LIFE HISTORY, MATING BEHAVIOR, AND MULTIPLE PATERNITY IN *OCTOPUS OLIVERI* (BERRY, 1914) (CEPHALOPODA: OCTOPODIDAE)

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI´I AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

ZOOLOGY

DECEMBER 2014

By

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Keywords: Cephalopod, Octopus, Sexual Selection, Multiple Paternity, Mating

DEDICATION

To my family, I would not have been able to do this without your unending support and love.

Thank you for always believing in me.

ACKNOWLEDGMENTS

I would like to thank all of the people who helped me collect the specimens for this study, braving the rocks and the waves in the middle of the night: Leigh Ann Boswell, Shannon Evers, and Steffiny Nelson, you were the hard core tako hunters. I am eternally grateful that you sacrificed your evenings to the octopus gods. Also, thank you to David Harrington (best bucket boy), Bert Tanigutchi, Melanie Hutchinson, Christine Ambrosino, Mark Royer, Chelsea Szydlowski, Ily Iglesias, Katherine Livins, James Wood, Seth Ylitalo-Ward, Jessica Watts, and Steven Zubler.

This dissertation would not have happened without the support of my wonderful advisor, Dr. Les Watling. Even though I know he wanted me to study a different kind of "octo" (octocoral), I am so thankful he let me follow my foolish passion for cephalopod sexual selection. Also, he provided me with the opportunity to ride in a submersible, which was one of the most magical moments of my graduate career.

The following people helped me through the dissertation process with support and patience (and phone calls): Dr. Ingrid Knapp, Annick Cross, Melanie Hutchinson, Megan Ross, Claire Lager, Rebecca Prescott, Sonia Rowley, Abby LaPointe, Julio Rivera, Ben Wainwright, Jon Whitney, Garret Lynch, Mike Burns, Emi Yamaguchi, and Bryan Lantz. And thank you Dr. Chris Bird for introducing me to this species and pointing me in the right research directions. Dr. Zac Forsman, thank you for your patience in teaching me over and over again how to use Geneious. I am grateful to Jeffrey Yamada for the additional set of eyes to analyze video mating analysis.

Many thanks go to Dr. James Wood, the octopus expert on my committee. He gave me extremely helpful critiques on my work and opened my eyes to even more studies in cephalopods. Dr. Rob Toonen, I am eternally grateful for all of the time you gave me to talk about octopuses, sexual selection, genetics and data analysis. Thank you Dr. Tom Oliver, I am so appreciative of your patience and your wealth of knowledge in octopuses, data analysis and genetics. Thank you to Dr. Jeff Drazen always asking the hard questions and keeping me on my toes in every committee meeting. Dr. Chuck Birkeland, thank you for reading my dissertation and providing me with insightful edits and comments. Dr. Dave Carlon, thank you for introducing me to the world of molecular ecology and putting up with my infinite questions. Thank you Dr. Alan Friedlander for your support into the fisheries side of my work. Even though it didn't pan out, I am still grateful for the help and I hope some day to follow up with that research.

I would also like to thank Dr. Roger Hanlon who started me on the path to cephalopod biology. The Marine Biological Laboratory in Woods Hole was my introduction into the true practice of science and the harsh realities (and rewards) of cephalopod behavioral research.

Thank you to HIMB for allowing me to live on Coconut Island for three years so I could carry out my experiments without having to commute in the middle of the night. Thank you to the Holland lab for letting me use the outdoor tanks for four years. Without those tanks, I wouldn't have had a home for the (over 100) octopuses in this study. And thank you to the ToBo lab for letting me use all the genetics equipment and resources that made my Chapter 4 possible.

This work was partially supported by NOAA National Marine Sanctuaries Program award (MOA grant No. 2005-008/66882) to Dr. Rob J Toonen. Thank you to the University of Hawai'i and the Edmondson Grant for providing additional funding. Thank you to the Malacological Society and the travel grant that enabled me to go to a conference in the Azores.

Thank you to Windfall Films and National Geographic for fulfilling my dream of being on T.V. talking about octopuses.

If I have forgotten anyone in these acknowledgments, I sincerely apologize.

ABSTRACT

Extremely little is known about *Octopus oliveri*, its life history, distribution, or behavior. It was originally found in the Kermadec Islands, and since then has been described in Japan and Hawai´i. This study establishes the identity of *Octopus oliveri* through the use of genetic testing (COI marker) and morphological characteristics. In addition, mating behavior, brooding time, egg development, and paralarvae of *Octopus oliveri,* are described for the first time. Females lay approximately 5000 eggs and brood them for 33 to 47 days. Females and males mate with multiple partners when housed communally in tanks. As is true with most octopuses, female *O. oliveri* have two oviducts for sperm storage and may be able to store viable sperm for at least 100 days and up to 10 months. This suggests that sperm selection or competition may be occurring, however it has rarely been studied in octopods and never in *O. oliveri*. Through a combination of behavioral and genetic studies, this research found multiple paternity to be the rule in *Octopus oliveri*, both in the field and in captivity. Four sets of behavioral experiments were recorded wherein six females were mated with three males in varying order, for a total of 24 females and 12 males. Mating pairs mated in the reach, mount and beak-to-beak positions. In the behavioral trials, the largest females mated for the longest amount of time. Five microsatellite markers were developed and used to test paternity in eleven egg broods resulting from the behavioral trials. The results showed skewed paternity in most broods, suggesting that sperm competition is present in this species. The two predictive variables in determining male mating success were mate order and male size. The first male to mate with a female in behavioral experiments was more likely to sire more offspring, as were the largest males. This is the first study in an octopus that combines both behavioral and genetic information to determine fertilization success. This

study contributes to the growing research on cephalopod mating systems and in particular shows that octopus mating dynamics may be more complex than previously thought.

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CHAPTER 1. LITERATURE REVIEW OF SEXUAL SELECTION IN CEPHALOPODS

ABSTRACT

Cephalopods represent some of the more interesting animals in sexual selection studies as they have complex mating behaviors, multiple sperm storage sites, and potential for both sperm competition and female choice. The bulk of the scientific literature on cephalopod reproductive behavior is centered on squid and cuttlefish mating dynamics. Polyandry, the mating strategy adopted by cephalopods, lends itself to many sexual selection pressures resulting in the complex mating behavior observed in squid, octopus, and cuttlefish species today. Recently, genotypic studies on multiple paternity in cephalopods have shown that multiple males contribute to broods, but often in different proportions. Arguments for differences in sperm allocation, mating systems, sperm competition and female cryptic choice have all been made to explain the variance in rates of paternity. However, no clear pattern has yet emerged to explain whether some traits are selected over others. This review addresses current knowledge on cephalopod mating strategies, evidence for sexual selection within genera, and the gaps in current knowledge of cephalopod mating systems.

A BRIEF DESCRIPTION OF SEXUAL SELECTION

Sexual selection is one of the key mechanisms in determining morphological, behavioral, and genetic traits carried through the evolution of a species. It is the selection that arises through the competition and preference for a mate. Traits favored through sexual selection increase mating success and therefore pass on to future generations (Panhuis, Butlin, and Zuk 2001; Jennions and Kokko 2010).

Sexual selection was described by Darwin to explain why traits that are perceived as cumbersome or even harmful, such as the peacock tail, might be carried on through generations (Darwin 1871). Those traits arise through competition for mates and mate choice and may shorten lifespan by increasing possibilities for predation, parasitism, or disease, but because they increase mating success, they are said to have evolved through sexual selection (Jennions and Kokko 2010). Over time, we have come to learn that sexual selection is not limited to traits that characterize sexual dimorphism and competition. Sexual selection includes many mechanisms that can co-occur and may be intersexual, intrasexual, direct, indirect, precopulatory, or postcopulatory (Davies and Krebs 1993).

An important factor in determining the extent of sexual selection in a population is the level of investment from either parent in offspring development. Beginning with the production of gametes, females invest a large amount of energy in generating large, food rich eggs, while males have small, plentiful, and easily manufactured sperm. According to traditional sexual selection theory (Bateman 1948), male fitness increases the more mates he is able to obtain. Male reproductive success is determined by how many offspring he can produce, given that male

sperm is energetically cheap to create. The female becomes the limiting resource, encouraging males to use their energy for mating effort and competition for females (Davies and Krebs 1993; Jennions and Kokko 2010). Theoretically, as the number of mates increases for a male of a species, so do the number of his genetically produced offspring. Conversely, a female of the same species may mate with several males without adding to her potential offspring produced. She has a certain number of eggs she will generate and presumably, her fitness will not change with more mating events.

Contrary to Bateman's theory however, we are learning that multiple mating by both males and females tends to be the rule and not the exception in nature. Research is showing that polyandry (females mating with multiple males) may be more beneficial both directly and indirectly than previously thought (Birkhead and Møller 1998; Arnqvist and Nilsson 2000; Jennions and Petrie 2000; Squires et al. 2012).

MECHANISMS OF SEXUAL SELECTION

Intrasexual selection, or sexual selection occurring between two individuals of the same sex, occurs though contests, coercion, infanticide, and sperm competition (Andersson and Iwasa 1996; Jennions and Kokko 2010). Mating contests occur when males (and less frequently, females) compete amongst each other through physical fighting, intimidation, or display behaviors. Those males that have developed the most successful traits will also be the males with access to females for copulation. Traits favored may include large size, strength, weaponry, agility and threat signals (Andersson and Simmons 2006). Among seahorses and pipefish, the roles are reversed with the females competing to deposit eggs on the ventral surface of the male.

This in turn results in marked sexual dimorphism (more pronounced secondary sexual characteristics) in females (Jones and Avise 2001).

In the case of coercion, males will copulate with females by force, therefore minimizing the energy expended in courting a female, but potentially lessening rates of fertilization. Males may also kill previous offspring from competing mates to ensure all parental care is given to their own offspring. Sperm competition between males can be both precopulatory and postcopulatory. Males may guard or sequester the female after insemination to prevent rival male copulation. They may also create sperm plugs or flush the female of previously obtained sperm (Birkhead and Møller 1998; Andersson and Simmons 2006; Jennions and Kokko 2010).

Intersexual selection occurs between individuals of the opposite sex. Typically, intersexual selection is thought of in terms of female mate choice. Females may choose males as a result of direct male phenotypic effects. If the male has a particularly attractive ornament that also reflects his ability to provide material advantages, the female may choose him for the resources he will provide, whether they are food, territory, parental care, or protection (Andersson and Simmons 2006). Another mechanism that has been suggested in sexual selection is sensory bias. A particular ornament or male phenotypic trait may have arisen under natural selection, but then becomes preferred by females and favored by mate choice, thus preserving the trait in the population (Panhuis, Butlin, and Zuk 2001). In 1930, R.A. Fisher proposed a hypothesis of sexual selection suggesting that both the female preference for a trait and the male trait itself will be passed along to the offspring, reinforcing coevolution between sexes (Jennions and Kokko 2010). This hypothesis is sometimes referred to as the Fisherian sexy sons hypothesis because

the female should produce "sexy" or phenotypically attractive sons to increase her chances of maintaining her genes in the population and would therefore pick a male with the traits she wishes her sons to inherit (Andersson and Simmons 2006). Sometimes the benefit to the female or her offspring may not be directly apparent. In such cases there may be indicator mechanisms that direct female mate choice to select males with high quality genes. When the trait seems potentially detrimental or cumbersome to a male, such as the large tail of the peacock, the handicap may indicate the male is strong enough to overcome its effects and therefore is genetically superior to other males (Birkhead and Møller 1998; Jennions and Kokko 2010). Another indirect method of sexual selection is in the genetic compatibility between individuals. When alleles of mates are complementary and beneficial to the genome of the offspring, those alleles will be maintained in the population. An example of this mechanism occurring at the genetic level is in the major histocompatibility complex (MHC), which contributes to individual health and ability to fight off disease (Edwards 1998).

Sexual selection of certain secondary sexual characteristics may not always be advantageous to the species. There are costs associated with producing traits to attract females. Males with conspicuous mating displays or ornaments may be more prone to predation. In addition, males that are selected for large size may require more food and therefore expend more energy on foraging (Andersson and Iwasa 1996). However, the act of mating itself is generally seen as more costly to females as she must invest time and energy in the production and care of eggs. The balance between energy expended and offspring produced is constantly fluctuating in sexual selection and greatly influenced by natural selection. The study of sexual selection facilitates an

understanding of how mating systems evolved, how they are maintained and how to conserve future populations.

SEXUAL SELECTION IN CEPHALOPODS

Cephalopods are excellent organisms to study sexual selection because they mate multiply, meaning that both males and females mate multiple times with different individuals through their lifetimes. This behavior leads to increased potential for both intrasexual (competition for mates, sperm competition) and intersexual (female choice, female cryptic choice) sexual selection. Furthermore, the study of sexual selection in cephalopods is becoming increasingly relevant given our mounting awareness of their importance to fisheries. Cephalopods play an important role in the marine ecosystem, both as predators and prey and the more is understood about their mating systems, the more they can be managed as part of an ecosystem and as a resource. The aim of this review is to assess what has been concluded in cephalopod sexual selection literature and what is lacking and needs to be determined.

ANATOMY

From the relatively few species of cephalopods whose life cycles have been fully described, a general pattern of a short life span and a single breeding season has been observed (excluding the Nautilus). Most live approximately 1-2 years, although the giant Pacific octopus can live from 3-5 years and it is unknown how long many deep-sea cephalopods live (Hanlon and Messenger 1998; Boyle and Rodhouse 2005). However, *Bathypolypus articus* will brood its eggs for over a year (Wood 1998) and a recent study found a species of deep-sea octopus (*Graneledone boreopacifica)* that has a brooding period of over four years, indicating that it lives at least 4-5

years (Robison et al. 2014). It is likely that it lives much longer given that individuals need to reach maturity before brooding can begin.

Male squid, cuttlefish, and octopuses have one (or two) (Sato, Kasugai, and Munehara 2013) modified arm, the hectocotylus, which they use to transfer many individual sperm bundled in sperm packets, called spermatophores to the female. The spermatophores are placed either in an internal oviduct, an external seminal receptacle, placed on the arms or head of the female, or in some cases in cuts made in the exterior mantle skin. Males are generally capable of mating early on in life (3-6 months maturity) (Hanlon and Messenger 1998) and will continue to mate until senescence (Anderson, Wood, and Byrne 2002).

Female cephalopods have one ovary where they produce and store eggs. In octopods, the female has two oviducts where she will store sperm up to several months. Most decapods have one oviduct internally and a seminal receptacle around the buccal mass where they can store sperm as well (Boyle and Rodhouse 2005). They can begin mating early in life, just as the males, however eggs are not generally laid until full maturity is reached. In most species there is one spawning event where the female will lay her eggs, however other mating strategies also occur; polycyclic spawning, multiple spawning (Rodaniche 1984) intermittent terminal spawning, and continuous spawning (summarized in Rocha and Guerra 2001; Barratt, Johnson, and Allcock 2006). Females lay multiple eggs (from tens to hundreds of thousands) by extruding them from the ovary and they become fertilized as they pass by the oviduct or in the arms near the seminal receptacle. The eggs are generally attached to a benthic substrate either in strands, capsules containing multiple eggs, or individually and may be laid in communal spawning events or in

small clutches separately. Eggs vary in size (<1mm to 40mm) and hatch into planktonic paralarvae or benthic hatchlings (Hochberg, Nixon, and Toll 1992). Parental care is observed almost exclusively in octopods where the female will brood eggs after laying them, blowing water over them and protecting them from predators. Some squid have also been found to brood eggs, although it appears to be less common (Bower 2012). Females die shortly after spawning or as in the case of octopuses, after eggs have hatched.

MATING SYSTEMS

Reproductive strategies of cephalopods vary across species (Rocha and Guerra 2001). In general, but not exclusively, squids mate in large shoaling groups, cuttlefish in small, loose aggregations, and octopods individually. As a result of these varying systems, different mating behaviors and tactics have evolved.

Squid

Typically, squids mate in large shoaling aggregations with multiple males competing for limited egg laying females (Sauer et al. 1997). Male agonistic behavior is characterized by complex chromatophore displays such as the white flashing of *Loligo vulgaris reynaudii* (Hanlon, Smale, and Sauer 2002), the Zebra Spread Display and Lateral Silver Display of *Sepioteuthis sepioidea,* or the Lateral flame in *Loligo plei* (Hanlon and Messenger 1998), to name a few. This behavior is considered to repel other males while establishing dominance and indicating to the female that he is an interested male. These intense visual displays may sometimes result in physical contact with males either pushing or attempting to bite one another. Females may also develop body patterning to repel males or attract suitors (Jantzen and Havenhand 2003).

Male squids may be described as paired "consort" males, unpaired males, or "sneaker" males. The consort males are generally larger and mate parallel to the mature female, depositing spermatophores in the mantle near the oviduct. Consort males typically guard the female before, during and after she lays her eggs, presumably ensuring paternity. Large unpaired males may displace the consort male and gain access to the female before spawning (Iwata and Munehara 2005). Small sneaker males compete with the consort male by mating head-to-head or in the male-upturned position with the female and depositing sperm around the buccal mass or placing sperm directly on the eggs as the female lays them (Wada et al. 2005; Hanlon, Smale, and Sauer 2002). These are termed "extra-pair" copulations because the sneaker male will mate with the female while the consort male is already mating. In some cases the sneaker male may mimic a female in order to gain access without provoking male agonistic behavior (Jantzen and Havenhand 2003).

Cuttlefish

Cuttlefish mate in smaller aggregations than squid, but have similar agonistic displays between competing males. The Intense Zebra Display chromatophore pattern is observed in several species (Boal 1997; Adamo and Hanlon 1996). This display between competing males can escalate into physical fighting, with one male trying to bite another. The winning male will then court and copulate with the female if she is receptive to mating. Cuttlefish most commonly mate in the head-to-head position and spermatophores are deposited around the buccal membrane of the female or in an internal seminal receptacle called the pharetra. The spermatophores that are placed on the buccal mass will extrude the spermatangia (sperm mass) that can then be used for

fertilization. Sneaker males are common in cuttlefish reproductive systems, and female mimicry by sneaker males is a strategy used to avoid agonistic male interactions (Naud et al. 2004).

Octopus

Octopuses are solitary animals and mating typically occurs between two individuals at a time, with little courtship behavior observed. There have been accounts of multiple males simultaneously mating with a single female in some species, but these appear to be exceptions (Hanlon and Messenger 1998). Octopuses mate in the open, possibly to minimize the risk of sexual cannibalism (Hanlon and Forsythe 2008). Males may mate with a female by mounting her or sitting next to her and extending the hectocotylus into the mantle and depositing spermatophores into the distal oviduct (Huffard and Godfrey-Smith 2010). Head-to-head mating is rare, although it has been observed in *Octopus chierchiae* (Rodaniche 1991). Mate guarding and male-male agonistic behavior has been described in *Abdopus aculeatus*, but is not generally associated with octopus mating behavior (Huffard, Caldwell, and Boneka 2010). However, because many octopuses are difficult to observe in the field, it is possible that this behavior is more commonplace than originally thought.

MULTIPLE MATING

Cephalopods have multiple mates during their reproductive period. There are costs and benefits to this mating strategy for both males and females. Mating is energetically taxing and often increases potential for predation. The agonistic displays observed in several cephalopod species are extremely conspicuous, making them easy targets as prey. However, the benefits of polyandry may include nourishment provided by substances in the seminal fluid (Mann, Martin,

and Thiersch 1966; Squires et al. 2012). Additionally, offspring may be more genetically fit when sperm competition or female choice occurs (good genes hypothesis, Jennions and Petrie 2000). Multiple mating leads to higher potential for genetic recombination, therefore increasing genetic diversity and possibly brood survival rate.

A recent study on dumpling squid *Euprymna tasmanica* showed that females that mated with multiple males had larger eggs at a faster rate than those that mated with only one male (Squires et al. 2012). This suggests that the female may benefit from multiple matings by obtaining nutritional supplements from the male's sperm, therefore enabling her to invest more yolk into her eggs.

SPERM COMPETITION

In animals where many males mate with a female, there is the possibility of sperm from multiple males competing to fertilize the female's egg. This is known in sexual selection literature as sperm competition (Parker 1998). Cephalopods have many characteristics that suggest sperm competition is likely: multiple mating by both sexes, sperm storage by females up to 10 months in some species (Wells 1978), delays between mating and egg laying, and varying mating systems (Baur 1998).

Sperm competition in cephalopods generally occurs in one of three ways. Males may guard or sequester the female after insemination to prevent rival male copulation, a male may actively remove sperm that was deposited earlier by a previous male, or he may increase the volume of the ejaculate to displace sperm from a previous male and increase his chances of fertilization.

Mate guarding is common in squid and cuttlefish (Wada, Takegaki, and Mori 2005; Iwata and Munehara 2005; Hanlon and Ament 1999; King, Adamo, and Hanlon 2003; Wada et al. 2010; Wada et al. 2006; Emery, Wilson, and Craig 2001; Hanlon, Smale, and Sauer 2002; Jantzen and Havenhand 2003; Naud et al. 2004) and has even been observed in a species of octopus (Huffard, Caldwell, and Boneka 2010). In general, mate guarding consists of the male, typically a larger "consort" male, remaining with the female as she lays eggs, presumably to ensure paternity (Hanlon, Smale, and Sauer 2002).

In *Sepia oficinalis, Sepia apama, Sepiella japonica,* and *Sepia lycidas* a conspicuous sperm flushing behavior has been observed in previously mated females. The cuttlefish mate in a head-to-head position and males will deposit sperm around the buccal mass, where the sperm is then stored in seminal receptacles. When a male encounters a female, he will flush the buccal area with water, thereby dislodging sperm from previous males (Hanlon and Ament 1999; Hall and Hanlon 2002; Wada et al. 2006; Wada et al. 2010). In *Sepia lycidas*, it was found that males would increase both the time spent on sperm removal and the number of ejaculations per mating when mating with a previously mated female. The size of the male also influenced which behavior he would exhibit, indicating that sperm allocation occurs to maximize mating success. If the male were small and likely to be interrupted by a competing male, he would spend more effort in increasing the number of ejaculations per mating, while large males might put more effort into sperm removal (Wada et al. 2010). Sperm removal has also been shown in *Sepia esculenta*, however the male will use his hectocotylus to directly remove sperm from the buccal area of the female, instead of the "flushing" behavior described above. It was found that even

when the male was the last male to mate with the female, he would still perform this sperm removal behavior, though in all instances he did not remove all of the sperm in the buccal mass (Wada, Takegaki, and Mori 2005).

Currently, there has only been one study in octopus that looked at sperm competition. In 1995, Cigliano looked at mating behavior in a pygmy octopus. He found that the initial mating period would increase when a female had mated with a previous male, indicating that the male could possibly detect female mating history. The reason for the increased mating time was predicted to be a result of males removing or displacing sperm from previous mates from within the female (Cigliano 1995). The ligula, or tip of the hectocotylus, is modified into a spoon shape that could possibly be used to remove sperm from the spermatheca of oviducal gland. Currently, this type of sperm removal has not been observed in octopus.

MULTIPLE PATERNITY

Beginning with Shaw and Boyle in 1997, cephalopod biologists began using microsatellites to determine multiple paternity. Since then, multiple paternity studies have been done in five species of squid (*Loligo forbesi,* Shaw 1997; Emery, Wilson, and Craig 2001, *Sepioteuthis australis, Loligo pealeii,* Buresch, Hanlon, and Maxwell 2001; Buresch et al. 2009; van Camp et al. 2004, *Loligo vulgaris reynaudii,* Shaw and Sauer 2004, *Loligo bleekeri,* Iwata and Munehara 2005) one cuttlefish (*Sepia apama* Shaw 2003; Naud et al. 2004; Naud 2005) and recently, two octopus species (*Graneledone biopacifica* Voight 2009, *Octopus vulgaris* Quintero et al. 2011). All studies found multiple paternity was prevalent. This is not surprising given that most cephalopods mate multiply, although mating does not necessarily ensure fertilization. In many

cases, the proportion of eggs fertilized by each male was skewed with some males siring more offspring than others, indicating the potential for sperm competition or female cryptic choice (Eberhard 1996).

There is some evidence for sperm precedence in cephalopods (Iwata and Munehara 2005; Hanlon, Smale, and Sauer 2002; Shaw and Sauer 2004). It is predicted that the males who mate with the female last before she lays her eggs will sire more offspring, which would account for mate guarding and sperm removal behavior in many species. Last male precedence was confirmed genetically in *Loligo bleekeri* with the last male fertilizing 85-100% of the eggs in four broods tested (Iwata and Munehara 2005) and 48% of eggs in a single egg strand of *Loligo vulgaris reynaudii* (Shaw and Sauer 2004). However, a study of precedence in *Loligo pealeii* showed high paternity of the first male in instances where time between mating with a second male and egg laying was short (40 minutes or less) (Buresch et al. 2009) indicating that perhaps mate order is not the most important factor in determining paternity. *Sepia apama* eggs were also genetically analyzed and showed no significant last male precedence, nor did sperm flushing behavior or mate guarding result in more offspring sired (Naud et al. 2004).

FEMALE CHOICE

Female choice can be precopulatory when a female directly rejects or accepts a males advances for mating, or postcopulatory in the form of female cryptic choice where a female may select one male's sperm over another (Eberhard 1996). Some examples include active sperm ejaculation by females and physical or chemical barriers within the female that may impede sperm success

(Birkhead and Møller 1998). In species where sperm storage exists, female cryptic choice is likely (Bussiere et al. 2010).

In 1997, Boal observed female choice of males in the cuttlefish *Sepia officinalis*. Males of this species have conspicuous chromatophore displays, termed "zebra" displays. Females showed less preference for males with strong zebra display, indicating that this behavior is likely agonistic. The females tended to chose males that had recently mated; suggesting chemical cues might be involved in female choice (Boal 1997).

In *Sepia apama* females may reject males by swimming or pulling away from them, or even by biting the males (Naud et al. 2004; Hall and Hanlon 2002). However, the particular characteristics that the female uses in choosing a mate are unknown. Females will mate with both large and small males and multiple males successfully fertilize eggs (Naud et al. 2004).

Caribbean reef squid, or *Sepioteuthis sepioidea*, will pair off after a complex courtship that involves vivid displays from both males and females. A male will only mate with a female if she allows him to approach close enough to "strike" her with his hectocotylus. Females have to actively move spermatophores placed on their forehead directly to the seminal receptacle, suggesting possible female choice. In one instance, a female was seen to remove the spermatophore of a male and throw it away with her arm. Some aggregations of *Sepioteuthis sepioidea* will result in multiple males attempting to mate with a female at once, but in general, males and females will pair off briefly to mate and then move on to mate with the next partner (Moynihan and Rodaniche 1982).

One recent paper described a remarkable behavior by *Idiosepius paradoxus,* the Japanese pygmy squid, in which the female will elongate the buccal mass to pick off sperm deposited by males. In some cases, she then ate the spermatangia, possibly to absorb nutrients and prepare for egg brooding. This behavior was less frequent in virgin females, suggesting that previously mated females may be more selective of mates (Sato, Kasugai, and Munehara 2013).

Octopus diguetti appears to select males in response to male ligula length. The ligula is located at the end of the hectocotylus and in the case of *Octopus diguetti*, it can inform the female on male maturity. Thus, the female exhibits choice when she allows a male with an appropriately sized ligula to mate with her (Voight 1991).

FUTURE DIRECTIONS

Polyandry, the mating strategy adopted by cephalopods, lends itself to many sexual selection pressures resulting in the complex mating behavior observed in squid, octopus, and cuttlefish species today.

Recently, genotypic studies on multiple paternity in cephalopods have shown that multiple males contribute to broods, but often in different proportions. Arguments for differences in sperm allocation, mating systems, sperm competition and female cryptic choice have all been made to explain the variance in rates of paternity. However, no clear pattern has yet emerged to explain whether some traits are selected over others. Further studies are needed to determine to what

extent female cryptic choice occurs, if at all. The combination of both behavioral and genotypic studies will provide a clearer picture of what factors are at play in *Octopus oliveri*.

CHAPTER 2. THE LIFE HISTORY OF *OCTOPUS OLIVERI* (BERRY, 1914) (CEPHALOPODA: OCTOPODIDAE) AND CONSIDERATIONS FOR ITS USE AS A STUDY ORGANISM.

ABSTRACT

Several shallow water octopuses can be found among the reef flats of the Hawaiian Islands. *Octopus oliveri* is particularly interesting as it inhabits the rocky intertidal zone and spends much of its time out of the water. Very little is known about this octopus and this study establishes a baseline of information on this species. This work describes habitat and activity patterns as well as egg development. Chromatophore patterns are used to distinguish *Octopus oliveri* (Berry, 1914) paralarvae from other Hawaiian cephalopod paralarvae. Preservation times before and after death are compared to illustrate variation in founder chromatophore patterns even among individuals of the same brood. *Octopus oliveri* is relatively easy to find and collect from the rocky intertidal region, it mates readily in captivity, lays eggs, and because of its small size, many can be kept at one time, making it a good species of octopus for study. This study served to identify distinguishing characteristics of *Octopus oliveri* and its offspring.

INTRODUCTION

Cephalopods are fascinating creatures that have captivated people for hundreds of years (Aristotle 350 B.C.E). They are legends of popular folklore, monsters of the deep, and masters of disguise. They are also important predators and sources of protein for countless marine species as well as a common food in restaurants around the world. Cephalopods are abundant from the equator to the poles, and from tide pools to the deep sea (Boyle and Rodhouse 2005).

In Hawai´i, octopuses are an essential part of the rich culture. In the Kumpulipo, the Hawaiian creation chant, one of the three males born at the dawn of human life is in the body of a "hot-striking octopus." Octopuses are mentioned multiple times through the chant, always as important and powerful beings throughout creation (Beckwith 1981). Many species are fished for local consumption, for bait, or to be shipped to other countries. The variety of octopus and squid species throughout the islands is remarkable and makes for an excellent research site for those interested in studying cephalopods.

Despite their relative abundance and their obvious importance in the Hawaiian Archipelago, many octopus species remain undescribed or understudied. Of the approximately 16 species of benthic octopods that have been described in Hawai´i from depths of 0-2500m, only 7 have been named (Huffard 2005; Young and Harman 1989). It is likely that there are still many species of octopods unknown in Hawai´i given that new species continue to be reported in the tropical Pacific region (Kaneko and Kubodera 2007; Huffard 2005).

One of the aforementioned understudied species is *Octopus oliveri*. Originally described by Berry in his 1914 report on the Cephalopoda of the Kermadec Islands, it has since remained relatively absent in scientific literature. Extremely little is known about *Octopus oliveri*, its life history, distribution, or behavior. It is a small, intertidal octopus locally known as the "rock tako" or "he´e pali." Fishermen will commonly collect this species as bait, although it is also occasionally eaten (personal comm. Bert Taniguchi). This study aims to present comprehensive information on the morphology, habitat, and life cycle of *Octopus oliveri*.

MATERIALS AND METHODS

Octopus oliveri individuals were collected from Kaka´ako Waterfront Park, and Kewalo Basin Marina, Honolulu, Hawai´i in the fall of 2010 through the summer of 2013 (over 100 individuals collected, 70 different excursions). Two to three people would walk along the rock wall during the evening hours for one to three hours (between 7pm-12am) with a flashlight. When an octopus was found, it was collected by hand and transferred to a five-gallon bucket. The males and females were kept in separate buckets. Adult octopuses were weighed on a platform scale and transferred to tanks on Coconut Island, Kāne´ohe. All octopuses were kept in large outdoor tanks at the Hawai´i Institute of Marine Biology (HIMB) with constant saltwater flow and ambient ocean temperature. The octopuses were fed frozen shrimp and live crabs on a regular schedule and the tanks were cleaned after each feeding. Water temperature records were obtained though NOAA Tides and Currents databases from the station located closest to the collection site in Honolulu (Station ID 1612340) and at Coconut Island (Station ID 1612481).

SPECIES VALIDATION

Adult morphology

Measurements were recorded from ten specimens collected (6 males, 4 females). Adult octopuses were weighed on a platform scale, photographs were taken of the specimens and measurements were collected by analyzing the photographs with the program ImageJ. Measurements were then compared with those recorded by Berry in 1914, Sasaki in 1929, and O'Shea in 1999. Morphological characteristics were also compared with other local shallow water octopuses in Hawai´i to determine that this was indeed *Octopus oliveri* and not a previously described or undescribed Hawaiian octopus.

Genetics

The mitochondrial cytochrome c oxidase subunit I (COI) was sequenced from three individual specimens using Sanger sequencing. *S*equences from two Hawaiian specimens were compared with sequences obtained from an *O. oliveri* specimen collected at the type locality in the Kermadec Islands (Raoul Island, Fishing Rock, AM C.477708).

HABITAT

During the collection of the octopuses, photographs were taken of the area to determine characteristics of typical habitat for this species. The time of collection was also recorded. Temperature, tide and moon phase data were acquired from NOAA Tides and Currents Databases (Station ID 1612340).

FEEDING BEHAVIOR

Octopuses were observed on the rocks during collection to discover what they were eating. Notes on the species being eaten and location of octopus were made. Feeding behavior in captivity was also observed and recorded.

EGG BROODING, MORPHOLOGY, AND DEVELOPMENT

Seventeen females laid eggs while in captivity at HIMB. Females and their eggs were monitored over the course of development. Eggs were collected from five females at varying times of development to study the embryonic stages before hatching. Temperature at time of spawning, through the brooding period, and at hatching was recorded.

PARALARVAE

Hatchlings of the first three females to lay eggs were fixed in 95% ethanol. Some hatchlings were collected while alive, whereas others were collected between one and five hours after death. After fixation in ethanol hatchlings were weighed on an analytical balance.

RESULTS

SPECIES VALIDATION

Morphology

Octopus oliveri was originally described by Berry in 1914 from the Kermadec Islands. Since that description, more morphological characteristics were explained by Sasaki's monograph of cephalopods from Japan in 1929 and Okutani et al. in 1987. In 1999, O'Shea elaborated on the original description by Berry, incorporating all previous descriptions (excluding Okutani et al.,

1987) and adding more materials from the Kermadec Islands. Most recently (2010), in a comprehensive report on the morphology of Octopodidae, Garcia added to the description presented by O'Shea.

Octopus oliveri is described as a small octopus (total length under 300mm) with an ovoid mantle, squared arms of varying length (longest arms ~90% of total length, Table 2.1), and covered in many papillae. The head is short and narrower than the mantle. The funnel is long (up to 40% mantle length) and the eyes are small and located above the base of arm pairs 1 and 2. Suckers number from around 100-200 per arm. The males have a third, right, hectocotylized arm that can be either long or short, with a strongly defined spermatophoral groove (Toll and Voss 1998; O'shea 1999; Garcia 2010). For a full description, see Garcia 2010.

The octopuses collected in Hawai´i and discussed in this paper share the same characteristics as those described by O'Shea in 1999 (Table 2.1). This, along with the following genetic information leads one to the conclusion that it is likely the same species. There are some small differences but they are likely due to variability among individuals. For example, the total length of those reported by Sasaki, Takashi et al., and those in this paper exceed 300mm, while those collected by Berry (1914) and O'Shea (1999) do not. It is possible that those collected in the Kermadec islands are not a complete representation of the species if there were only juveniles. The raw data of measurements are available for ten individuals in the O'Shea description and it is likely that there are smaller or larger individuals in other populations.

Table 2.1 *Octopus oliveri* measurements. TL (total length), ML (mantle length), MWI (mantle width index=mantle width/mantle length percent), HdLI (head length index=head length/mantle length percent), HdWI (head width index=head width/mantle length percent), FuLI (funnel length index=funnel length/mantle length percent), ALI (arm length index=arm length/total length percent), WDI (web depth index=web depth/longest arm length percent).

	Berry (1914)	Sasaki (1929)		O'Shea (1999)		Ylitalo	
N	2	1	3	6	4	6	3
Sex	F	M	F	M	F	M	F
TL (mm)	175-190	290	330-360	61-233	172-262	94-306	263-308
ML (mm)	40-45	62	52-60	15.7-49.5	38-69	24-82	48-112
MWI	68.9-92.5	50	66.6-80.0	74-91.5	65.3-69.6	39.6-78.66	45.2-72.8
HdLI	----	----	----	23.4-32.0	21.7-34.1	19.2-24.7	19.7-30.6
HdWI	51.1-60.0	41.9	41.6-58.3	52.8-71.3	45.8-57.1	31.1-55.6	31.5-49.0
FuLI	35.5-42.5	----	----	28.4-35.4	30.0-40.5	31.7-37.5	20.8-39.7
ALI	60.0-75.8	70.7-75.9	63.6-81.4	50.8-91.2	72.7-86.7	40.1-81.8	51.7-83.6
WDI	14.4-22.2	10.0-10.9	7.7-15.5	12.3-33.9	11.2-30.6	7.8-24.5	8.6-22.4

---- Indicates no information available

A body pattern that is observed in live specimens is a mottled white and brown coloration that is generally seen when the animal is at rest (Figure 2.1 A). The animal will often flush white when startled and then will turn a uniform dark red-brown color, possibly an aggressive (or deimatic (Andrews, Darmaillacq, and Dennison 2013)) behavior (Figure 2.1 B & D). In the museum specimens previously described, a uniform dark purple coloration was the only pattern reported (O'shea 1999). A characteristic that is not mentioned in previous descriptions is that when alive, this species shows regular dispersal of green papillae across its body, presumably to camouflage with the algae that is abundant on intertidal rocks (Figure 2.1 C).

Of the octopus known in Hawai´i, six have been found to live in the shallow waters around the island. *Octopus cyanea,* or the day octopus, may be the most well known as it is heavily fished by local fishermen and it is highly abundant throughout open reef flats and among coral rubble. *Callistoctopus ornatus* is another large, locally fished species known regionally as the “night

tako," and can be found in shallow sand and reef flats at night. The lesser known octopuses include the small to moderate sized *Octopus hawaiiensis, Amphioctopus arenicola, Octopus oliveri* and the crescent octopus.

Octopus hawaiiensis was originally described by Berry in 1914 (previously *Polypus hawaiiensis*) and it is characterized by a smooth globulose body with no cirri or tubercles. The type was thought to be lost but was recently rediscovered in the Paris museum of natural history and the genus validated (Norman 2005). *Octopus oliveri* has many papillae over both its body and arms, indicating that it would not likely be confused with *Octopus hawaiiensis.*

Amphioctopus arenicola was only described in 2005, and this a small octopus (approximately 250 grams) lives on sandy substrates. One of the distinguishing characteristics of this genus is the lack of stylets (Huffard 2005). As *Octopus oliveri* has commonly been found in the rocky intertidal zone and has stylets, it is unlikely that it would be mistaken for this species.

As far as is known, there is no scientific name ascribed to the crescent octopus, a small octopus that is found on open reef flat and tide pools. A brief description was given in the dissertation of Becky A. Houck in 1977 that characterized this species as small (all under 90g in wet weight) with two crescent-like patches on the dorsal surface of the mantle. Other identifying characteristics include scalloped suckers, numerous tubercules, and a white stripe between eyes to mantle tip when agitated (Houck 1977). *Octopus oliveri* has been found exclusively in exposed rocky intertidal areas in Hawai´i, does not have scalloped suckers or crescent pigmentation, and can be considerably larger than 90g (up to 280g wet weight).

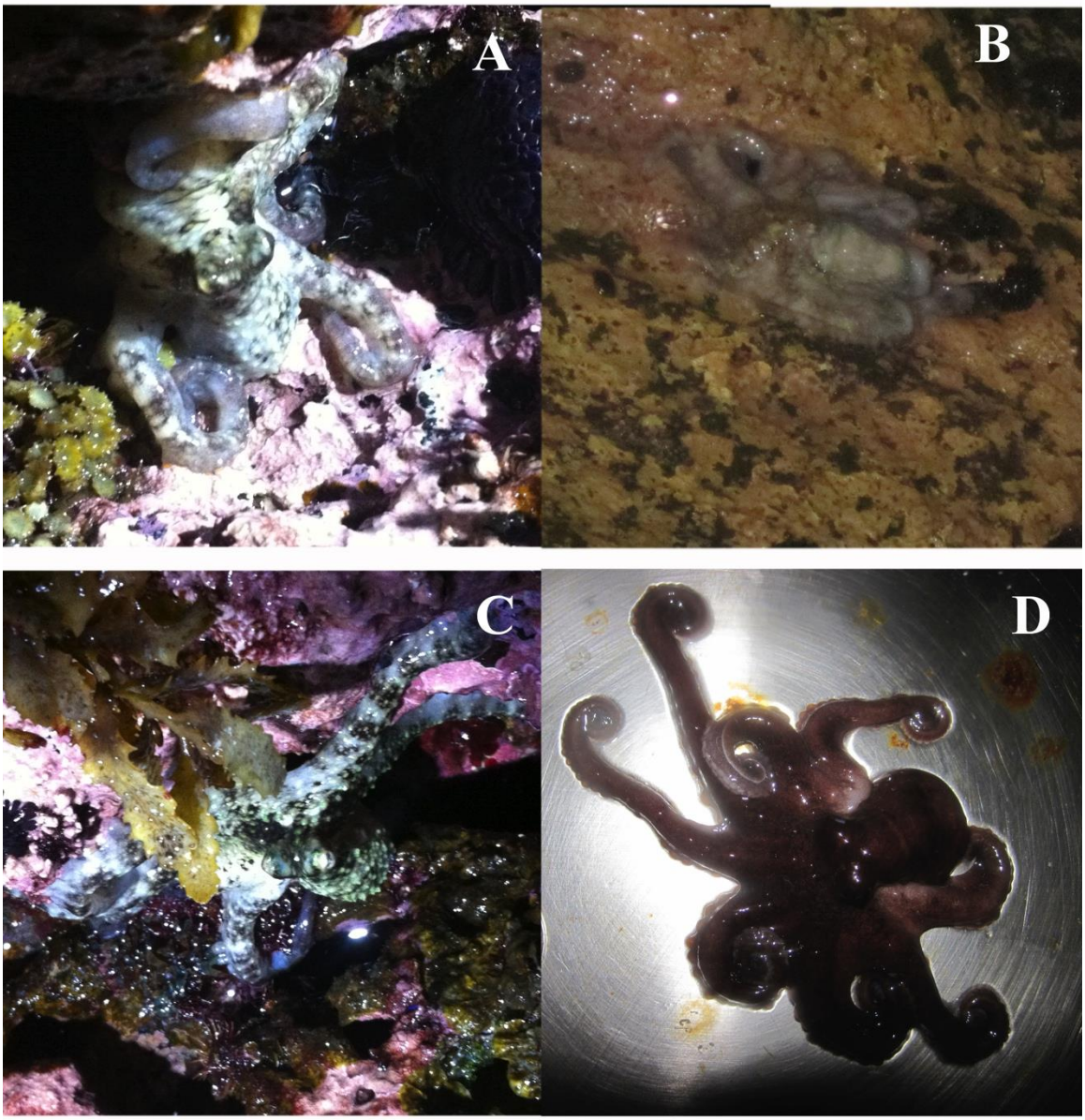

Figure 2.1 Body patterns in *Octopus oliveri*. (A) Mottled brown and white "resting" state. (B) White flushing behavior after being spotted on rocks. (C) Raised green papillae dispersed over brown mottled pattern. (D). Dark red-brown uniform coloration.

All of these octopuses differ considerably from that described here as *Octopus oliveri*. Therefore, it is likely a separate species from those previously described from the Hawaiian Islands.

Genetics

Of the three mitochondrial cytochrome c oxidase subunit I (COI) sequenced, two were identical and the other differed by only one base pair (accession codes KC848885 and KC848886). The two sequences differed at most by 3-7 bp from the specimen collected at the type locality in the Kermadec Islands (Raoul Island, Fishing Rock, AM C.477708) (N. Wilson, Australian Museum, personal comm.), thus strongly supporting the identification as *Octopus oliveri* (Figure 2.2). In addition, the sequences were almost identical (99% similarity) to two sequences from purported *O. oliveri* specimens recorded in Genbank with accession numbers AB430532.1 collected from Japan (vouchered at Tsunemi Kubodera National Museum of Nature and Science, NSMT: Mo.75913) and GQ900744.1 from Hawai´i (Kaneko, Kubodera, and Iguchis 2011; Huffard and Godfrey-Smith 2010). In contrast, the multitude of *Abdopus* spp. and *Octopus* spp. in the NCBI database are at best 88% similar to our sequences and together our sequences and those putative *O. oliveri* in Genbank are each 12% or more divergent from any other species in the database.

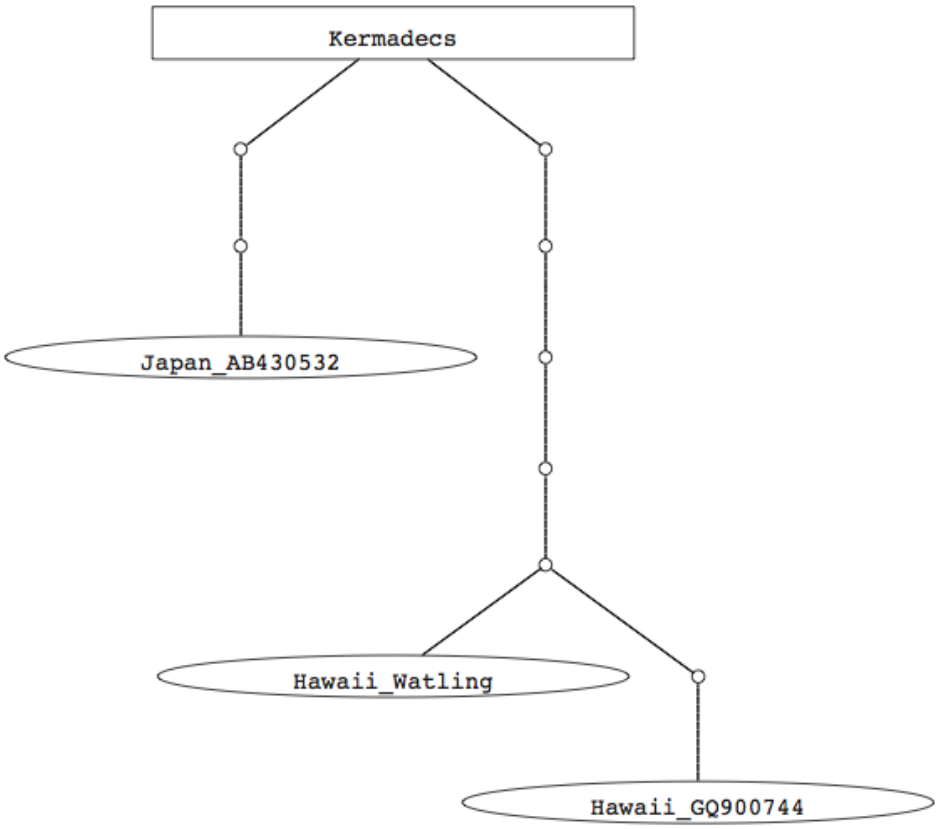

Figure 2.2 Haplotype network of *Octopus oliveri* for COI marker provided by Nerida Wilson, Australian Museum. Hawaii_Watling is the *Octopus oliveri* specimen collected for this study.

HABITAT

In the original description of *Octopus oliveri*, it was stated that the individuals were collected from among the intertidal rocks in the Kermadec Islands (Berry 1914). In Sasaki's description, they are said to be common in the littoral habitat living in crevices in the reefs (Sasaki 1929). O'Shea also states that this species is intertidal, however he includes their range to be several meters depth. In this study, specimens were found exposed in the rocky intertidal region down to approximately 20cm depth in the subtidal zone.

Octopus oliveri were seen at Kaka´ako Waterfront Park, Kewalo Basin Marina and Makapu´u Beach on Oahu. They have also been found in Kahului, Maui, Hilo, Hawai´i, and the Northwest Hawaiian Islands (Chris Bird, personal comm.) (Figure 2.3). In all instances, they were found in rocky intertidal regions exposed to high wave action. Hawaiian wave dominated rocky intertidal shores are characterized by environmental stressors that include temperature fluctuations, air exposure and extreme hydrodynamic force from waves (Bird et al. 2013).

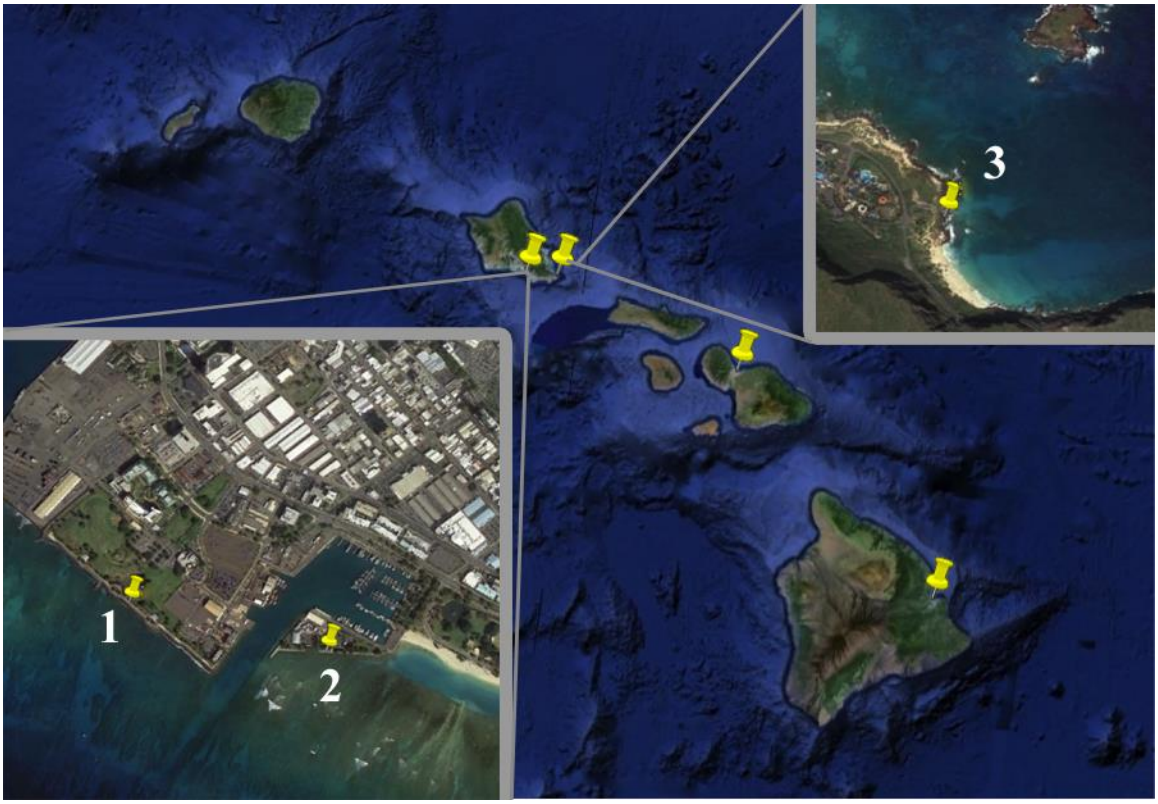

Figure 2.3 Distribution around the Main Hawaiian Islands and collection sites around Oahu. (1) Kaka´ako waterfront park, (2) Kewalo Basin Marina, (3) Makapu´u Beach.

Specimens were found exclusively during night hours, at least one hour after sunset. Multiple locations were surveyed during daylight hours as well to determine whether they could be found amongst the rocks. There was no correlation between when the octopuses were found and the water temperature ($R^2 = 0.04$), tide level ($R^2 = 0.03$), or moon phase ($R^2 = 0.02$).

The two main collection sites were Kaka´ako Waterfront Park and Kewalo Basin Marina. These two sites have rocky intertidal zones characterized by basalt boulder break walls covered in crustose coralline algae (Figure 2.4 A). The biota includes herbivorous limpets, urchins and snails (*Colobocentrotus atratus, Cellana exarata, Cellana sandwicensis, Cellana talcosa, Littoraria pintado, Echinometra mathaei, Echinometra oblonga,* and *Cypraea mauritiana*), omnivorous scavengers (*Grapsus tenuicrustatus, Bathygobius* spp.) and predatory snails, crabs and anemones (*Purpura aperta, Drupa ricina, Drupa morum, Morula uva, Morula granulata, Thais intermedia, Neothais harpa, Plagusia depressa tuberculata,* and *Cladactella manni*). The octopuses were found exposed on the tops of the rocks or in small holes (generally formed by *Echinometra mathaei)* within the wave action zone.

The Makapu´u Beach intertidal region has a higher diversity and density of algae growth (Figure 2.4 B). The algae that is found in this region includes *Acanthophora spicifera, Laurencia* sp., *Padina australis, Sargassum echinocarpum, Turbinaria ornata, Ulva fasciata, Ulva reticulata, Asparagopsis taxiformis, Coelothris irregularis, Amansia glomerata, Colpomenia sinuosa, Codium edule,* and crustose coralline algae. Other than abundance of algae, the biota do not differ much from that listed above. The octopuses are still found among holes dug by rock boring urchins and out on the tops of rocks.

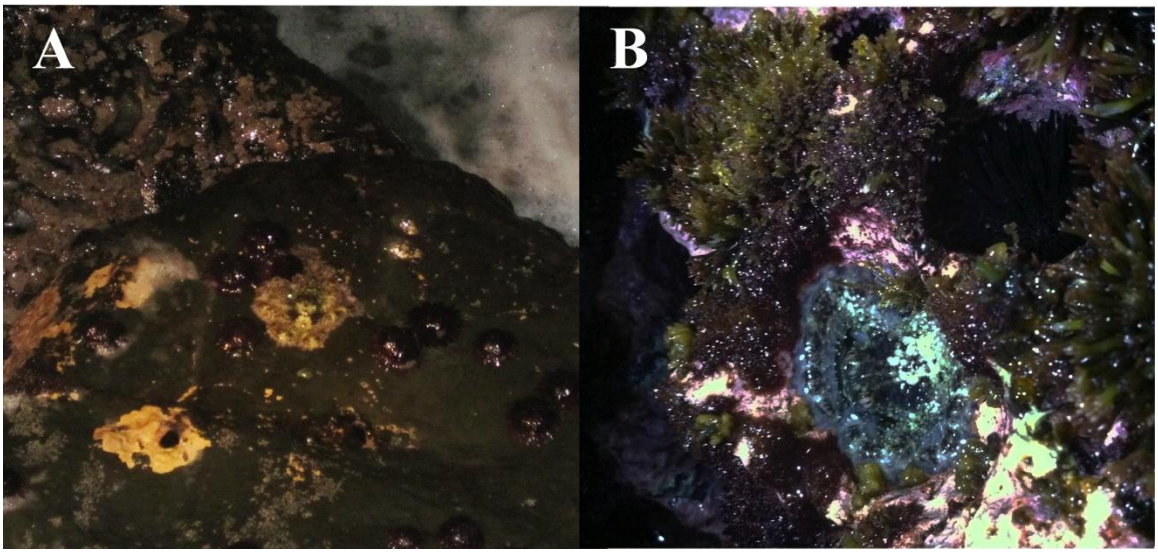

Figure 2.4 Habitat of *Octopus oliveri*. (A) Wave-dominated smooth rocky intertidal characterized by crustose coralline algae and *Colobocentrotus atratus* (B) Algae dominated rocky intertidal, Makapu´u Beach

FEEDING BEHAVIOR

During the collection of the octopuses, they were observed eating two species of grapsid crabs: *Plagusia depressa tuberculata*, the brown crab and *Grapsus tenuicrustatus,* the a´ama crab. In many cases, the crab was as large, if not larger than the octopus it was being consumed by. In all cases, the octopus was found while it was in the process of eating the crab and therefore was not seen in the process of hunting the crab. When in captivity, occasionally the octopuses were given brown crabs to eat. In these instances, the octopus would quickly reach out three or four arms then lunge at the crab with its entire body, landing on top of the crab and pulling it inwards towards the beak.

EGG BROODING, MORPHOLOGY, AND DEVELOPMENT

Females would stop feeding generally one to two days before laying eggs and would not eat after eggs were laid, as is typical in octopus brooding behavior (Rocha and Guerra 2001; Guerra,

Allcock, and Pereira 2010). The smallest weight of a female to lay eggs was 68 grams with the largest being 278 grams (Table 2.2). Following brooding, females had lost up to 50% of their body weight from the start of laying eggs, with a weight loss of roughly 35% being the average (σ =14%).

Eggs were laid over the course of two to three days. The female would attach strands of eggs to a nearby hard substrate (generally overhead) and then protect the eggs by wrapping her arms around and blowing water on them (Figure 2.5). There was a very slight correlation between mean water temperature and brooding time ($R^2 = 0.27$, $p = 0.02$) (average temperature 25.45°C ± 0.56) (Table 2.2, Figure 2.6). Eggs were laid between the months of October and April.

Figure 2.5 Female *Octopus oliveri* with brood. Eggs attached overhead and female wrapped around strands.

During brooding, the female would darken in color and spread her arms out towards any observer who approached within 20cm of her eggs. She would push and pull on tweezers that

were used to collect egg strands. In one instance, the female abandoned the eggs temporarily before returning a day later. Unfortunately, that female died before the eggs hatched. During the subsequent necropsy of the mother, many immature unfertilized eggs were found in the ovary.

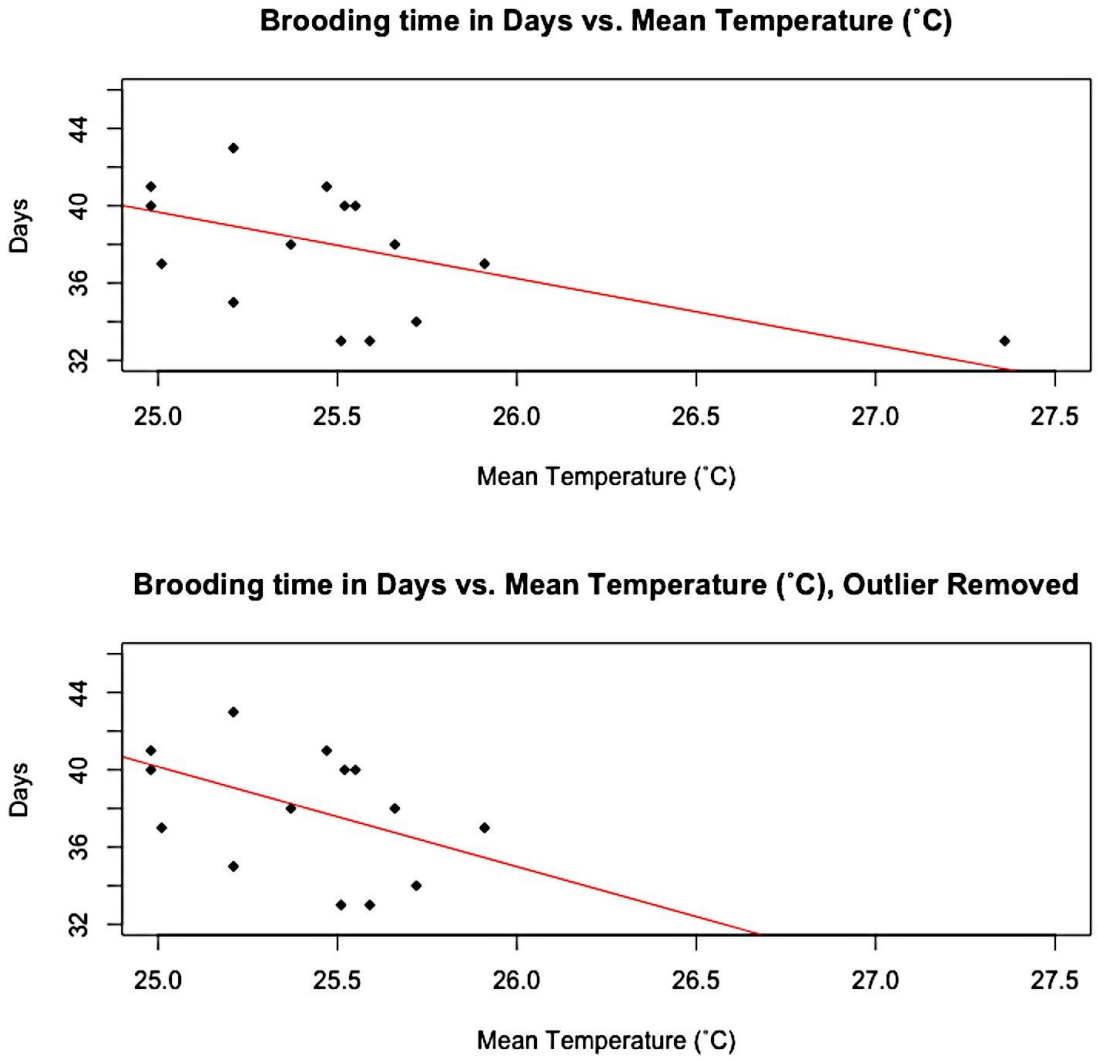

Figure 2.6 Distribution of brooding time for each female plotted against mean water temperature with ($R^2 = 0.27$, $p = 0.02$) and without an outlier ($R^2 = 0.22$, $p = 0.06$).

Eggs hatched approximately 38 days after being laid (range of 33-47 days) (Table 2.2). The female was observed blowing water forcefully on the hatchlings, possibly aiding in their release from the egg. The paralarvae swam up into the water column, suggesting a planktonic lifestyle,

which is consistent with their morphology. There was no yolk visible when they hatched. After approximately four days, all hatchlings would perish. Females remained alive after hatching for up to 28 days (Table 2.2).

Morphology of eggs and egg strands

Octopus oliveri eggs were laid in strings attached to a hard substrate, which the female covered with her mantle and arms. The females laid varying numbers of eggs and strings (average number of eggs 4200 ± 2600, average number of strings 85 ± 31)(Table 2.2). The chorion of the eggs was ovoid and elongated and the stalks were entwined in the center of the string around a substance secreted by the oviducal glands (Figure 2.7).

Egg development

A general pattern of embryonic development was observed from the four sets of eggs that were collected intermittently though the brooding process. Eyes are visible beginning between day 15 and day 17. On the 19th day of development definition between the yolk and tentacles is seen. Chromatophores are visible starting from day 23 and the embryo rotates around the 27th day of development. In some broods the embryo did not turn until day 30. Full development took between 33 to 47 days (Figure 2.7).

Table 2.2 Measurements of female *Octopus oliveri*: egg broods, dates and weights. (Continued on next page).

Octopus	Weight of female before laying eggs (g)	Date eggs Laid	Number of strands measured	Number of strands total	Average length of strands (mm)	Date eggs began hatching	End of hatching period
1	278	11/2/10	31	-	16.98 ± 3.59	12/10/10	12/14/10
2	172	11/10/10	-	-	-	12/15/10	12/16/10
3	140	12/4/10	-	-	-	1/13/11	1/20/11
4	114	3/15/12	29	75	14.87 ± 4.53	5/3/12	5/9/12
5	148	4/15/12	25	42	14.67 ± 2.08	5/23/12	5/25/12
6	92	4/18/12	27	77	19.27 ± 3.02	5/21/12	5/25/12
7	150	3/22/12	10	77	23.66 ± 0.58	5/3/12	5/11/12
8	180	3/26/12	27	75	27.17 ± 6.51	5/3/12	5/11/12
9	118	4/15/12	-	-	-	5/18/12	5/21/12
10	174	4/11/12	10	-	32.6 ± 0.58	5/21/12	5/25/12
11	146	4/21/12	37	96	17.79 ± 4.39	5/25/12	5/30/12
12	86	10/7/12	11	117	14.90± 3.21	11/9/12	11/14/12
13	238	11/5/12	9	122	30.33± 4.24	12/16/12	12/18/12
14	70	11/22/12	10	78	16.8 ± 0.92	12/30/12	1/2/13
15	102	3/8/13	10	134	22.30 ± 3.02	4/20/13	4/23/13
16	68	3/14/13	10	27	19.80 ± 4.24	4/24/13	4/27/13
17	112	3/21/13	12	94	17.42 ± 4.32	4/28/13	5/2/13

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Table 2.2 (Continued) Measurements of female *Octopus oliveri*: egg broods, dates and weights.

Octopus	Brooding time in days	Average water temperature during brooding (°C)	Average number of eggs per strand	Approximate number of eggs	Average egg width (immediately before hatching) (mm)	Average egg length (immediately before hatching) (mm)	Date of Death
1	38	25.37 ± 0.98	55 ± 9	4000	1.51 ± 0.08	3.41 ± 0.19	1/6/11
2	35	25.21 ± 0.99	-	400	-	-	11/27/10
3	40	24.98 ± 1.29	-	2000	-	-	2/9/2011
4	47	24.88 ± 0.93	47 ± 20	3500	1.50 ± 0.11	3.89 ± 0.26	5/8/12
5	38	25.65 ± 1.43	55 ± 11	2500	1.66 ± 0.06	3.89 ± 0.25	6/20/12
6	33	25.58 ± 1.40	53 ± 11	4000	1.46 ± 0.16	3.30 ± 0.12	6/18/12
7	41	24.97 ± 0.86	48 ± 7	3500	1.55 ± 0.08	3.63 ± 0.25	-
8	37	25.01 ± 0.85	128 ± 31	10,000	1.61 ± 0.01	3.56 ± 0.07	-
9	33	25.51 ± 1.30	-	-	-	-	5/13/12
10	40	25.55 ± 1.37	65 ± 8	-	-	-	-
11	34	25.71 ± 1.47	70 ± 19	7000	1.62 ± 0.09	3.67 ± 0.05	6/20/12
12	33	27.36 ± 0.93	44 ± 6	5200	1.70 ± 0.06	3.55 ± 0.37	11/16/12
13	41	25.47 ± 1.33	67 ± 8	8200	1.33 ± 0.13	3.13 ± 0.12	1/13/13
14	38	24.75 ± 1.22	38 ± 7	3000	1.34 ± 0.02	3.10 ± 0.29	-
15	43	25.20 ± 1.51	45 ± 6	6000	1.35 ± 0.06	3.02 ± 0.35	5/6/13
16	40	25.51 ± 1.54	35 ± 7	1000	1.52 ± 0.18	3.07 ± 0.21	5/4/13
17	37	25.91 ± 1.59	36 ± 10	3500	2.08 ± 0.05	4.08 ± 0.22	-

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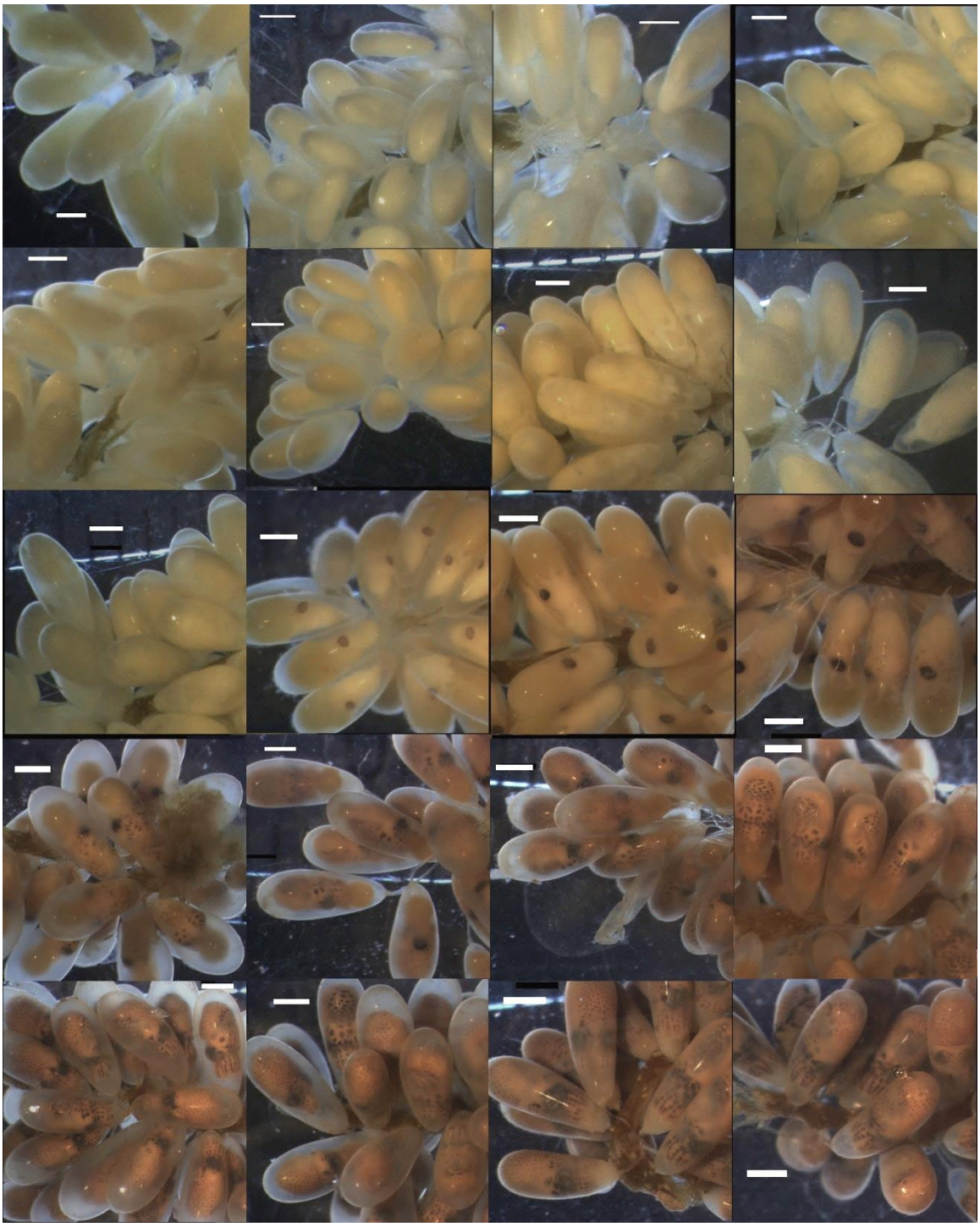

Figure 2.7 Egg development of *Octopus oliveri*. White bars represent 1mm measure bar. Top row from left to right: day 1, day 3, day 5, day 7. Second row from left to right: day 9, day 11, day 13, day 15. Third row from left to right: day 17, day 19, day 21, day 23. Fourth row from left to right: day 25, day 27, day 29, day 31. Fifth row from left to right: day 33, day 35, day 37, day 38.

PARALARVAE

Morphology of hatchlings

Hatchlings were collected and preserved while alive and after death to illustrate the difference in chromatophore patterns while relaxed vs. contracted. The morphological characteristics of the two groups of hatchlings were similar, however, there were some measurements and patterns that differed (Table 2.3, 2.4). Hatchlings are described using terminology suggested by Young et al. (1989) and Hochberg et al. (1992) when possible.

Table 2.3 Morphological measurements of *Octopus oliveri* paralarvae fixed while alive and fixed after death. Weights were recorded after preservation in ethanol.

	Fixed Alive (*n*=25)		Fixed after Death (*n*=14)	
	Mean ± SD	Range	Mean ± SD	Range
total length (mm)	2.43 ± 0.17	2.11-2.67	3.69 ± 0.29	3.28-4.31
mantle length (mm)	1.02 ± 0.11	0.88-1.20	1.79 ± 0.17	1.45-1.98
mantle width (mm)	1.69 ± 0.09	1.54-1.84	1.66 ± 0.18	1.43-1.94
arms length (mm)	0.82 ± 0.11	0.56-1.04	0.71 ± 0.09	0.57-0.87
funnel length (mm)	0.45 ± 0.08	0.33-0.64	0.64 ± 0.09	0.51-0.78
eye diameter (mm)	0.58 ± 0.06	0.41-0.69	0.58 ± 0.06	0.47-0.69
suckers per arm	3.96 ± 0.35	3-5	4.14 ± 0.36	4-5
buds	2.96 ± 0.68	2-4	2.64 ± 0.63	2-4
weight (mg)	3.03 ± 0.50	2-3.9	3.53 ± 0.58	2-4.3

Table 2.4 Chromatophore numbers on different areas of the body in *Octopus oliveri* hatchlings fixed while alive and fixed after death

	Fixed Alive (*n*=24)					
	Dorsal		Ventral		Aboral	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Mantle	78.96 ± 22.11	26-111	118.29 ± 19.02	91-150		
Visceral	13.58 ± 4.75	4-25				
Head	10.58 ± 3.24	4-18	7 ± 2.39	4-14		
Eyes			11.41 ±2.67	7-16		
Funnel			10.08 ± 3.39	4-17		
Arms					18.25 ± 2.88	14-22

			Fixed after Death (*n*=18)			
	Dorsal		Ventral		Aboral	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Mantle	68.11 ± 20.98	31-121	187.77 ± 17.83	163-218		
Visceral	37.05 ± 9.22	19-50				
Head	33.22 ± 6.57	21-42	14.33 ± 3.56	9-20		
Eyes			15.38 ± 2.03	12-20		
Funnel			13.94 ± 3.04	9-20		
Arms					17.66 ± 2.93	12-23

Young et al. (1989) described the paralarvae of several common octopodid species from Hawai´i. They suggested that there are two stages to paralarval development; stage one differing from stage two by the absence of sucker buds on the arms. Stage two is reached after considerable growth and development of clearly defined sucker buds (Young and Harman 1989). However, the paralarvae of *O. olivieri* examined in this study were collected immediately following hatching, before any growth could occur, and they all possessed sucker buds, indicating that this species differs developmentally from other Hawaiian octopus paralarvae (Table 2.3, Figure 2.8 A). This may indicate a shorter planktonic lifestyle as they have already reached stage two of development upon hatching.

Whether fixed alive or after death, the arms of the hatchlings were subequal and shorter than the globose mantle. The four main suckers on the arms were arranged linearly, with medium-sized suckers around the mouth, two larger suckers outside of those, and a few small suckers out toward the tip of the arms (Figure 2.8 A). In those octopuses fixed while alive, there were small suckers at the tips of the arms that were small buds, while those fixed after death had suckers that appeared to be "opened."

The arms constituted 34% of the total body length in the hatchlings fixed while alive, though in the hatchlings fixed after death the arms constituted 19% of the total body length (Table 2.3) suggesting considerable muscle contraction at time of death. The mantle length and funnel length in both hatchlings fixed while alive and after death were approximately 45% and 18% of the total body length, respectively. In addition, the eye diameters for both groups of hatchlings were identical (Table 2.3).

Chromatophore arrangement

In both groups of hatchlings, there are characteristics of the chromatophore patterns that are similar (Table 2.4). On the dorsal mantle, tegumental chromatophores are present on the anterior section in a simple band with a varying number of chromatophores (six to eight). In the midregion of the dorsal mantle, there are a few round chromatophores generally making a "Y" shape, leaving three segments of the dorsal mantle without any tegumental chromatophores. The posterior region of the dorsal mantle is characterized by three or four marginal bands of round chromatophores (Figure 2.8 B, D). The supravisceral chromatophores on the dorsal mantle are greatly expanded in the hatchlings preserved while alive, resulting in fewer being counted than for those preserved after death (Table 2.4). The chromatophores are arranged in a complex band down the center of the animal over the viscera (Figure 2.8 B, D).

The dorsal head was obscured in the hatchlings fixed while alive. The chromatophores are very large and the patterning is difficult to discern. However, it appears that there are two rows (four-four pattern) of extrategumental chromatophores (Figure 2.8 B). When looking at the hatchlings fixed after death, the extrategumental chromatophores are expressed in a more complicated four-four-two-three pattern in addition to tegumental chromatophores on the head (Figure 2.8 D).

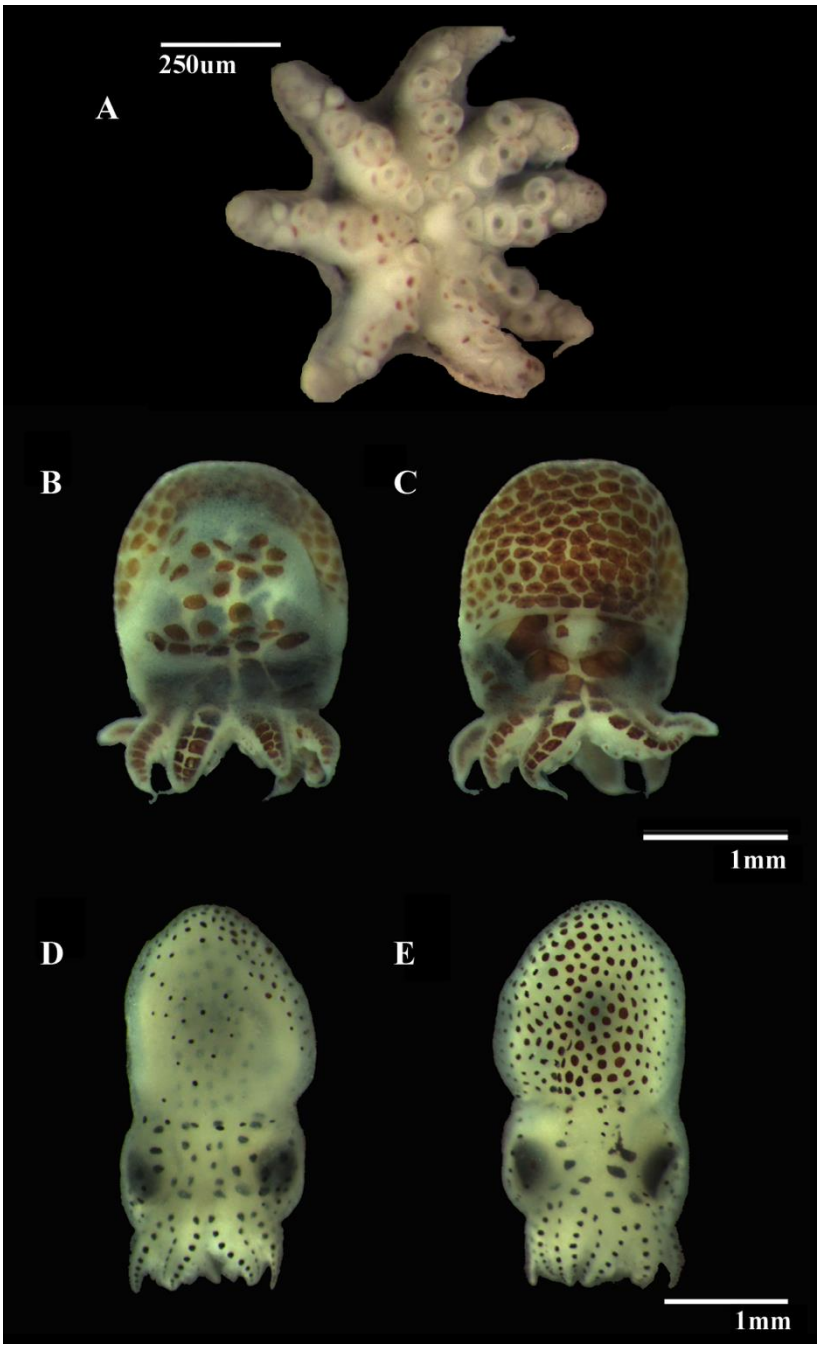

Figure 2.8 (A)*Octopus oliveri* hatchling mouth and arms showing sucker and bud arrangement. Scale bar: 250 μm. Dorsal (B) and ventral (C) view of *Octopus oliveri* preserved in 95% ETOH while alive, one day old. Scale bar: 1 mm. Dorsal (D) and ventral (E) view of *Octopus oliveri* preserved in 95% ETOH after death, one day old. Scale bar: 1mm.

The arms have rectangular tegumental chromatophores in a double line (eight or nine in each row) decreasing in size towards the tip (Figure 2.8, Table 2.4). Chromatophores are also present both on and between individual suckers on the arms (Figure 2.8).

The ventral mantle is characterized by densely, uniformly distributed tegumental chromatophores (Figure 2.8 C, E). They are round or hexagonal in shape. On the ventral head, there are four large tegumental chromatophores surrounding the funnel. Additionally, there are small chromatophores surrounding the eyes of the hatchlings. The funnel has a row of a varying number of chromatophores lining each side (Figure 2.8 C, E).

DISCUSSION

Both morphological data and analysis of the COI gene strongly indicates that this species is *Octopus oliveri*, originally described from the Kermadec Islands. Two additional observations of *O. oliveri* have been recorded in Japan, suggesting a wide distribution of this species across the Pacific (Kaneko, Kubodera, and Iguchis 2011) (Figure 2.9). Establishing the morphological characteristics of the paralarvae in addition to genetic tools will enable further research into the population connectivity and distribution of *O. oliveri*.

The females all laid eggs between the months of October and April, so it is possible that seasonality exists in this species (Table 2.2). However, since no eggs were found in the field, it cannot be certain.

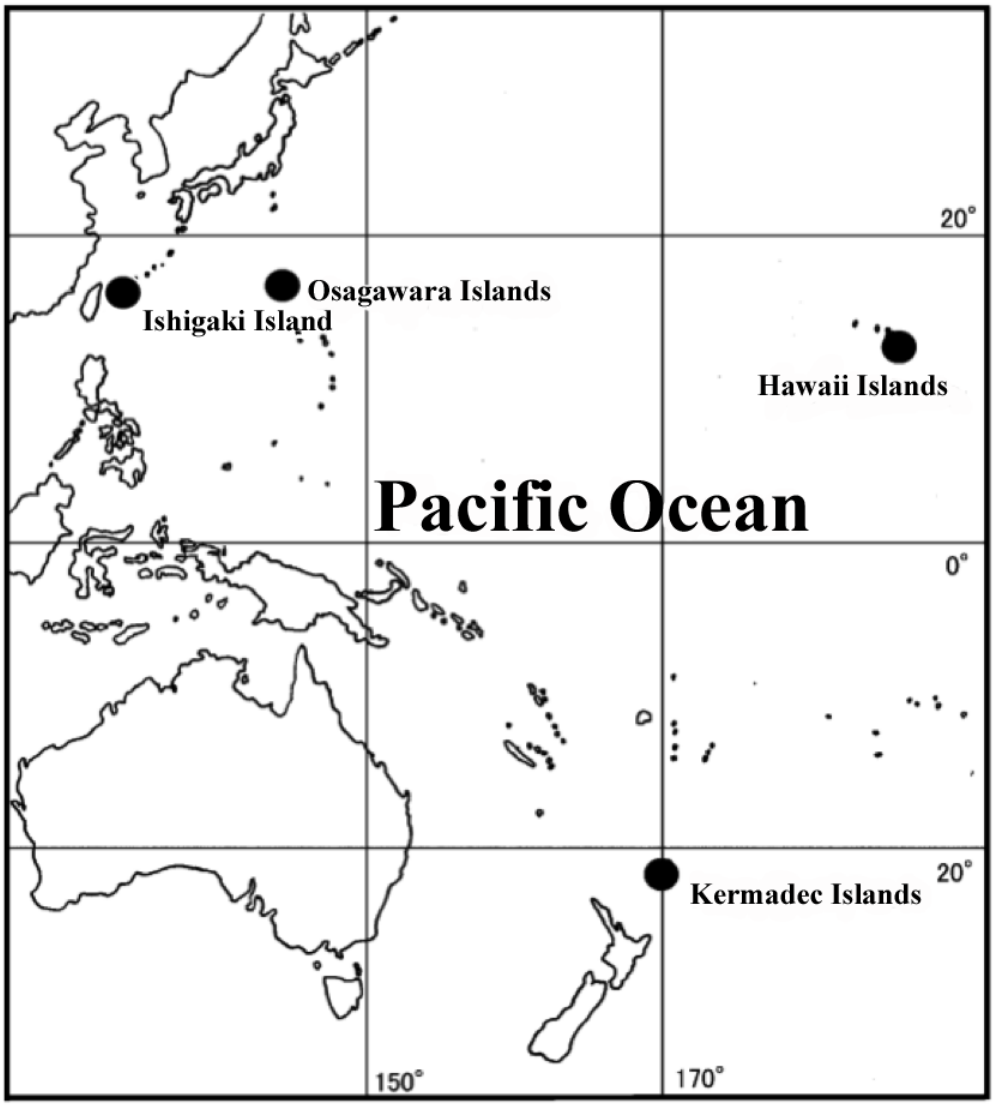

Figure 2.9 Distribution of *Octopus oliveri.* Kermadec Islands, Berry, 1914 and O'Shea, 1999. Ishigaki Island, Takashi *et al.*, 1987. Osagawara Islands, Sasaki, 1929. Hawai´i Islands, Kaneko and Kubodera, 2007 and current paper.

The paralarvae of this species are distinctive from other Hawaiian octopus hatchlings currently described (Hochberg et al. 1992; Young et al. 1989). *Octopus oliveri* hatchlings most closely resemble paralarvae type B collected by Young et al. in 1989, however they can be distinguished by the higher number of tegumental chromatophores on the midregion of the dorsal mantle. In addition, the posterior region of type B is characterized as almost bare, while in these *O. oliveri* specimens there are numerous large and small chromatophores.

The hatchlings of *Octopus oliveri* can be identified by their founder chromatophores and characteristic measurements. However, there were slight variations in these characteristics depending on the time of preservation. The ratio of arm to body length is a common measurement used to identify species of paralarvae. In *O. oliveri*, these measurements differed greatly depending on whether the paralarvae were fixed while alive or after death. In addition, in *O. oliveri* hatchlings fixed after death, the chromatophores are appreciably smaller than those in the hatchlings fixed while alive. The chromatophores in hatchlings fixed after death are relaxed and therefore reduced, allowing for a greater number of supravisceral and extrategumental chromatophores to be visible. These variations in chromatophore arrangements within single brood populations have been noted in other species (Hochberg et al. 1992; Huffard and Gentry 2009). Consequently, the variations discussed should be considered when identifying octopus species in early stages of development. In addition to these morphological characteristics, genetic testing is proving extremely useful in identifying species. The combination of both methods would likely be the best methodology in determining species identification.

CHAPTER 3. MATING BEHAVIOR OF *OCTOPUS OLIVERI* (BERRY, 1914) (CEPHALOPODA: OCTOPODIDAE): THE EFFECT OF MALE ORDER, SIZE, AND FEMALE CHOICE IN OBSERVED MATING SUCCESS

ABSTRACT

Mating behavior among octopuses can be complex and varied, although only a small number of species have been studied in detail. It is believed that sperm competition occurs in octopuses, as there are two oviducts, lengthy sperm storage, and multiple mating partners. However, no clear mechanism for sperm competition has yet been discovered among octopuses. In addition, the role of female choice has rarely been studied in octopus mating behavior. *Octopus oliveri* is widespread in the rocky intertidal zone of Oahu, Hawai´i and mates readily in laboratory conditions making it an ideal candidate to study octopus reproductive behavior. Four sets of behavioral experiments were recorded wherein six females were mated with three males in varying order, for a total of 24 females and 12 males. Video analysis of mating behavior shows the duration of mating, fighting, and resting for 62 successful experimental trials. Mating behavior for this species was recorded for the first time. Mating was observed for all males regardless of size or order. Females were seen to remove intact sperm packets of some males, however it is probable that the males simply failed to direct the spermatophore directly into the oviducal gland. Multiple observations of females initiating mating occurred, suggesting female choice.

INTRODUCTION

Reproductive success is dependent on a broad range of physical, behavioral and genetic characteristics generally as a result of competition for mates, or sexual selection. Individuals that are competitively superior will often gain access to more mates and increase their chances of passing on gametes. The traits that determine whether an individual is successful vary among taxa, but can include both pre- and postcopulatory mechanisms of sexual selection (Birkhead and Møller 1998).

Among marine organisms, cephalopods represent some of the more interesting species in which to study reproductive behavior. Squid and cuttlefish often have complex mating displays in large conspicuous aggregations with highly skewed operational sex ratios, lending themselves to interesting sexual selection studies (Hanlon and Messenger 1998). While studies on sexual selection in squid and cuttlefish appear in the literature with some frequency, those on octopus remain few and far between. The reason for this may be that octopuses are typically solitary animals that seem to mate opportunistically, which would suggest low rates of sexual selection. However, as the mating behaviors of more species of octopus are being described, systems appear to be more complex than previously thought (Huffard, Caldwell, and Boneka 2010; Huffard and Godfrey-Smith 2010; Mohanty, Ojanguren, and Fuiman 2014).

Within the class Cephalopoda, sperm competition has been observed in a variety of squid and cuttlefish species in the form of mate guarding, sneaker males, sperm flushing and increased

sperm allocation (Wada et al. 2010; Wada et al. 2005; Iwata and Munehara 2005; Naud et al. 2004; King, Adamo, and Hanlon 2003; Jantzen and Havenhand 2003; Emery, Wilson, and Craig 2001; Hanlon and Ament 1999; Hanlon, Smale, and Sauer 2002).

In octopuses, sperm competition is generally believed to occur due to the presence of multiple mating, two oviducts with which to store sperm, and long-term sperm storage capabilities (Hanlon and Messenger 1998; Birkhead and Møller 1998; Wigby and Chapman 2004). Yet, mate-guarding and sneaker behavior has only been described in one species of octopus (Huffard, Caldwell, and Boneka 2010), and direct sperm competition in the form of sperm precedence has only been inferred in one unnamed pygmy octopus species and even then, the mechanism behind the sperm competition remains unknown (Cigliano 1995).

Male sperm precedence is the nonrandom utilization of one males sperm over another (Birkhead and Møller 1998). This can occur through female cryptic choice within the oviduct of the female, overt female rejection of sperm packets, or through male displacement of previously placed sperm packets by rival males. In nature, some animals such as spiders and salamanders show first male sperm precedence (Tennessen and Zamudio 2003), where the first males to inseminate a female are most successful in fertilizing the female gametes. Others exhibit a "last in, first out" strategy, as seen in damselflies removing the sperm packets of previous males (Birkhead and Møller 1998). In those cases, the last male to mate with the female is the first male to fertilize the eggs. No clear pattern of sperm precedence in cephalopods has been discovered to date, but it has been predicted in several species (Iwata and Munehara 2005; Shaw and Sauer 2004; Buresch et al. 2009; Naud et al. 2004; Cigliano 1995).

In 1995, Cigliano performed behavioral experiments with an unnamed pygmy octopus and found that the time between first inserting the hectocotylus into the female mantle and the first arch and pump between succeeding males increased, suggesting that the second male was somehow removing sperm from a previous male. In cuttlefish, males can be seen shooting jets of water and flushing sperm from the female buccal mass (Wada et al. 2010; Hanlon and Ament 1999), however because octopus sperm is placed internally in the distal oviduct, flushing with water is unlikely to be the mechanism of sperm removal. It is predicted that the third arm of the octopus (the hectocotylus) can be used to remove the sperm of previous males (Cigliano 1995).

The tip of the hectocotylus is characterized by a ligula and calamus (Figure 3.1). The male passes a spermatophore down the groove of the hectocotylized arm to either of the two distal oviducts of the female. Once the spermatophore reaches the female, the spermatophoric reaction begins wherein the spermatophore enlarges with diffusion of saltwater across the membrane, ultimately bursting and releasing the individual spermatozoa into the oviduct. The sperm is then stored within the spermathecae in the oviducal gland, along with the sperm from previously mated males (Wodinsky 2008; Mann 1984).

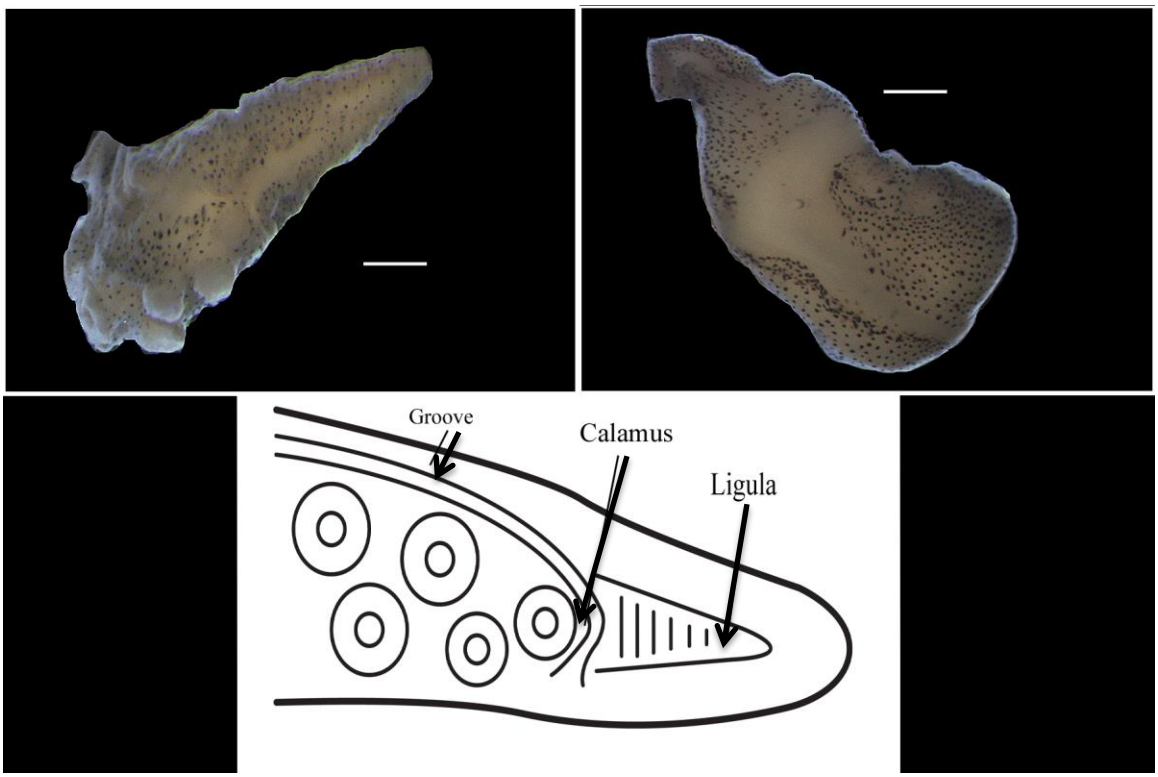

Figure 3.1 The structure of the tip of the hectocotylized octopus arm. The spermatophore is passed down the groove on the top right hand ridge of the diagram and passed to the calamus and ligula to the oviducal gland of the female octopus. The top two pictures are examples of *Octopus oliveri* hectocotyli, the white bar represents 1mm.

One of the goals of this study was to determine whether sperm precedence in the manner Cigliano described could be found in another small octopus species. *Octopus oliveri* is a small, tropical, intertidal octopus that mates readily in lab conditions, making it an ideal candidate for studies in cephalopod reproduction. Initial observations of this species in a large communal tank indicated that females of this species do mate with multiple males, however, not simultaneously.

Another goal of this study was to observe whether any conspicuous male trait was suggestive of observed mating success. Large body size can be a predictor in determining mating success as larger males will generally win in precopulatory competitive contests, not only in octopuses, but across many taxa (Birkhead and Møller 1998; Huffard, Caldwell, and Boneka 2010; Andersson

and Iwasa 1996). In addition, larger body size may be an indicator to females of genetic superiority in survivability and trigger female choice. By conducting mating experiments that remove the potential for precopulatory male-male competition, one can observe whether size is a predictor of mating success.

This study serves to describe the mating behavior of a minimally studied intertidal octopus and observe on a small scale whether there were any apparent behavioral indications of sperm competition or female mate choice. Do females differentially reject copulation attempts based on male size or mate order? Or are all mating attempts successful? Can sperm precedence in the manner Cigliano described be seen in this species? If all mating attempts are successful, it would indicate that females are not choosy, that they are mating with any male presented to them, possibly to ensure high levels of genetic diversity in broods. It is also possible that the mating events are risky, and refusing a partner could endanger the female and rather than denying male access, she would mate with whoever is presented to her.

MATERIALS AND METHODS

COLLECTION

Octopus oliveri individuals were collected from Kaka´ako Waterfront Park, and Kewalo Basin Marina, Honolulu, Hawai´i in the fall of 2010 through the summer of 2013 (over 100 individuals collected, 70 different excursions). Two to three people would walk along the rock wall during the evening hours for one to three hours (between 7pm-12am) with a flashlight. When an octopus was found, it was collected by hand and transferred to a five-gallon bucket. The males

and females were kept in separate buckets. Adult octopuses were weighed on a platform scale (wet weight) and transferred to tanks on Coconut Island, Kāne´ohe. Each octopus was housed in an individual tank (38cm x 21cm x 23.5cm) with a piece of coral or PVC pipe for shelter and a plastic well-ventilated lid. These tanks were then placed in a large outdoor tank at the Hawai´i Institute of Marine Biology (HIMB) with constant saltwater flow and ambient ocean temperature. The octopuses were fed frozen shrimp and live crabs on a regular schedule and the tanks were cleaned after each feeding. Water temperature records were obtained though NOAA Tides and Currents databases from the station located closest to the collection site in Honolulu (Station ID 1612340) and at Coconut Island (Station ID 1612481).

EXPERIMENTS

The mating experiments were designed so that multiple sets of trials could be run, thereby increasing the power of analysis. Six females and three males were chosen randomly from the available pool of collected octopuses. Each female was paired with each of the three males (one male at a time) for a total of 18 trials per set of experiments. The males were chosen with maximum variation in size, one being the largest, one smallest, and one midsize. Each of the six females had a different order of mates (i.e., female 1 with male A, B, C, female 2 with male B, C, A etc.) allowing for every possible combination of pairing.

All mating trials occurred at night, as this species is nocturnal (Ylitalo, Watling, and Toonen 2014). Three 15-gallon (61 cm x 32 cm x 32 cm) tanks were set up with constant seawater flow and separated by black plastic to ensure that adjacent pairs did not influence the other octopuses. Sessions were recorded using a 6 LED USB PC Web Camera with the infrared filter removed. A

camera was mounted 100 centimeters above each tank (measured from the floor of the tank). A 48-LED Illuminator Infrared Light was placed in front of each tank to illuminate the video without disturbing the octopuses.

The female was always placed in the tank first and allowed to settle for approximately 10 minutes. Then, the male was introduced and the trial would begin. Three pairs were filmed simultaneously, each pair with its own camera, during each experimental night. Trials would last at least two hours and would end when the mating pair separated anytime after. Also, if a female tried to escape from the tank three times, the trial was ended as it was predicted the female would have escaped the male in the field. In some cases, this would mean the trial would last less than two hours. Videos were analyzed after all trials were completed.

Spermatogenesis after mating has been explored in several cephalopods, often with sperm production occurring immediately following copulation (Hanlon and Ament 1999; Van Heukelem 1976). However, the rates of sperm production vary across individuals. Given this knowledge, the males were allowed to rest one day between sessions to allow for sperm regeneration.

Each female had trial history recorded to analyze whether mate order or mate size influenced the observed mating success. Mating success was described as the amount of time a male spent mating with a female and the number of times he was able to complete the arch and pump movements.

Sixty-two trials were completed and over 125 hours of video were analyzed twice by the same observer (H. Ylitalo) and once by another observer (Jeffrey Yamada) to ensure continuity between evaluations of behavior. Three central behaviors were recorded: mating, fighting (agonistic behavior) and resting. A trial was considered successful when any or all of these behaviors between the two octopuses were recorded. Within these general categories, more specific interactions were described as follows.

Mating was described as the period starting with the male approaching the female and feeling around her mantle and arms, attempting to insert the hectocotylus. When the hectocotylus was inserted, the male would begin arch and pump movements. During the "arch" movement, the male lifted the groove on the hectocotylized arm to the mantle, lining it up with the penis inside the mantle cavity, giving the male a hunched appearance. This was followed by the "pump", when the male inflated the mantle in a deep respiratory movement and exhaled explosively, sending the spermatophore down the ridge of the arm and into the oviducal gland of the female (Wodinsky 2008; Wells and Wells 1972). The number of times a male completed each arch and pump movement was recorded as well as the time between first inserting the hectocotylus to the first arch and pump.

Fighting (agonistic behavior) was described as the period when at least one octopus appeared to be trying to escape the other. Writhing arms (grappling), suckers pulling on skin (arm pulling), and biting were observed, however no inking was ever noted. During fighting, the hectocotylus was clearly not inserted in the female, but physical contact was necessary for fighting to be

recorded. In some instances, fighting would result in mating (generally in the mount position), while in others the octopuses would separate and a resting period would begin.

Resting behavior was described as the period of time when neither octopus was touching the other. They could be swimming away from each other, moving around the tank, or lying still. They had to be apart from each other and the male could not have any arm inside the female for resting to be recorded.

In addition to these three main behaviors, any instances of female behavior that could be perceived as female choice were recorded. For instance, if a female was seen to approach the male to begin mating, if a female did not mate with one male but did mate with others, or if a female was seen to overtly remove a sperm packet during any trial, the act was recorded.

DATA ANALYSIS

To determine whether there was a significant difference between the amount of time spent mating, fighting or resting between the first, second or third male to mate with the female, the non-parametric Friedman rank test (FR_X) was used. Only females that mated with each of the three males in their set were included in this analysis.

To analyze the effect of mate size on mating, fighting, and resting duration, the non-parametric Kruskal-Wallis [T(x)] test was used. For this analysis, all trials were included except those of the females that did not mate in any of their three trials. Male size relative to female was calculated by dividing female weight by male weight (grams). Males that were within 15% of female

weight were considered equal in size, those below 15% were small, and those above 15% were classified as large males. Similarly, male size relative to average male size in the sampled population and female size relative to average female size in the sampled population were calculated. The sampled population consisted of all octopuses used in this study, 24 females and 12 males.

The Chi-square test (χ^2) was used on a subsample of trials in which females successfully mated with all three paired males and were observed exhibiting behavior resembling female choice. This test was used to test the significance of observed female choice on experimental male success.

RESULTS

MATING PATTERNS IN *OCTOPUS OLIVERI*

In all, 62 successful trials between 36 individuals (24 females and 12 males) were performed. Of those, 46 trials included mating. During the course of the experiments, three females died due to water contamination in the holding tanks, resulting in only one experimental trial each. Three females laid eggs before completing all three trials, one laying eggs after only one trial and two laying eggs after two trials. Three more females only completed two of three trials because two died of unknown causes before their last trials and one escaped.

As with most octopuses, the mating behavior observed between and within individuals was varied (Huffard 2007; Wells and Wells 1972). However, a general pattern could be seen among

mating pairs in the trials where mating occurred. The average time it took for the male to approach the female and begin mating was 18 minutes (σ = 17 minutes), with the shortest amount of time being 8 seconds and the longest 1 hour and 7 minutes. No obvious courtship was seen in either behavior or body patterns for either male or female octopuses.

The male would touch the female all over her mantle and arms while searching for the oviduct with his hectocotylus for approximately 30 seconds to one minute. Most mating occurred in either the arm reach or mount position (Wells 1978) (Figure 3.2), however in 12 trials, beak-to-beak mating (Rodaniche 1984) was observed (Figure 3.3). After a brief period where the hectocotylus was inserted (Cigliano 1995), the male would begin the conspicuous arch and pump movement (Wells and Wells 1972), making it very easy to record the number of times the arch and pump occurred. The most a male was able to arch and pump in one mating trial was 74 times, the least was 5 times, with an average of 25 times during a single mating session (σ = 18 arch and pumps). The average time between each arch and pump was 2 minutes and 12 seconds (σ = 1 minute 26 seconds). During mating, the male was generally a dark brown-red color and the female was a pale white, although this was not always seen (Figure 3.2).

Mating would end when either the male or the female would detach from the other, either to begin fighting or resting. The longest a male and female spent mating uninterrupted was 1 hour and 33 minutes. In general, each trial was characterized by many short bouts of mating, the shortest being approximately 1 minute in duration. The average time spent mating (all short bouts added together) per trial was 1 hour (σ = 45 minutes). In the 16 trials where no mating occurred, variable times and combinations of both fighting and resting were observed. The data

from these trials was used in the size analysis but not in precedence as only females who mated in all three trials were used for the latter analysis.

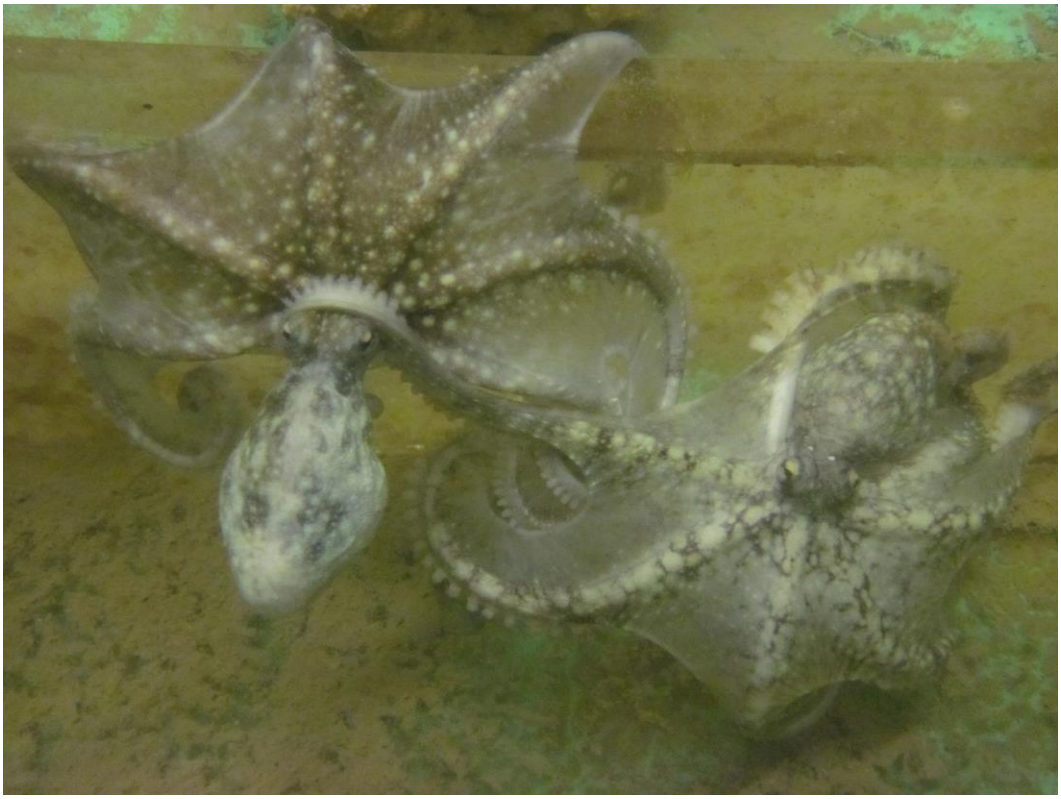

Figure 3.2 Mating in the reach position between *Octopus oliveri.* The male is on the right with his hectocotylus stretched and inserted into the female on the left.

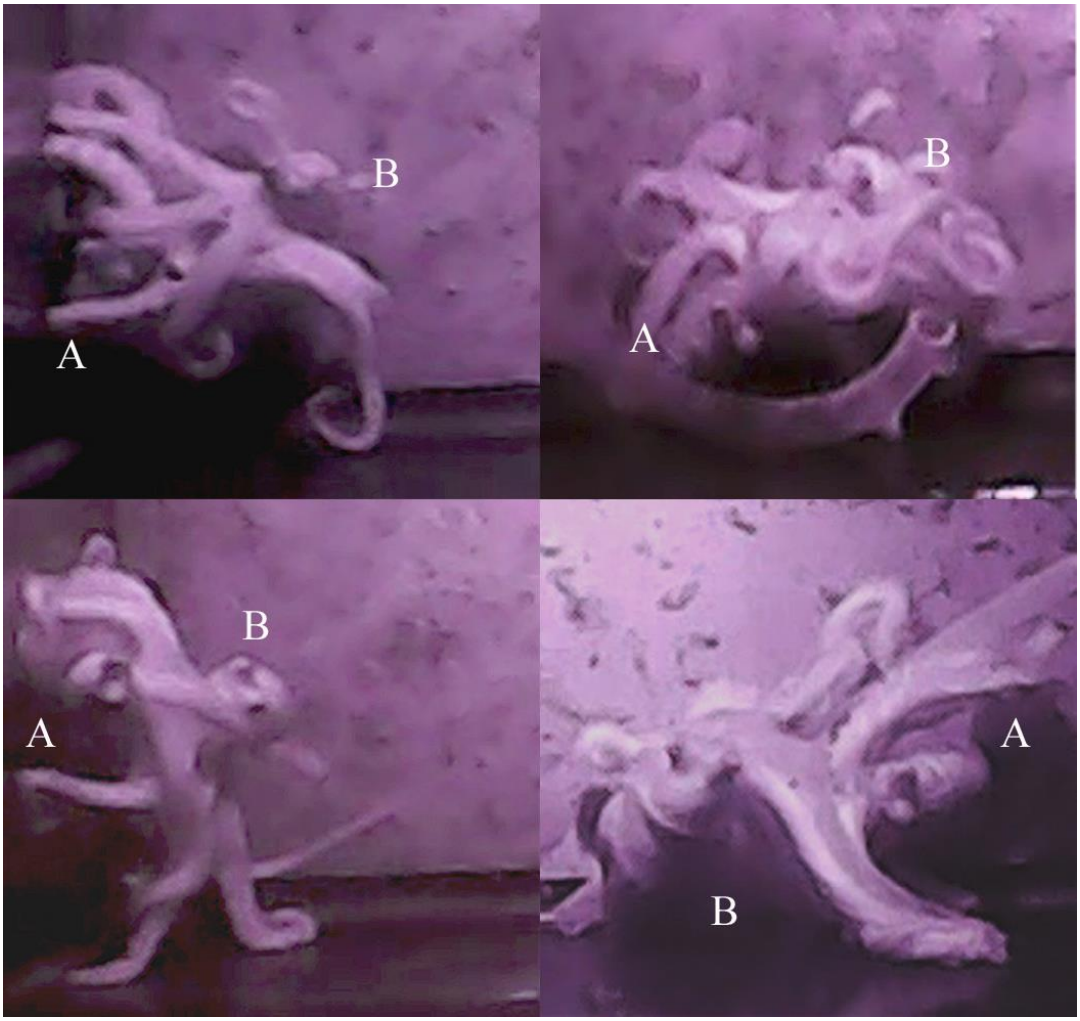

Figure 3.3 Video stills of four mating pairs in the beak-to-beak mating position. Females are indicated as the letter A and males as the letter B.

PRECEDENCE

Fifteen of the 24 experimental females successfully completed all three trials. The data from these trials were used to analyze whether precedence influenced observed behavior. The Friedman rank test showed no significant difference between the mating, fighting, or resting time of the first, second or third male mated with an individual female (mating $FR_X = 0.43$, $p = 0.82$, fighting $FR_X = 1$, $p = 0.61$, resting $FR_X = 1$, $p = 0.81$, $n = 15$) (Figure 3.4). Nor was there a

difference in the number of arch and pumps seen in successive mating trials ($FR_X = 0.32$, $p = 0.81$, $n = 15$).

To determine whether there was a difference between the amount of time the male spent with his hectocotylus inserted in the female before the first arch and pump in each successive trial, only females that mated in all three trials were included in analysis. There was no significant difference in the time it took for the male to begin the first arch and pump between successive mating trials ($FR_X = 1.56$, $p = 0.46$, $n = 9$).

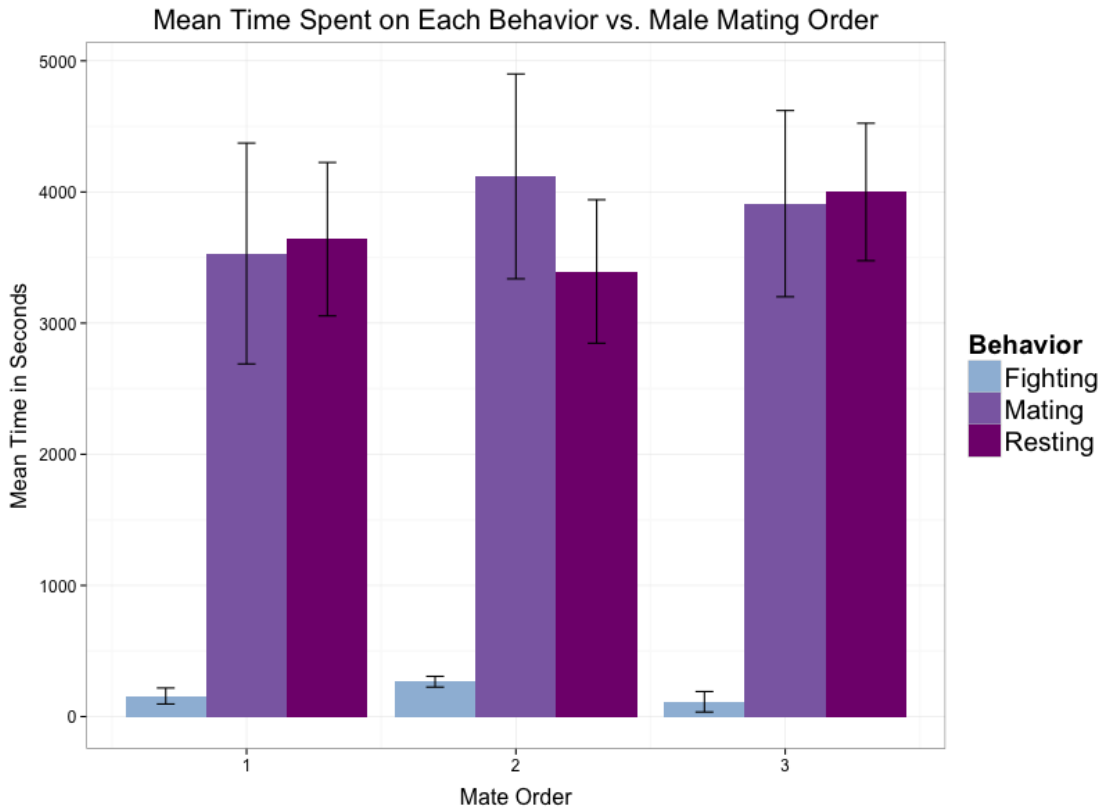

Figure 3.4 Mean time (in seconds) spent on fighting, mating, or resting for all males from each category of male order. Error bars represent standard error (σ_χ).

When analyzing the individual behavioral patterns of a single male, again no significant difference was found (mating: $FR_X = 0.4$, $p = 0.81$, fighting: $FR_X = 0.93$, $p = 0.61$, resting: $FR_X =$

0.4, $p = 0.61$, number of arch and pumps: $FR_X = 0.43$, $p = 0.85$, time from start of mating to first arch and pump: $FR_X = 2$, $p = 0.37$, $n = 9$) (Figure3.5).

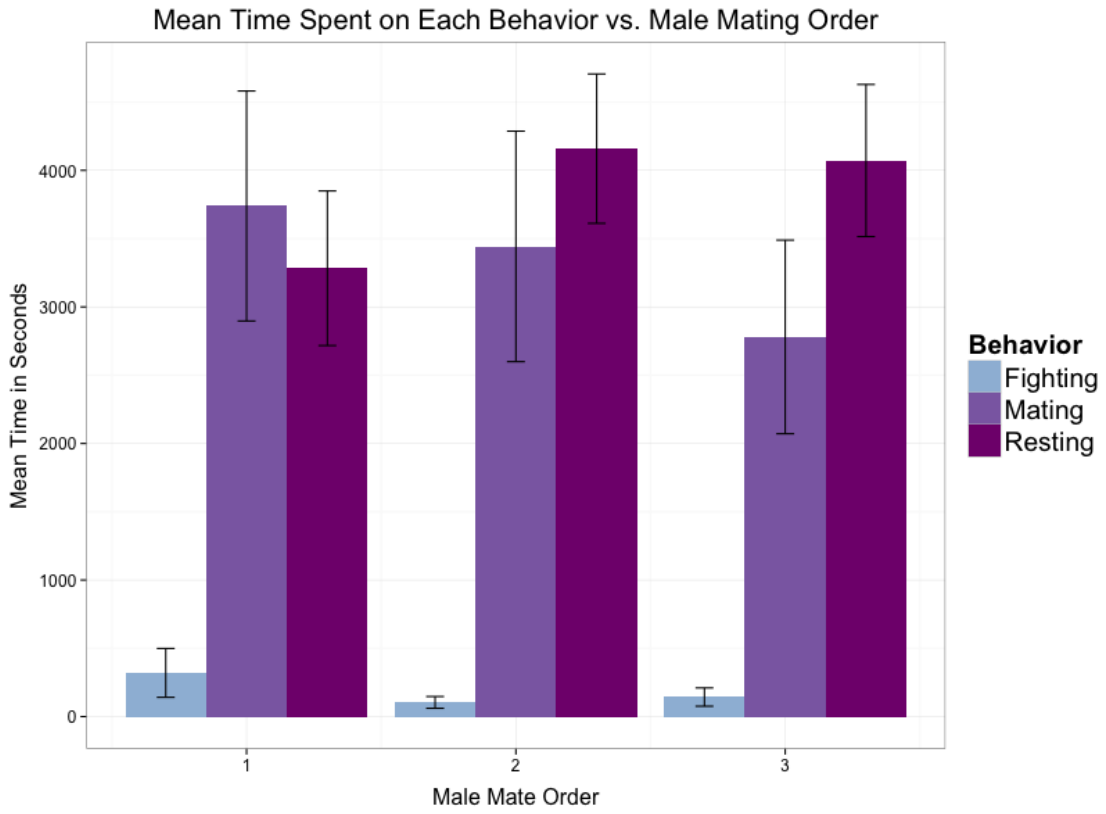

Figure 3.5 Mean time (in seconds) spent on fighting, mating, or resting in individual males between given mate order. Error bars represent standard error (σ_{χ}).

SIZE

The Kruskal-Wallis test showed no significant trend between mating and relative male size (T(x) = 0.31, $p = 0.85$, $n = 52$). Nor was the amount of time spent resting (T(x) = 1.22, $p = 0.54$, n = 52) or fighting (T(x) = 0.06, $p = 0.97$, $n = 52$) found to be significant (Figure 3.6). Male size relative to the female did not appear to affect the number of times a male would arch and pump (T(x) = 3.21, $p = 0.2$, n = 52).

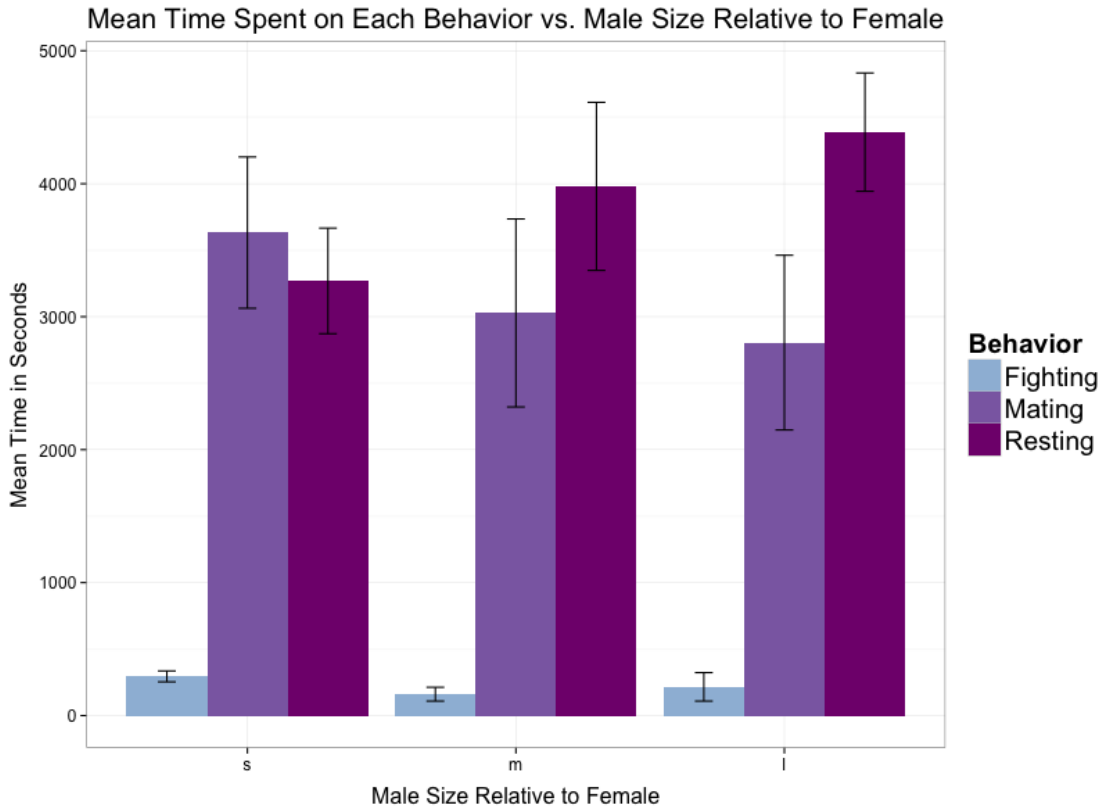

Figure 3.6 Mean time (in seconds) spent on fighting, mating, or resting when comparing male size relative to female. S: small, m: medium, l: large. Error bars represent standard error (σ_{χ}).

Male size relative to other males, was not significant in any of the behaviors (mating: T(x) = 1.92, p = 0.38, fighting: T(x) = 2.32, p = 0.31, resting: T(x) = 0.44, p = 0.8, number of arch and pumps: T(x) = 0.37, p = 0.83, n = 52) (Figure 3.7). However, female size relative to other females in the population was significant in influencing mating time (T(x) = 6.7, p = 0.03, n = 52) and the number of arch and pumps (T(x) = 8.38, p = 0.01, n = 52), while resting (T(x) = 3.36, p = 0.18, n = 52) and fighting (T(x) = 1.08, p = 0.58, n = 52) were not significant (Figure 3.8).

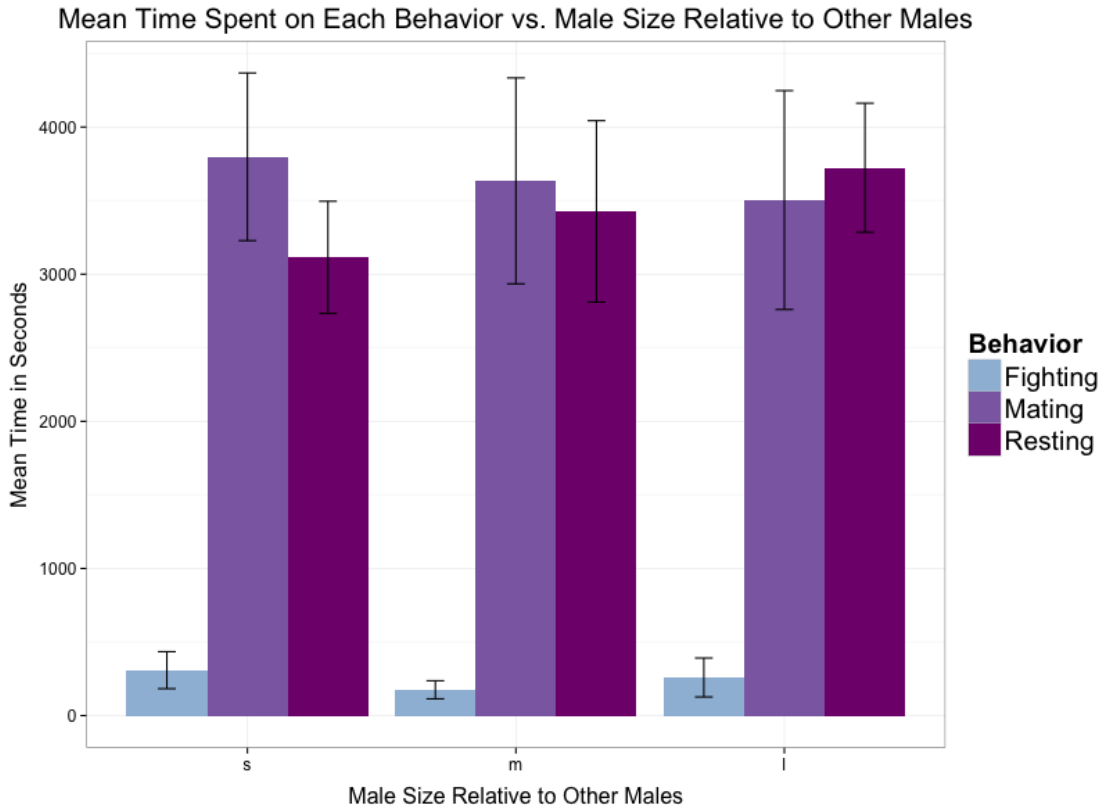

Figure 3.7 Mean time (in seconds) spent on fighting, mating, or resting when comparing male size relative to other males in the population. S: small, m: medium, l: large. Error bars represent standard error (σ_{χ}).

FEMALE CHOICE

There were 9 experimental females who had at least one trial where no mating occurred. Mate order, male size relative to the female, male size relative to other males, or female size relative to other females was not significant in predicting whether mating would not occur (mate order χ^2, $p = 0.79$, $n = 18$, male size relative to female χ^2, $p = 0.53$, $n = 23$, male size relative to other males χ^2, $p = 0.98$, $n = 23$, female size relative to other females χ^2, $p = 0.39$, $n = 23$).

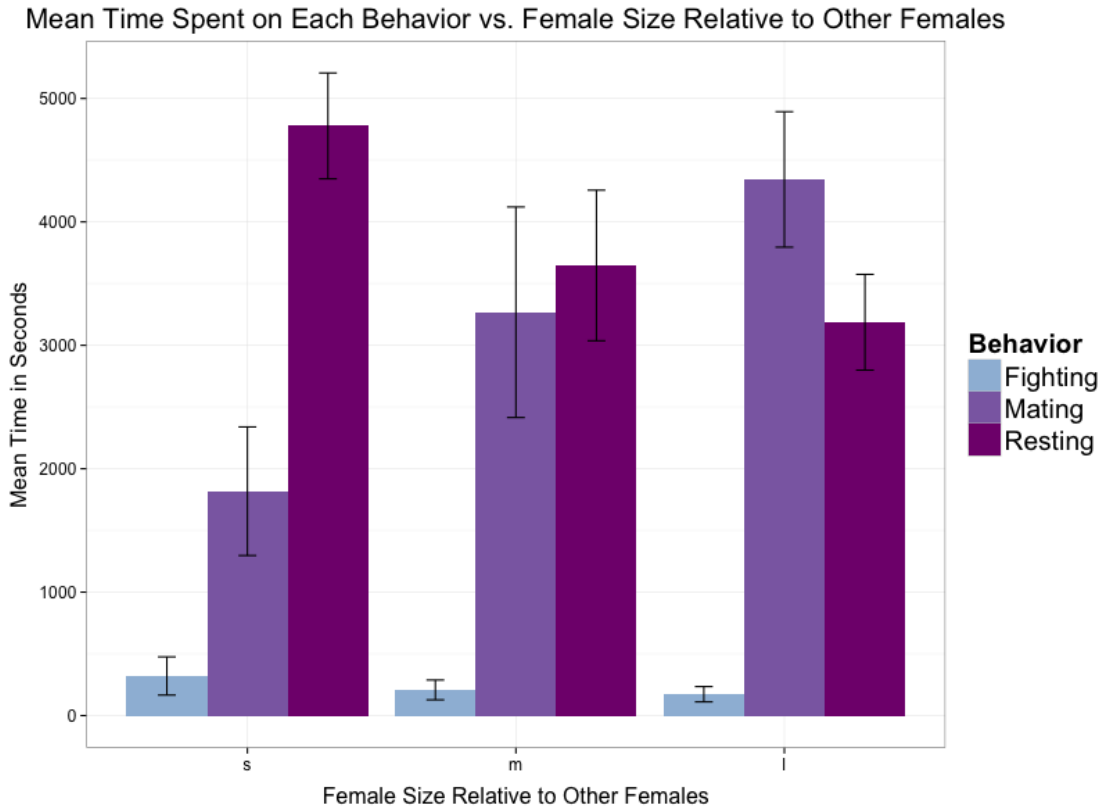

Figure 3.8 Mean time (in seconds) spent on fighting, mating, or resting when comparing female size relative to other females in the population. S: small, m: medium, l: large.

In 13 of the 46 trials where mating occurred, the female was the one to approach the male to begin mating (χ^2, $p = 0.003$, $n = 46$). The female would either move herself under the male or grab the male and pull him on top of her, followed by mating. This behavior was exhibited by 9 of the 24 trial females. Eight of these instances occurred with the first male to mate with the female, 5 with the second male, and none with the third, a significant difference (χ^2, $p < 0.01$, $n = 27$). The size of the male relative to the female did not appear to be a factor in whether the female would display this behavior as it occurred 6 times when the male was larger, 5 times when the male was smaller and twice when the male was approximately equal in size to the female (χ^2, $p = 0.34$, $n = 27$). The size of the female relative to the other experimental females also did not appear to be a factor as 2 small, 3 mid-size, and 4 large females demonstrated this

behavior (χ^2, $p = 0.62$, $n = 27$). Six of the nine females that exhibited this behavior laid eggs after the trials were concluded.

In 19 of the mating trials, the female was observed removing an intact sperm packet. This happened either by the female exhaling forcefully and expelling the spermatophore (32 instances total), or the female moving her arms over her mantle, dragging them close to the mantle opening and "pulling" out the sperm packet (2 observations). Thirteen of the 24 trial females displayed this behavior. There did not appear to be any pattern in whether the female would remove the sperm packet. In 8 instances, females removed sperm packets from the first male, 7 instances from the second male and 4 from the third (χ^2, $p = 0.61$, $n = 24$). The size of the male was also not a significant factor in sperm removal (χ^2, $p = 0.81$, $n = 24$).

DISCUSSION

In general, the mating behavior of *Octopus oliveri* appears typical of the order Octopoda, with the only remarkable deviation being the presence of beak-to-beak mating, albeit uncommon (~25%). Rodaniche (1991) was the first to describe beak-to-beak mating in the larger Pacific striped octopus; however in his observations, beak-to-beak mating was the only mating position exhibited by that species. In *Octopus oliveri*, the mount, reach, and beak-to-beak mating positions were all observed, possibly suggesting that all positions result in successful fertilization. Sexual cannibalism has been observed in a number of octopus species, implying that beak-to-beak mating would be a dangerous position for a male (Hanlon and Forsythe 2008). Cannibalism did occur among non-experimental *Octopus oliveri* when housed in a large

communal tank but it was unclear if it was specifically sexual cannibalism or for competitive or other reasons. Two females and three males were cannibalized when placed in the large communal tank. Still, the fact that cannibalism occurs at all would suggest that males might be wary of mating in a position that would make them vulnerable to consumption. However, it is possible that the drive to mate outweighs the potential risk of cannibalism. Of the nine females who had trials where beak-to-beak mating occurred, five laid eggs. The males in these trials may have been responding to a chemical cue from the female inviting more risky mating behavior. Chemical cues have been found to be very important in both squid and cuttlefish mating, indicating that they may play a role in octopus mating as well (Cummins et al. 2011; Boal 2006; Buresch et al. 2004). No cannibalism was observed in any of the experimental trials but that does not rule out the possibility that it may occur in the wild.

Unlike Cigliano's experiments in 1995, these experiments did not show any evidence of a sperm competition mechanism between males. There was no significant change in the amount of time between when the male would insert his hectocotylus and when he would begin the arch and pump movements, regardless of the mate order or size. There are several possible reasons for this result. Variation among the tissue of the ligula and calamus of octopuses may be a factor in dictating whether sperm competition occurs in the manner Cigliano suggested (Thompson and Voight 2003; Voight 2009). Erectile tissue has been found in the ligulae of some octopuses, while others were found to have muscular hydrostats (Thompson and Voight 2003). Whether one type of tissue would be more effective in removing the sperm of a previous male is unclear. However, it is possible that the ligula structure is instrumental in sperm removal and that of

Octopus oliveri, being very short (Garcia 2010), lacks the flexibility to remove sperm deposited by previous males.

It may also be possible that the time between mating sessions was sufficient to allow spermatozoa to penetrate deep into the spermatheca (De Lisa et al. 2013), therefore rendering sperm removal unlikely if not impossible. Spermatozoa have been found in the oviducal gland of *Octopus tetricus* one day (24h) after mating, although whether it was the sperm of the experimental male or a previous male from the field was unclear (Joll 1976). Nonetheless, it would appear to take at least one day for spermatozoa to travel from the distal oviduct to the spermatheca, suggesting that the time between mating in these trials with *Octopus oliveri* may have been extensive enough to limit sperm competition, at least outside of the spermatheca.

The statistically significant behavior responses of this study were positively correlated with female size relative to other females. The larger the females were, the longer the mating duration and the higher the number of arch and pumps by males. The largest females were likely to spend more time mating, possibly because they were more sexually mature and therefore close to brooding. Size in octopuses is generally dependent on environmental factors such as food quality and temperature and it can therefore be difficult to determine what size determines sexual maturity in a female (Semmens et al. 2004). However, in some octopuses size can be a predictor of fecundity, which may indicate that males are more likely to invest time in mating with larger females (Leporati, Pecl, and Semmens 2008; Mohanty, Ojanguren, and Fuiman 2014). In the case of *Octopus oliveri*, it appears that larger females are more amenable to mating, possibly because they are close to spawning. While female octopuses can mate and store sperm months

before laying eggs (Wells 1978), it may be that the quality of the sperm is reduced over time (Reinhardt 2007), making it likely that smaller females would delay mating until they are closer to spawning.

There appears to be some evidence for female choice, both in females that approached males to mate and females that removed sperm packets. While it was a relatively rare occurrence for females to approach males initially for mating (~28%), it is interesting to note that more than 60% of the time, a significant value, it was with the first male presented to the female, regardless of size difference. This may indicate that mature females isolated from males would be more amenable to mating with any male that is presented to them, as they may initially be sperm limited. A recent study of *Octopus bimaculoides* mating behavior found a similar pattern with large females mating for longer periods with the first male to approach them (Mohanty, Ojanguren, and Fuiman 2014). As more mates are presented to them, however, they may become more selective.

In the instances where females remove sperm packets, it is possible that this would have been a signal to the male that the female was not receptive to mating. Because these experiments were done in a laboratory setting, the mating behavior may not have been typical of that in the field. These females were placed in a tank with one male and they were unable to escape for two hours. In the wild, if a female removed a sperm packet, it could be an indication that she has exhibited female choice and rejected the male. Since there was not an obvious correlation between aggression, size or mating order in determining whether a female would remove a

sperm packet, it is possible there were other factors involved that were not measured, such as chemical cues.

Also, of the nine females who exhibited primary approach behavior, 2/3 of them laid eggs at the end of the experiments. In the three others, two died during a water contamination event, and the last died unexpectedly before laying eggs. If these females were nearing brooding, they may have been trying to acquire as much sperm as possible, making them less likely to fight with males. Again, this was a pattern found by Mohanty (2014), where the more mature females spent more time mating with males. In the current study, maturity of females could not be determined before brooding because a necropsy would have been required to measure gonadal development and the eggs were needed for genetic testing.

In instances where females removed sperm packets, it is possible that there was a mechanistic explanation for its removal. Wodinsky (2008) described the spermatophore transfer process in two *Octopus* species. In his experiments, he noted that females were seen to expel spermatophores before the spermatozoa within the spermatophore had ejaculated and concluded it was a result of a disconnection between the calamus and the distal oviduct. If this is the case, the removal of sperm packets may have merely been a result of misplaced anatomy than actual female choice. As there was no pattern among male size or precedence in incidences where the females removed sperm packets, it is likely that all males are capable of placing the ligula incorrectly. Still, those males who are more adept at ligula placement may be more successful in fertilizing the female if they maximize the number of spermatophores transferred to the female in each mating encounter. The production of spermatophores is not without cost to the male,

suggesting that loss of sperm packets would be detrimental to their fitness and selective pressure for correct placement would be high. The males in trials with sperm packet removal were not wholly unsuccessful in mating with females, as there were often many arch and pumps following a removal. This again suggests a possible misfire on the part of the male.

Aggression between males and females in the trials was frequent, but occurred quickly, without much visible injury to either party. This may indicate that fighting is used only to ensure no cannibalism occurs between the mating pair. Or it may serve as a test of the mates mating ability by showing the female he is aggressive. However, because it occurred across mating pairs, it is unlikely that male-female aggression was indicative of mate choice. Aggression did not result in more arch and pumps, longer mating duration or any other advantageous behavior in the trials.

These experiments indicate that among *Octopus oliveri* individuals, females mate indiscriminately with males in any order and of any size, showing minimal behavioral evidence for sexual selection. However, it is possible that sexual selection occurs through contests and agonistic behavior between males in the field before copulation. Clearly studies in the field to observe this octopus mating would be extremely beneficial, but field observations of this species are rare and somewhat difficult as they live in high wave action zones with dangerous rocky terrain. Future studies should include potential for male-male contests, which may provide behavioral evidence of precopulatory sexual selection. In addition, although there was no behavioral evidence for sexual selection, it does not preclude the presence of postcopulatory sperm competition or female cryptic choice. Genetic analyses of the female broods will illuminate whether sperm precedence and multiple paternity are occurring in this species.

CHAPTER 4. MICROSATELLITE DEVELOPMENT AND MULTIPLE PATERNITY IN *OCTOPUS OLIVERI* (BERRY, 1914) (CEPHALOPODA: OCTOPODIDAE)

ABSTRACT

This study shows that octopus mating dynamics may be more complex than previously thought. Through a combination of behavioral and genetic studies, multiple paternity was found to be the rule in *Octopus oliveri*, both in the field and in captivity. Five microsatellite markers were developed and used to test paternity in eleven egg broods. The results showed skewed paternity in most broods, suggesting that sperm competition is present in this species. The two predictive variables in determining male mating success were mate order and male size. This is the first study in an octopus that combines both behavioral and genetic information to determine fertilization success.

INTRODUCTION

To detect features that undergo sexual selection in a species, both phenotypic and genotypic studies are necessary. Traditionally, studies of sexual selection were limited to top down strategies; inferring genetic causes from phenotypic patterns (Hall et al. 2010). Behavioral observations of female choice and manipulations of male traits were used to determine rates of sexual selection in a population. With the development of molecular techniques, bottom-up studies of sexual selection at the genetic level are possible.

Developments in detection and sequencing of microsatellite sequences have allowed for connections to be made between the genetics of mating systems and sexual selection.
In species where females have the potential ability to select a particular male's sperm over others, either through manipulation of internal musculature during copulation (directing sperm to a "dead end") or rejection of sperm after copulation, those sperm selected are evidence for sexually selected traits. Comparing the genetic sequences of the contributing males with the competitive PCR results would allow one determine preferred sperm and therefore characteristics of mate preference (Hall et al. 2010). Similarly, in systems where sperm competition occurs, genetic markers can indicate which males are most successful in fertilizing offspring.

Multiple paternity, or the presence of numerous males fertilizing offspring in one brood, is common across many taxa, in both vertebrates and invertebrates (Cutuli et al. 2013; Yue et al. 2010; Borkowska, Borowski, and Krysiuk 2009). In mating systems where multiple paternity

occurs, it is often common to have high rates of sperm competition. Sperm competition occurs when sperm from two or more males compete to fertilize the ova of a female (Birkhead and Møller 1998; Birkhead and Pizzari 2002).

Within the Cephalopoda, sperm competition has been observed in a variety of squid and cuttlefish species in the form of mate guarding, sneaker males, sperm flushing and increased sperm allocation (Wada et al. 2010; Wada et al. 2005; Iwata and Munehara 2005; Naud et al. 2004; King, Adamo, and Hanlon 2003; Jantzen and Havenhand 2003; Emery, Wilson, and Craig 2001; Hanlon and Ament 1999; Hanlon, Smale, and Sauer 2002). In octopuses, sperm competition is generally believed to occur with the presence of multiple mating, two oviducts with which to store sperm, and long-term sperm storage capabilities (Hanlon and Messenger 1998; Birkhead and Møller 1998; Wigby and Chapman 2004). Yet, mate-guarding and sneaker behavior has only been described in one species (Huffard, Caldwell, and Boneka 2010), and direct sperm competition in the form of sperm precedence has only been inferred in one unnamed pygmy octopus species and even then, the mechanism behind the sperm competition remains unknown (Cigliano 1995).

Male sperm precedence is the nonrandom utilization of one males sperm over another (Birkhead and Møller 1998). This can occur through female cryptic choice within the oviduct of the female, overt female rejection of sperm packets, or through male displacement of previously placed sperm packets by rival males. In nature, some animals show first male sperm precedence (Tennessen and Zamudio 2003), where the first males to inseminate a female are most successful in fertilizing the female gametes, while others exhibit a “last in, first out” strategy, as seen in

damselflies removing the sperm packets of previous males (Birkhead and Møller 1998). No clear pattern of sperm precedence in cephalopods has been discovered to date, but it has been predicted in several species (Iwata and Munehara 2005; Shaw and Sauer 2004; Buresch et al. 2009; Naud et al. 2004; Cigliano 1995).

The male octopus has a modified third right arm called the hectocotylus, which he uses to transfer sperm packets (spermatophores) to the female. A sperm mass is encapsulated along with an ejaculatory organ in each spermatophore. As the spermatophore is passed down through the penis and into the groove of the hectocotylus, osmotic pressure begins to force water through the outer tunic of the spermatophore. The male reaches into the mantle of the female with his hectocotylus and transfers the spermatophore to the distal oviduct where the ejaculatory process begins. The sperm mass is released from the spermatophore and it travels up the oviduct and is stored in the spermathecae in either of the two oviducal glands (Mann 1984; Hanlon and Messenger 1998; Wells 1978; Wodinsky 2008). Females will mate with multiple males before laying eggs and can store the sperm for up to 10 months in some species (Mangold 1987). The eggs become fertilized as they travel through the oviducal gland and down the oviduct (Forsythe and Hanlon 1988).

Octopus oliveri is a small, tropical, intertidal octopus that mates readily in lab conditions, making it an ideal candidate for studies in cephalopod reproduction (Ylitalo, Watling, and Toonen 2014; Berry 1914). Initial observations of this species in a large communal tank indicated that females of this species do mate with multiple males, however, not simultaneously.

Microsatellites are short tandem repeats of nucleotides that tend to occur in non-coding regions of DNA. Microsatellite markers are particularly useful in paternity studies because they are polymorphic, codominant, locus-specific, and require very little DNA to amplify. Still, because they are generally species specific, developing appropriate primers can be a labor-intensive process. Only two previous studies have been conducted using microsatellites to determine whether multiple paternity was present in octopus broods, one with *Graneledone boreopacifica* (Voight and Feldheim 2009)and the other with *Octopus vulgaris* (Quintero et al. 2011). These studies confirmed that multiple paternity was occurring in these species, however they did not observe mating prior to collecting the eggs, so it is unknown if mating behavior affected fertilization success.

This study attempts to combine both behavioral and genetic information to determine whether multiple paternity is present in *Octopus oliveri* and if so, to assess the ratios of paternity for each male. Equal rates of paternity would suggest no female choice and no sperm competition between males. A skew in paternity rates would indicate some males are more successful than others in siring offspring. The aim is to discover the factors that influence fertilization success in *Octopus oliveri*.

MATERIALS AND METHODS

ANIMAL COLLECTION AND CARE

Octopus oliveri individuals were collected from Kaka´ako Waterfront Park, and Kewalo Basin Marina, Honolulu, Hawai´i in the fall of 2010 through the summer of 2013. Two to three people

would walk along the rock wall during the evening hours for one to three hours (between 7pm-12am) with a flashlight. When an octopus was found, it was collected by hand and transferred to a five-gallon bucket. The males and females were kept in separate buckets. Adult octopuses were weighed on a platform scale (wet weight) and transferred to tanks on Coconut Island, Kāne´ohe. Each octopus was housed in an individual tank (38cm x 21cm x 23.5cm) with a piece of coral or PVC pipe for shelter and a plastic well-ventilated lid. These tanks were then placed in a large outdoor tank at the Hawai´i Institute of Marine Biology (HIMB) with constant saltwater flow and ambient ocean temperature. The octopuses were fed frozen shrimp and live crabs on a regular schedule and the tanks were cleaned after each feeding. Water temperature records were obtained though NOAA Tides and Currents databases from the station located closest to the collection site in Honolulu (Station ID 1612340) and at Coconut Island (Station ID 1612481).

DNA EXTRACTION

Arm tip muscle tissue was collected from 11 adult females and 9 adult males.

Egg strings from each clutch were collected one or two days before hatching and fixed in separate vials of 90% ethanol. Individual eggs were sampled from 9-12 randomly selected strands from each of the 11 broods of females. Eggs were randomly sampled from the top, middle, or bottom section of the egg strand and their locations were recorded. The paralarvae were almost fully developed at this time to provide the most DNA possible. DNA extractions were performed using the HotSHOT protocol on each embryo and adult muscular tissue sample (Truett et al. 2000).

PCR AMPLIFICATION

Microsatellite loci developed for *Octopus vulgaris* and *Graneledone boreopacifica* (Greatorex et al. 2000; Quintero et al. 2011; Voight and Feldheim 2009) were tested for use in *Octopus oliveri,* however they failed to amplify. Therefore, specific microsatellites and primers for the microsatellite loci were designed through the "Post-sequencing bioinformatics pipeline" for *Octopus oliveri* (Fernandez-Silva et al. 2013). Initially, 50 putative loci were tested, but after screening, only the 5 best sets of primers were optimized (Table 4.1) (Selkoe and Toonen 2006). The three-tailed primer method described by Gaither et al. (2009) was used in PCR amplification.

Two primer mixes were prepared for each individual sequenced. Primer mix A consisted of 10µl each of 100mM primer Octoli_3R, Octoli_7R, Octoli_10R, Octoli_11R, fluorescent yellow (NED), red (PET), green (VIC), and blue (6-fam) dye. In addition, there were 2.5µl of 100mM primer Octoli_3F-**T1**, Octoli_7F-**T2**, Octoli_10F-**T4**, and Octoli_11F-**T3** (Table 4.1). The rest of the mixture comprised of 410µl of RNAse free water (H_2O). Primer mix B used the same ratio of solutions as listed above for Primer mix A, however primers Octoli_17, Octoli_18, Octoli_22, and Octoli_23 were used. Octoli_10, Octoli_11, and Octoli_18 were not used in the final analysis, but they were kept in the primer mixes to ensure no differences in amplification among samples would occur. Each individual PCR reaction mix contained 3µl 2X Multiplex MasterMix, 0.6µl 10X Primer mix, 1.4µl RNAse free water, and 1µl 1:10X template DNA for a total of 6µl for each reaction.

PCR amplification was completed on a Bio-Rad iCyler as follows: 95°C for 15 minutes (1 cycle), 95°C for 30 seconds, 60°C or 62°C (see Table 1) for 90 seconds, 72°C for 60 seconds (35 cycles), followed by a final extension of 72°C for 30 minutes. Amplified PCR products were genotyped on an Applied Biosystems 3730X Genetic Analyzer at the University of Hawai´i at Manoa. The fragments were scored on Geneious version 6.7.1 created by Biomatters (Kearse et al. 2012) according to guidelines laid out by Selkoe and Toonen (2006).

DATA ANALYSIS

The maternal genotypes from each of the 11 broods were compared with the embryo genotypes manually to ensure that at least one maternal allele was found at each loci, confirming Mendelian inheritance. Then, after excluding the maternal alleles one can make a conservative estimate of the number of sires contributing to a brood by using the single-locus minimum (SLM) method. This involves counting the number of paternal alleles at each locus in the progeny array and dividing the largest number by two (assuming all males are heterozygotes) and rounding up. Given that each parent can only contribute two alleles (ignoring mutation) the number of parents cannot be smaller than this conservative estimate (Jones 2005).

Table 4.1 Microsatellite markers developed for this study; their sequences and levels of polymorphism.

Locus	Motif	Primer Sequence (5'-3')	T_a(°C)	Size Range (bp)	N_A	H_O	H_E	Freq of Nulls
Octoli_003	$(TAGA)_{12}$	F: **T1-**GCACGTTGTACGCGATTC	62	154-200	11	0.888	0.856	0.018
		R: ATATGCATGAAGACGCAACTC						
Octoli_007	$(TATG)_{12}$	F: **T2-**CGCAGACGAGGAATCAATAG	62	152-184	9	0.718	0.816	0.063
		R: GGAGAACAGACACAAGAACACAG						
Octoli_017	$(TATG)_8$	F: **T2-**AGCAACACGATGGCCTCTAC	60	180-202	5	0.569	0.521	0.048
		R: AGTCCAACAAGCTTCGATCC						
Octoli_022	$(TGA)_{21}$	F: **T1-**AGCCATGTGGTTGAGAACG	60	239-287	14	0.943	0.902	0.022
		R: GCGTGCCTCTCTTCATCAG						
Octoli_023	$(GAT)_{20}$	F: **T3-**GCCATGAATTCCAAGTAACTAACC	60	160-199	15	0.856	0.846	0.007
		R: CATCGTCATACGCCATCATC						

T1: PET-5'-GGCTAGGAAAGGTTAGTGGC-3'; **T2**: 6-Fam-5'-TCATACATGTCTCTCAGCGTAAAC-3'; **T3**: VIC-5'-GACTATGGGC GTGAGTGCAT-3'; **T4**: NED-5'-ACCAACCTAGGAAACACAG-3' , T_a(°C): Annealing temperature in degrees centigrade N_A: Number of alleles, H_O: Observed heterozygosity, H_E: Expected heterozygosity

The program GERUD v. 2.0 was then used to evaluate the broods with an exhaustive algorithm along with population allele frequencies to find the minimum number of paternal genotypes to explain the array (Croshaw, Peters, and Glenn 2009; Jones 2005). For five of the experimental broods analyzed, the genotypes in the array proved too complex for GERUD, and the analysis resulted in the software crashing. The locus with the lowest expected exclusion probability (Octoli_17, Table 4.2) was then removed from those five broods and analysis was run again. The removal of one locus proved successful in simplifying the genotype arrays for analysis for only two females, while the remaining three arrays continued to freeze. GERUD was also used to calculate the expected exclusion probability for each locus and for the combined loci.

Table 4.2 Expected exclusion probabilities of all 5 loci given known or unknown paternal genotypes.

	Expected exclusion probabilities		
	Neither parent known	One parent known with certainty, one unknown	Parent pairs known
Octoli_3	0.552	0.714	0.881
Octoli_7	0.468	0.643	0.826
Octoli_17	0.138	0.257	0.393
Octoli_22	0.665	0.799	0.936
Octoli_23	0.530	0.695	0.866
All Loci	0.968	0.995	1.000

Parentage was assessed using the maximum likelihood ratio program in CERVUS v. 3.0 (Slate, Marshall, and Pemberton 2000; Marshall et al. 1998; Jones et al. 2010). The likelihood ratio is the probability that the candidate parent is the true parent compared with the probability of an alternate unrelated candidate parent. The program uses this ratio to determine the most likely father given a known maternal genotype, a set of candidate paternal genotypes, and the brood

genotypes. CERVUS incorporates typing error, unsampled candidate parents, and missing genotypes into the program analysis. I used both strict (95%) and relaxed (80%) confidence in paternal assignment. However, all subsequent analysis was performed on the data generated at the 95% confidence level as recommended by Marshall et al. (1998).

The offspring that were not assigned paternity at 95% confidence were then rerun through GERUD to find potential paternal genotypes from the wild. The assumption was that wild males who mated with females before collection sired the unassigned offspring. GERUD also calculates how many offspring are assigned to each wild type male. To corroborate the number of eggs assigned to paternal genotypes generated by GERUD, CERVUS was run again using only unassigned eggs (at a 95% confidence level).

The program f_{mm} was used to assess the frequency of multiple mating in the natural population of *Octopus oliveri* using the genotypes of broods of non-experimental females (Neff 2002). This program considers the number of loci, the number of alleles and their frequencies, and reproductive skew. These results were used to corroborate multiple paternity through the SLM and GERUD and to extrapolate rates of multiple paternity in wild populations.

The Chi-squared test (χ^2) was used to test for significance in the paternity ratios observed. To determine the significance of mating time (in seconds), male order, male size (in grams), number of arch and pumps, and frequency of females observed removing sperm packets on the number of eggs sired by each experimental male, ANOVA tests and Pearson's product-moment correlations were run. Data on behavioral factors contributing to each brood are listed in Chapter

3. The best model of predictors was calculated using marginal likelihood ratio tests and AIC (Akaike Information Criterion) model selection tables. All tests were run using the program R (2014).

RESULTS

GENETICS

Multiple paternity was found in all experimental broods with at least 2 to 4 males contributing when analyzed manually with the conservative single-locus minimum (SLM) method. In the non-experimental broods, multiple paternity was found in all but one array. However, when GERUD was used to analyze the broods, at least 2 sires were determined for both experimental and non-experimental females, indicating multiple paternity in all broods (Table 4.3).

Table 4.3 Number of non-maternal alleles at each locus and the minimum number of males needed to account for those alleles.

Experimental Females	Locus Octoli_3	Locus Octoli_7	Locus Octoli_17	Locus Octoli_22	Locus Octoli_23	Min no. of males (SLM)	Min no. of males (GERUD)
F1	6	4	2	7	5	4	5+
F2	4	2	1	4	3	2	4
F3	4	3	2	5	4	3	5
F4	4	2	1	5	4	3	5
F5	5	4	0	5	5	3	5*
F6	3	1	1	4	6	4	5+
F7	5	1	1	6	5	3	5*
F8	4	2	3	4	4	3	5+
Non-Experimental Females							
F9	2	1	0	2	4	2	3
F10	3	3	0	5	2	3	4
F11	1	1	1	1	1	1	2

+ indicates GERUD unable to calculate number of sires higher than listed number due to program freezing, *locus Octoli_17 removed from analysis and genotype array rerun

GERUD generates the most likely paternal genotypes for each brood given allele frequency in the population. These genotypes were then compared with the genotypes of known experimental males. In all but one of the broods, no exact matches of paternal genotypes were found. This is likely due to GERUD calculating the most likely and minimum number of genotypes not every possible paternal genotype.

The program f_{mm} calculated an expected frequency of multiple mating in the population at 37%, with a 95% confidence interval starting with at least two sires.

Analysis of broods in CERVUS showed a trend of first mating precedence in egg fertilizations. When analyzed with both a 95% and 80% confidence interval, the results suggested that the female used significantly more sperm from the first male to mate, either in the field (if assuming one previous mate), or in the behavioral experiments (χ^2 95% CI; $p < 0.01$, $n = 8$, χ^2 80% CI; $p = 0.01$, $n = 8$) (Table 4.4, Figure 4.1). The number of offspring sired by first males to mate differed significantly from the number sired by the last males ($p < 0.01$)(Figure 4.2).

There was no pattern of male dominance within strands. Multiple males were found to have sired offspring within a single strand. Distribution of paternity among strands appeared to be random (Figure 4.3).

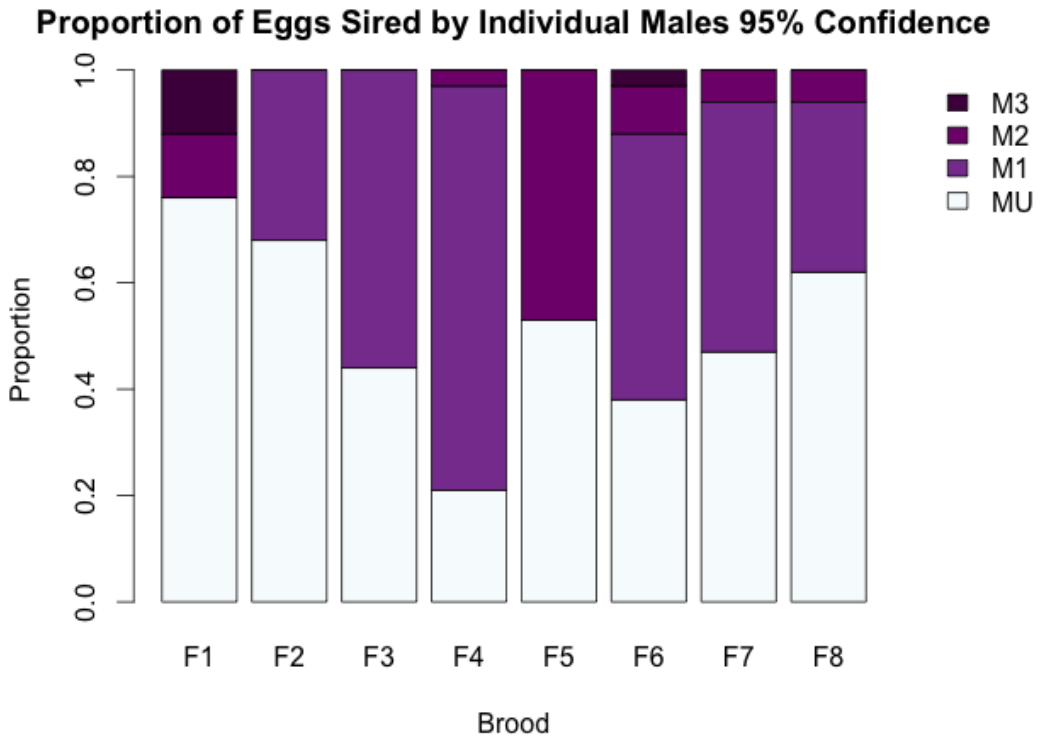

Figure 4.1 Percentage of eggs sired by males ranked by order for each of the female broods. MU: Unknown males, M1: First male to mate in experimental trials, M2: Second experimental male, M3: Third male.

When the unassigned eggs were rerun in GERUD and CERVUS, the category of "other" was split up into much smaller subsets (Table 4.5, Figure 4.4). The number of fathers that accounted for the unassigned eggs ranged from 2 to 6. Rerunning the analysis shows a significant difference in the proportion of eggs sired by the wild males and first experimental males versus the second and third experimental males (Figure 4.5).

Figure 4.2 Percentage of eggs sired vs. male order, before rerunning genotypes of unassigned eggs. $p < 0.01$ for all between 0, 2 and 3, Residual Std. Error= 0.19, DF=23, R^2=0.51.

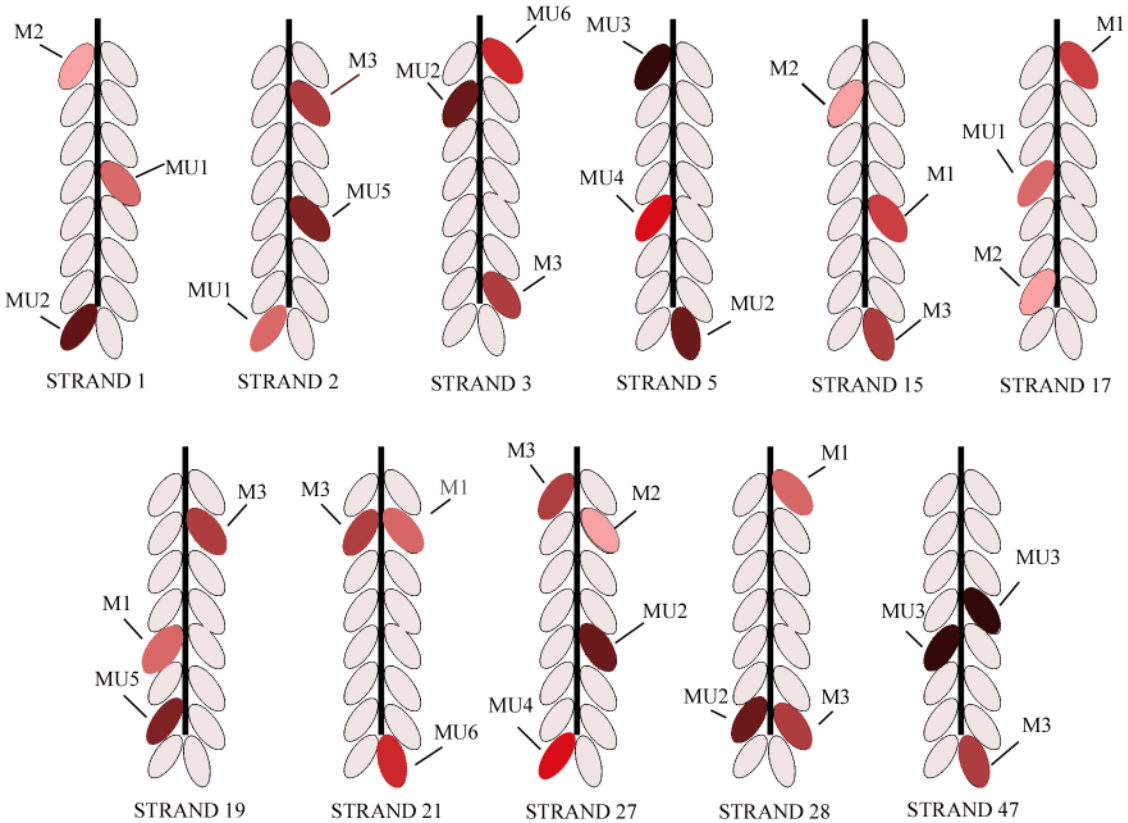

Figure 4.3 An example of paternity distribution among egg strands collected from each female brood. Female 1 brood strands illustrated here.

Table 4.4 Number of eggs per brood sired by each male calculated by CERVUS, with 95% and 80% confidence levels. If none of the candidate fathers matched the offspring genotype, they were counted as sired by "other."

	95% Confidence				80% Confidence				
Female	1st male	2nd male	3rd male	Other	1st male	2nd male	3rd male	Other	N
Set 1									
F1	0	4	4	26	5	4	10	15	34
F2	--	11	0	23	--	12	8	14	34
Set 2									
F3	--	--	19	15	--	--	22	12	34
Set 3									
F4	26	**	1	7	28	--	4	2	34
F5	0	**	16	18	2	--	23	9	34
Set 4									
F6	17	3	1	13	25	4	5	0	34
F7	16	2	0	16	21	2	0	11	34
F8	11	2	0	21	21	4	3	6	34
Average	11.67	3.14	5.13	17.38	17	5.2	9.38	8.63	34
Total	70	22	41	139	102	26	75	69	272

-- indicates no mating occurred, ** indicates paternal genotype missing

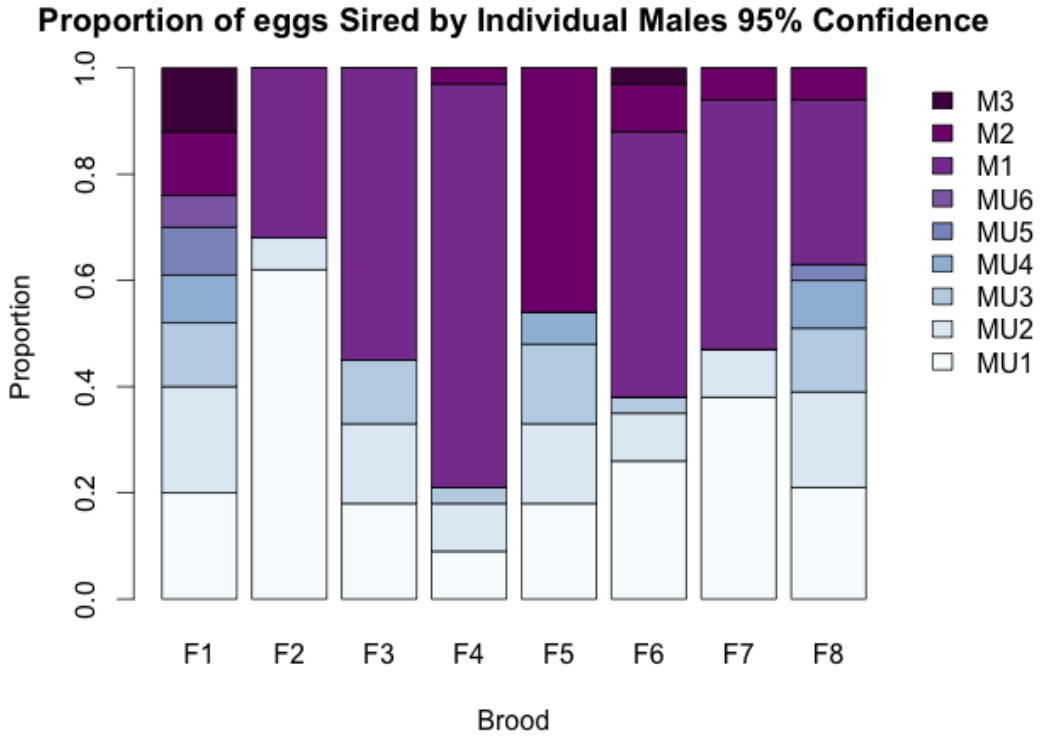

Figure 4.4 Percentage of eggs sired by all possible males, both experimental and wild type. MU1-MU6: All unknown wild males, M1-M3: Experimental males.

The next step was to see which of the behavioral or characteristic variables, if any, were the most explanatory in determining paternity. Only the experimental males were used in this analysis because there was no available data on size, mate order, or any behavioral characteristics from any wild male the female may have mated with prior to experiments. Data on behavior and characteristic variables from experimental trials are listed in Chapter 3. The trials where no mating occurred were removed from the data analysis, as these did not result in any possible offspring for that male.

Figure 4.5 Percentage of eggs sired versus male order after GERUD rerun on "Other" putative males from matings, which occurred in the field before collection. $p > 0.001$ for both 0 and 1. Residual Std. Error= 0.15, DF= 44, $R^2 = 0.28$.

Table 4.5 Number of unknown candidate males that contribute to each brood and number of eggs attributed to each male.

Female ID	Number of Unassigned Eggs	Number of Sires	Distribution of offspring among sires
F1	26	6	7, 7, 3, 2, 3, 4
F2	23	2	21, 2
F3	15	3	6, 5, 4
F4	7	3	3, 3, 1
F5	18	4	6, 5, 5, 2
F6	13	3	9, 3, 1
F7	16	2	13, 3
F8	21	5	7, 6, 4, 3, 1
Non-Experimental Females			
F9	34	3	18, 11, 5
F10	24	4	9, 9, 4, 2
F11	34	2	30, 4

The marginal likelihood ratio tests help to visualize patterns in male-female behavior and proportion of eggs sired based on several variables: male mating order, male size in grams, number of arch and pumps during each trial, time spent mating during a trial, the number of instances where a female was seen removing a sperm packet in a trial, and the male size relative to the female (small, mid-size or equal to the female, or large) (Figure 4.6). Running an ANOVA and plotting each of the variables alone against the percentage of eggs sired showed a positive correlation in the size of the male in grams ($p < 0.001$), the number of arch and pumps in a trial ($p < 0.05$), and the removal of sperm packets during a trial ($p < 0.05$). In mate order ($p < 0.001$) and mating time ($p < 0.01$), however, there is a negative correlation. There was no significant correlation in male to female size and percentage of eggs sired ($p < 0.1$). This model uses all variables in the ANOVA, likely causing overfitting. The small sample size and large number of parameters lends itself to model error. The Aikake's Information Criterion was used to determine which variables would prove the most useful predictors. The size of the male relative to the female was removed in the model table because no clear relation was found in the ANOVA test. The simplest and highest weighted model included only the male order and male size in grams as predictive variables (Table 4.6, Table 4.7).

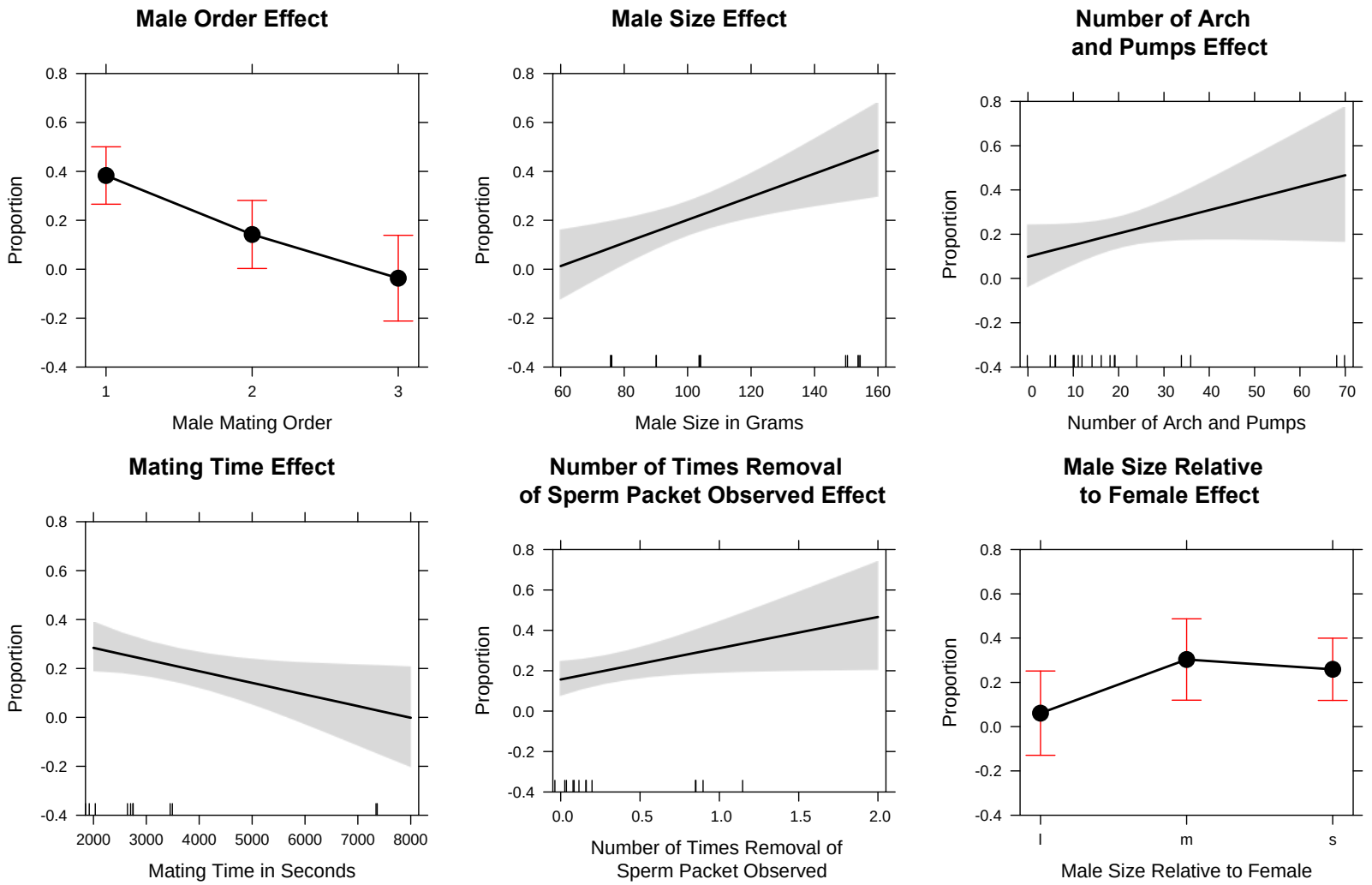

Figure 4.6 Single linear regression/ one-factor ANOVA plots of possible explanatory variables in paternity analysis. Male mating order (likelihood ratio χ^2= 23.3, DF = 2, $p < 0.001$), Male size in grams (likelihood ratio χ^2 = 11.8, DF = 1, $p < 0.001$), Number of arch and pumps observed in mating trial (likelihood ratio χ^2= 3.8, DF = 1, $p < 0.05$), Mating time in seconds (likelihood ratio χ^2 = 5.8, DF = 1, $p < 0.01$), Number of times a female removed a sperm packet (likelihood ratio χ^2= 5.1, DF = 1, $p < 0.05$), Male size relative to female (l: large, m: medium, or approximately equal to female size, s: small) (likelihood ratio χ^2= 3.9, DF = 1, $p = 0.13$).

Table 4.6 AIC Model selection table; variables listed in order of the highest to lowest weighted. (Continued on next page).

Explanatory variables	DF	AICc	Δ AIC	Weight
male order x male size	5	110.9	0	0.538
male order x number of arch and pumps x male size	6	114	3.06	0.116
male order	4	115.2	4.3	0.063
male order x mating time x male size	6	115.3	4.36	0.061
male order x male size x removal of sperm packet	6	115.3	4.38	0.057
male size	3	116.2	5.3	0.038
male order x number of arch and pumps	5	116.8	5.87	0.029
male order x mating time	5	117.5	6.62	0.02
mating time	3	117.8	6.92	0.017
number of arch and pumps	3	117.9	6.99	0.016
number of arch and pumps x male size	4	118	7.08	0.016
removal of sperm packet	3	118	7.12	0.015
male order x mating time x number of arch and pumps x male size	7	118.3	7.44	0.013
male order x removal of sperm packet	5	118.6	7.69	0.012
male order x removal of sperm packet x number of arch and pumps x male size	7	119	8.1	0.009
male size x removal of sperm packet	4	119.1	8.16	0.009

Table 4.6 (Continued) AIC Model selection table; variables listed in order of the highest to lowest weighted.

Explanatory variables	DF	AICc	Δ AIC	Weight
male order x mating time x removal of sperm packet x male size	7	120.4	9.51	0.005
mating time x number of arch and pumps	4	120.9	9.97	0.004
number of arch and pumps x removal of sperm packet	4	120.9	10.05	0.004
mating time x removal of sperm packet	4	121	10.14	0.003
mating order x mating time x number of arch and pumps	6	121.1	10.18	0.003
male size x number of arch and pumps x removal of sperm packet	5	121.7	10.84	0.002
mating time x number of arch and pumps x male size	5	121.7	10.84	0.002
mating time x number of arch and pumps x removal of sperm packet	5	121.7	10.84	0.002
male order x mating time x removal of sperm packet	6	121.8	10.93	0.002
mating time x arch and pump x removal of sperm packet	5	124.6	13.72	0.001
mating time x male size x removal of sperm packet	5	122.7	11.8	0.001
male order x mating time x number of arch and pumps x male size x removal of sperm packet	8	124.5	13.61	0.001
mating time x number of arch and pumps x removal of sperm packet x male size	6	126.1	15.22	0
male order x mating time x number of arch and pumps x removal of sperm packet	7	126.2	15.28	0

Table 4.7 Variable importance; the sum of the weights of all models that include a variable.

	Male Order	Size of Male (g)	Number of Arch and Pumps	Mating Time (sec)	Removal of Sperm Packet
Importance:	0.88	0.82	0.21	0.13	0.12
N containing models	16	15	16	15	16

DISCUSSION

The analysis of 11 broods with five microsatellite markers confirmed the presence of multiple paternity in *Octopus oliveri.* The inclusion of both experimental broods and broods from females who had mated in the field further supported this hypothesis. Given the presence of multiple paternity in the deep sea octopus *Graneledone boreopacifica* (Voight and Feldheim 2009) and the shallow water *Octopus vulgaris* (Quintero et al. 2011), it is possible this reproductive strategy is conserved among octopods.

One of the benefits of multiple paternity is higher genetic diversity in the brood, making offspring survival more likely. Additionally, it is possible that females will mate with multiple males because the effort it would take to reject the males would put the females in more danger than mating. Another explanation for multiple mating events is that the female is trading up, or finding males with better sperm quality to fertilize their eggs. In the case of *Octopus oliveri*, this last explanation does not fit with the results in this study.

A limited number of studies have been done to measure precedence with microsatellites in cephalopods and those have focused predominantly on loliginid squids and cuttlefish (Voight

and Feldheim 2009; Naud et al. 2004; Quintero et al. 2011; Shaw 1997; Shaw and Sauer 2004; van Camp et al. 2004; Iwata and Munehara 2005; Buresch et al. 2009; Emery, Wilson, and Craig 2001). Last male precedence was found in two squid species: *Loligo bleekeri* and *Loligo vulgaris reynaudii* (Iwata and Munehara 2005; Shaw and Sauer 2004), and while it was predicted in the cuttlefish *Sepia apama,* no clear precedence was found (Naud et al. 2004). In both squid and cuttlefish, males can deposit sperm packets (spermatangia) either inside the mantle, or around the buccal mass surrounding the mouth. This means that there is both external and internal fertilization, where in octopuses there is only internal fertilization. Possibly because squid and cuttlefish mate in large aggregations, the last male to encounter the female is able to ensure his paternity by guarding her when the eggs are laid.

Contrary to the patterns of last male fertilization dominance in the above listed species, this study suggests that there may be first male precedence in *Octopus oliveri*; although because none of the females collected could be considered virgin females, the order and characteristics of males from previous matings cannot be determined. Nonetheless, there does appear to be skew among all the broods, indicating that some males are fertilizing more offspring than others. Certainly the last males to mate in the experimental broods sired less offspring than the first males.

One possible explanation for the variation in paternity rates among the unknown males in the field is sperm quality. Since it is unknown when the female mated with these previous males, it is possible that some of the sperm had been stored for a long enough time that it began to decrease in quality. Then, when the female had new males presented to her, specifically the first experimental male, these males were able to displace some of the low quality sperm of previous

males. If this were the case, it is also possible that the male could have overwhelmed the spermathecae with sperm, making later sperm depositions by other males more difficult.

It has been suggested that the ligula on the tip of the hectocotylus is used to remove sperm deposited by previous males (Quintero et al. 2011; Cigliano 1995). This seems unlikely at least in the case of *Octopus oliveri,* given that the last few males to mate were generally less successful in siring a large proportion of offspring. In addition, there was no clear distribution of sires among the individual strings or among the whole brood. This is in contrast to what was found in the squid *Loligo vulgaris reynaudii* (Shaw and Sauer 2004), where percentage of offspring sired by one male changed along the length of the egg strand.

Size was the only other variable that could be considered predictive in this study. Although size did not appear to influence the ability of a male to mate with a female in the behavioral experiments (see Chapter 3), the use of the microsatellite markers indicates larger males sire more offspring. This may be due to a number of different factors. The most likely scenario is that the large males have larger spermatophores and are sperm loading, or overwhelming the spermathecae with their sperm (Simmons and Fitzpatrick 2012). Because there was no significant positive correlation between the number of arch and pumps and the number of offspring sired, this would indicate that males of all sizes were transferring approximately the same number of spermatophores to the female. However, since there is skewed paternity in the broods analyzed in this study it may indicate that larger males contribute higher numbers of individual spermatozoa, thereby increasing their chances of fertilization over smaller males.

Among octopodids, spermatophore size is highly correlated with mantle length; the larger the mantle size, the larger the spermatophore, with few exceptions (Mann 1984). Small or mid-sized octopuses of the genus *Octopus* have approximately 100-300 spermatophores at one time in the Needham's sac (Voight 2001; Silva, Sobrino, and Ramos 2002; Voight 2009). Spermatophore production is continuous upon male maturity (size and age varies among species) with dozens formed each day (Wells 1978; Mangold 1987). Each spermatophore contains a sperm reservoir, which contains the individual spermatozoa. Voight (2001) found that the sperm reservoir length is tightly correlated with spermatophore length, indicating that males are incapable of manipulating the size (and therefore the number of spermatozoa) of the spermatophore to maximize the amount of sperm delivered to the female.

If spermatophore length is correlated with mantle length, it stands to reason that a larger male would have larger spermatophores and therefore a larger sperm reservoir. This is indeed the case in *Octopus americana* (Drew 1919), however, Mangold-Wirz (1963) found spermatophore lengths reach a maximum size regardless of mantle size in several species of octopus. Therefore, one cannot conclude definitively that in *Octopus oliveri* larger males would have larger spermatophores. Given that larger *Octopus oliveri* males did statistically sire more offspring than smaller males does suggest that those males may have used a strategy of sperm loading (Simmons and Fitzpatrick 2012). If all of the spermatophores were the same size between males, one would expect males to arch and pump as many times as possible during a mating trial. Then, regardless of size, the number of arch and pumps should be strongly correlated with the number of eggs sired by a particular male.

Unfortunately, spermatophore size was not measured in the males of this study. Further research into octopus mating should include analysis of spermatophore length in relation to mantle length to determine whether maximum spermatophore size is reached across all octopus species. This would indicate that sperm loading might be a sperm competition strategy in octopods.

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