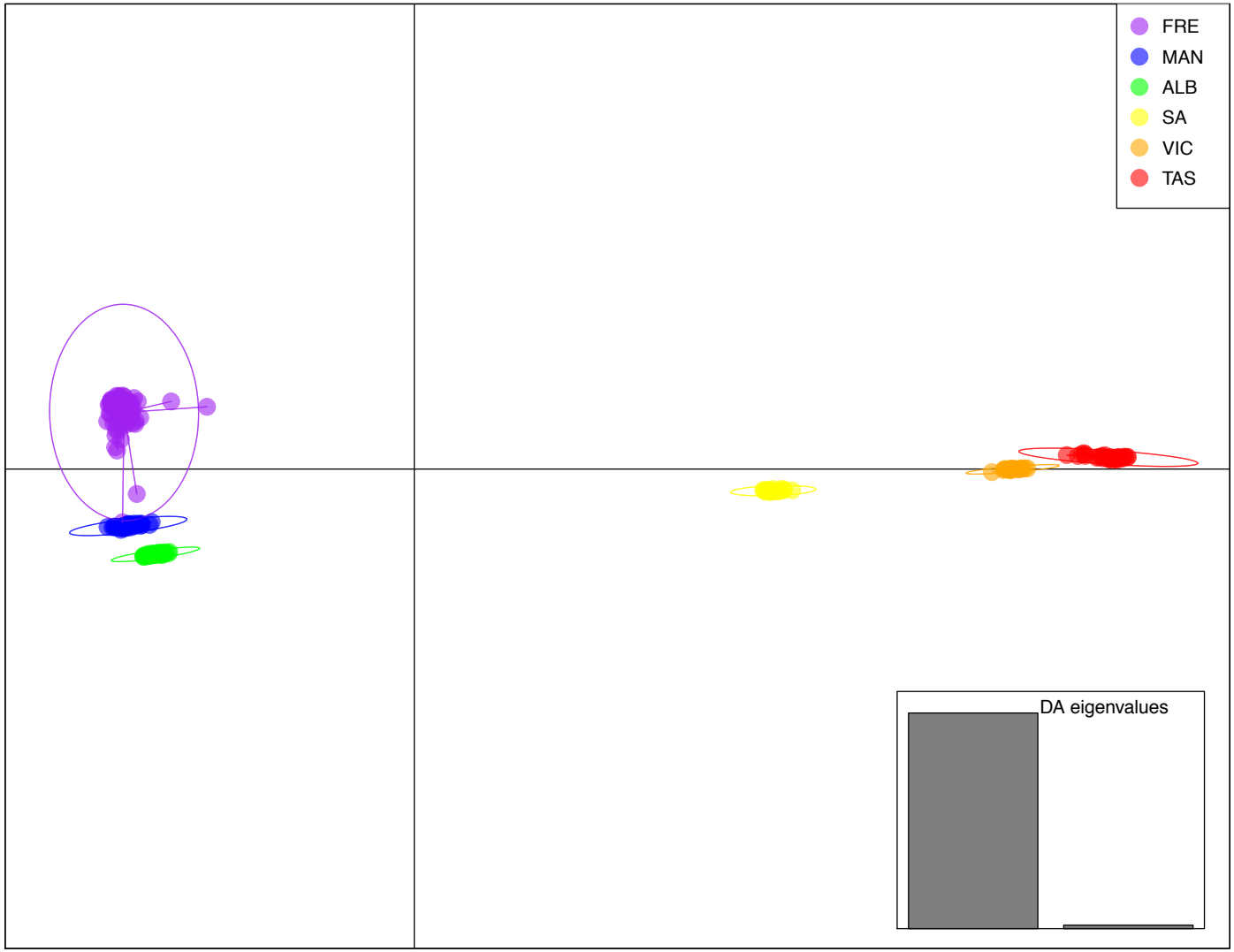
Appendix 2.1: The phylogenetic relationships and general morphology of major cephalopod groups mentioned in the text of chapter 2 (taken from Voss 1977 in Hanlon & Messenger 1998).

Appendix 5.1: A list of the animals used as receivers during odour trials in chapter 5 with their associated wet weights. The numbers of baseline and seawater treatments for which ventilation rates were recorded for each receiver are given in columns 3 and 4. The ID of each animal used as an odour source during male odour and female odour treatments for each receiver is given in columns 5 and 6. The animals that participated in each of the three corresponding focal-animal trials are listed in column 7.

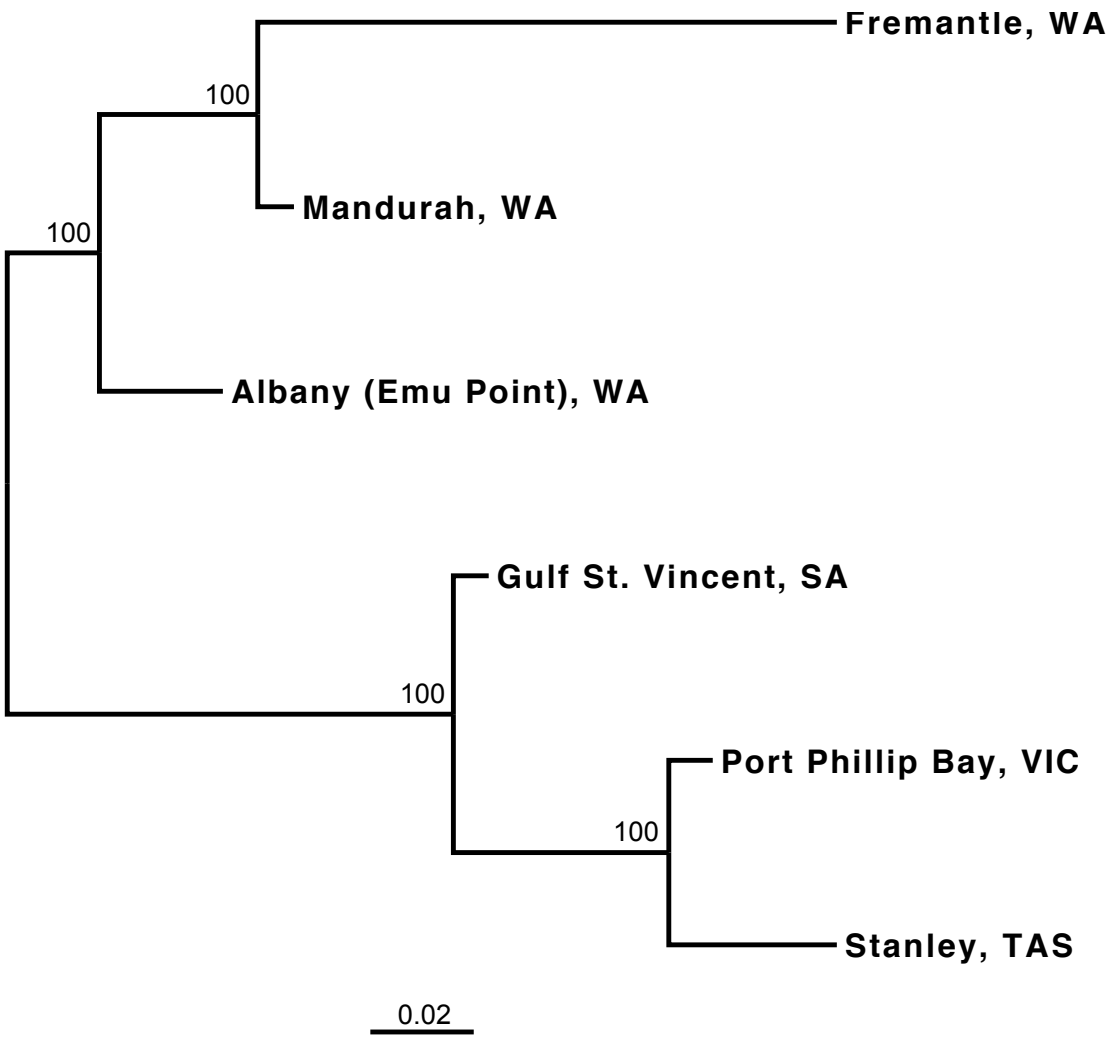
Receiver ID	Receiver Wet Weight (g)	Treatments during Odour Trials				Participants in Focal Animal Trials
Females		**Baseline (*N*)**	**Seawater (*N*)**	**Male Odours (ID)**	**Female Odours (ID)**	
F1	8	3	3	M1 M2	F4	Trial 1:
F2	3	3	3	M1 M2	F4	F1, F2, F3, F4 M1, M2
F3	5	3	3	M1 M2	F4	
F4	12	3	3	M1 M2	F1	
F5	6	4	4	M3 M4 M5 M6	-	Trial 2:
F6	5	5	5	M3 M4 M5 M6 M10	-	F5, F6 M3, M4, M5, M6
F7	5	6	6	M7 M8 M9 M11 M12	F8	Trial 3:
F8	9	6	6	M7 M8 M9 M11 M12	F7	F7, F8, F9 M7, M8. M9
F9	1	11	11	M7 M8 M9 M10 M11 M12 M13 M14 M15	F7 F10	
F10	1	4	4	M13 M14 M15	F9	
Males						
M1	7	2	2	M2	F4	
M2	4	2	2	M1	F4	
M7	3	2	2	M11	F7	
M8	5	2	2	M11	F7	
M10	2	2	2	M13	F9	
M13	3	2	2	M15	F9	
M14	1	2	2	M13	F9	
M15	1	1	1	-	F9	

Appendix 6.1: The results for the homogeneity tests of inbreeding coefficients (F_{is}) within each site (N > 20) are given below. Tests were run on subsets of loci that were stringently tested for HWE within each population to exclude the possibility of null alleles. F_{is} estimates were significantly heterogeneous among loci at all sites.

Site	X^2	d.f.	*p*
FRE	19,514.324	7,783	0.000
MAN	18,444.332	9,043	0.000
ALB	15,915.317	7,366	0.000
SA	10,340.797	5,897	0.000
VIC	127,737.807	5,332	0.000
TAS	7,873.197	4,574	0.000



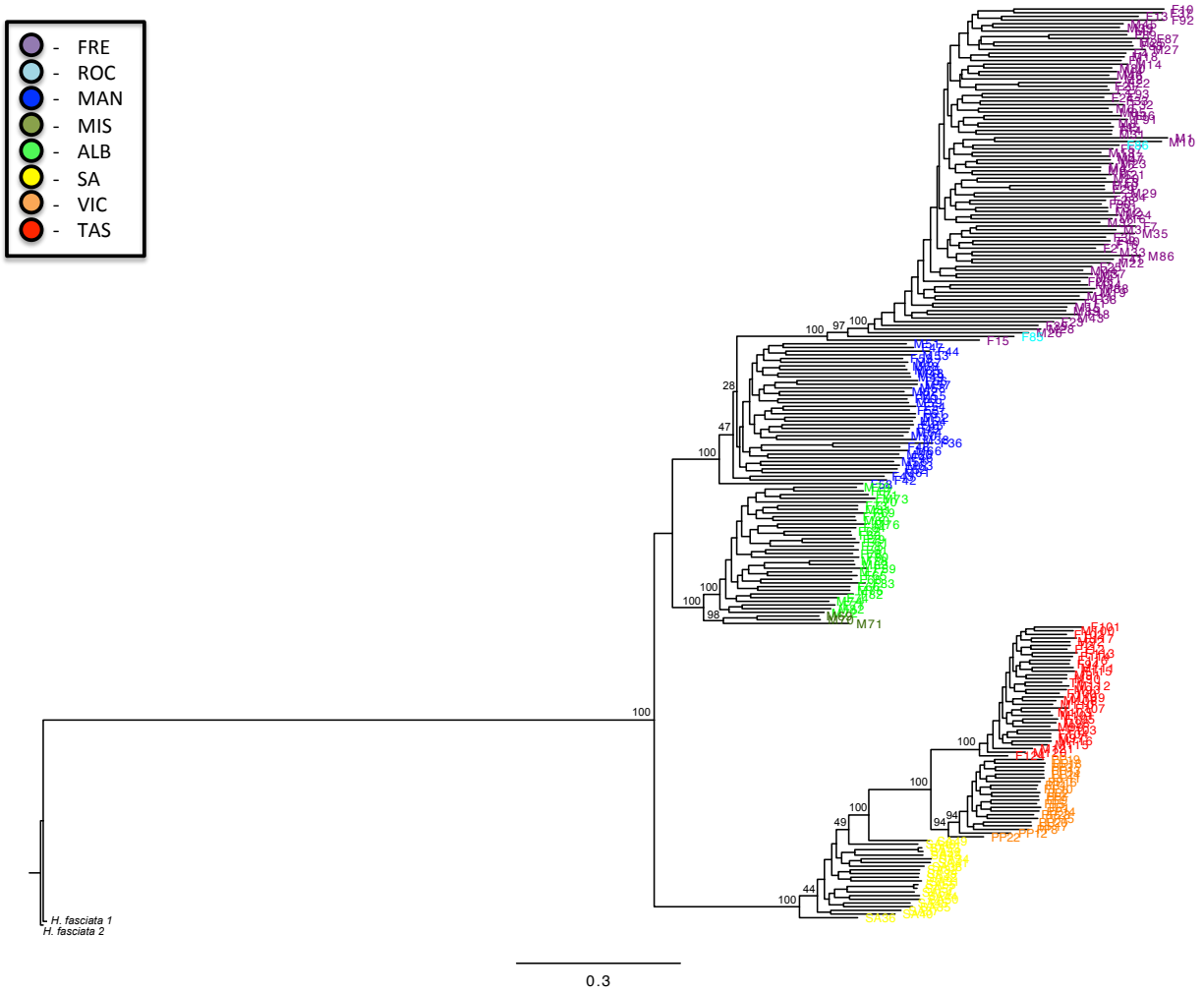
Appendix 6.2: A DAPC scatter plot, created with the R package *adgenet*, displays the extent of structuring between each sample site with $N > 20$ based on Discriminant Functions 1 and 2. The applicable A-score analysis revealed that all meaningful structuring between sites was explained by these two discriminant functions.



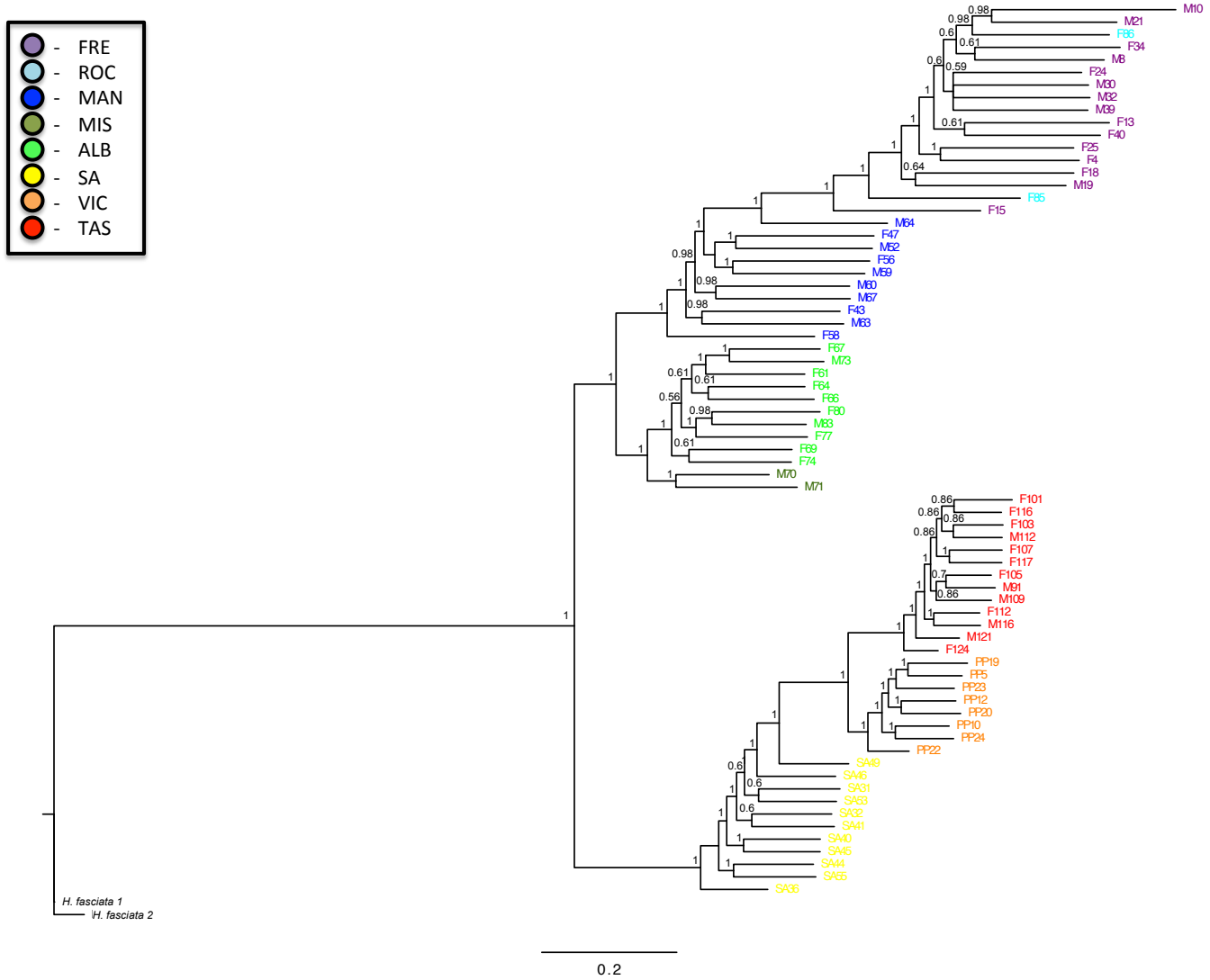
Appendix 6.3: The evolutionary relationships between sampled locations with $N > 20$ are illustrated using the Neighbour-Joining reconstruction method with Nei's standard genetic distances averaged over 1,000 permutations. The optimal tree is shown with the sum of branch lengths = 0.367 and bootstrap values to the left of each node.

Appendix 6.4: The numbers of outlier loci discovered by Lositan and BayeScan analyses are shown below. Overlapping directional outliers from both analyses at FDR = 0.01 were used for tree construction of the western sites in Figure 6.5. No overlapping outlier loci were detected by BayeScan among the eastern sites at low FDR thresholds. Therefore, directional loci identified by Lositan among the eastern sites at an FDR of 0.01 were used in tree construction if they were jointly identified by BayeScan with an FDR up to 0.36. These outliers among the eastern populations need to be interpreted with caution, however the high Fst values and alpha scores of these loci strongly suggest that they occur at diversifying regions of the genome for individuals sampled from these locations.

Compared Sites	Lositan Outliers (FDR = 0.01)		BayeScan Directional Outliers	Overlapping Directional Outliers used in Analyses	Average Fst of Overlapping Outliers (± S.E.)		Average BayeScan Alpha Score of Overlapping Outliers (± S.E.)
	Stabilising	Directional			Lositan	BayeScan	
FRE x MAN x ALB	2,065	1,181	540 (FDR = 0.01)	196	0.896 (± 0.003)	0.577 (± 0.002)	1.561 (± 0.009)
SA x VIC x TAS	422	729	12 (FDR = 0.36)	11	1.000 (± 0.000)	0.682 (± 0.018)	1.043 (± 0.092)



Appendix 6.5: A maximum-likelihood tree the 248 *H. maculosa* group samples from the eight sample sites used in this study based on 100,000 bootstraps with DArTseq PAV dominant markers. Two samples of the sister taxon *H. fasciata* are included as an out-group. The bootstrap values are listed to the top left of major nodes. Sample names are colour-coded to their sample site, as per the legend in the upper left, with the out-group samples left in black.



Appendix 6.6: A Bayesian reconstruction of a 74-sample subset of the *H. maculosa* group used in this study and two *H. fasciata* sister taxon samples based on PAV dominant markers. The posterior probabilities of each divergence are listed next to each node. Sample names are colour-coded to their sample site, as per the legend in the upper left, with the out-group samples left in black.