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New insights about barnacle reproduction: Spermcast mating, aerial copulation and population genetic consequences

by

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Abstract

Barnacles are mostly hermaphroditic and they are believed to mate via copulation or, in a few species, by self-fertilization. However, isolated individuals of two species that are thought not to self-fertilize, *Pollicipes polymerus* and *Balanus glandula*, nonetheless carried fertilized embryo-masses. These observations raise the possibility that individuals may have been fertilized by waterborne sperm, a possibility that has never been seriously considered in barnacles. Using molecular tools (Single Nucleotide Polymorphisms; SNP), I examined spermcast mating in *P. polymerus* and *B. glandula* as well as *Chthamalus dalli* (which is reported to self-fertilize) in Barkley Sound, British Columbia, Canada. Embryo masses of isolated individuals and isolated pairs of all three species had alleles that were not present in the genome of the parents or of immediate partners, which indicates that spermcast mating occurred. However, the rate of fertilization by spermcasting was higher in *P. polymerus* (100% and 24% in isolated individuals and pairs, respectively), followed by *B. glandula* (100% and 7.7%) and *C. dalli* (70% and 9.1%). The relatively shorter size of the penis — and hence lower copulation rates — in *P. polymerus* compared to the other two species could favor higher rates of spermcasting. Moreover, lower apparent spermcasting in *C. dalli* could reflect a higher incidence of self-fertilization. Surveys of the sperm release process in *P. polymerus* using a belt-transect method, indicated that tidal and weather conditions did not affect sperm release, however, lower barnacle density (less opportunity for copulation) and higher wave action of the low shore (more chance to disperse sperm) compared to

high shores was associated with more sperm release. By videotaping *P. polymerus*, I found that — unlike all other known barnacles — they copulate mostly in air, during the incoming tides. Extended times of aerial copulation were observed in barnacles on less wave-exposed shores. Using mitochondrial DNA markers, I tested for genetic divergence among *P. polymerus* populations experiencing different wave exposures and shore heights that might be associated with observed differences in spermcasting and copulation. However, none of the analyses indicated any genetic differentiation among populations or between shore levels. These novel observations raise many questions about some long-established beliefs regarding barnacle reproductive biology.

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Chapter 1. General Introduction

1.1 Evolution of mating systems in sessile organisms

Evolution of different mating patterns has been the subject of interest and research among evolutionary biologists since Darwin's transformational ideas about sexual selection (Darwin, 1859). Two major evolutionary forces for sexual selection are female choice and male-male competition, which often yield significant morphological variation among species (Darwin, 1859). In most sedentary animals, where males do not have the chance to compete directly for females, male competition is limited to sperm production (Parker, 1982). Based on sex allocation theories, as mating group size (the number of potential mates; MGS) increases, the resource allotment to sperm production increases and vise versa, insofar as the minimum or no sperm production happens in isolated individuals (Charnov, 1982, 1987; Yamaguchi, et al., 2007; Yamaguchi, et al., 2008). Similarly, simultaneous hermaphroditism (where individuals bear the reproductive resources for both male and female function) arises when fitness declines when additional resources are allocated to either male or female reproduction (Charnov, 1982, 1987). Hermaphroditism has an obvious advantage for sedentary organisms or animals with limited motility because they can potentially mate with all of the likely limited number of individuals they encounter during their life (Ghiselin, 1969). Most sessile and slow-moving marine organisms are broadcast spawners (those that have external fertilization, such as some corals, polychaetes, echinoderms, etc.) or spermcasters (those that release

sperm into the water to fertilize eggs retained by remote individuals, such as many sponges, corals, bivalves, tunicates, etc.), and hermaphroditism is quite common among them (Bishop and Pemberton, 2006; Ghiselin, 1969). However, due to rapid dilution and limited life-span of sperm after release, fertilization success is generally lower in both groups than in those with direct copulation (Bishop, 1998), so hermaphroditism permits larger mating group sizes and therefore higher fitness. Self-fertilization rarely happens in hermaphroditic animals, though, especially in broadcasters or spermcasters that are not usually prone to many barriers to gamete dispersal. Cross-fertilization decreases inbreeding and increases gene flow and hence yields higher fitness in these organisms (Bishop and Pemberton, 2006; Ghiselin, 1969).

Nonetheless, not all sedentary animals are broadcasters or spermcasters. Barnacles are among the few sessile aquatic organisms that supposedly copulate with neighbors to transfer sperm using very long penises and yet they are mostly hermaphroditic (Addison and Hart, 2005). However, dioecy (large females and small attached pure males called dwarf males) and androdioecy (large hermaphrodites and small complemental males, which are protandrous hermaphrodites whose sexual growth stops at male stage) also exist among burrowing and parasitic species (Anderson, 1994; Yamaguchi, et al., 2007). Therefore, barnacles have received extensive attention for studies on the evolution of mating systems and sex allocation models from Darwin onwards (Barnes, 1992; Charnov, 1987; Darwin, 1851, 1854; Kelly and Sanford, 2010; Yamaguchi, et al., 2007; Yamaguchi, et al., 2008). Despite decades of such studies, several

questions about barnacles mating patterns remain unanswered. The focus of my thesis was answering such questions and unraveling some of the puzzling reproductive behaviors in a few species of intertidal barnacles.

Intriguingly, most hermaphroditic flowering plants also cross-fertilize by a process similar to spermcasting called pollination, in which pollen is carried to the stigma of other flowers of the same species by wind or animals (Raven and Johnson, 2002). Although some interesting parallels exist between pollination in plants and spermcast mating in animals, these will not be considered further.

1.2 Mating system of barnacles

Among crustaceans, barnacles are quite unusual. First, they are sessile for their entire postlarval lives (Anderson, 1994), whereas most postlarval free-living crustaceans are mobile. Second, most free-living barnacles are hermaphroditic (Anderson, 1994), whereas separate sexes are the rule in other Crustacea. As mentioned above, barnacles exhibit several different patterns of mating, but the superorder Thoracica — which includes the free-living, non-boring stalked (gooseneck) and acorn barnacles — are known mostly as simultaneous hermaphrodites. In hermaphroditic barnacles, individuals acting as females send chemical signals to their neighbors and functional males start searching for a potential female by exploratory movements of their well-developed penises that can reach up to eight times their body lengths (longest known penis size relative to the body length; Neufeld and Palmer, 2008). Males then deposit sperm into the females' mantle cavity; this pattern is usually called pseudo-copulation because sperm is not released inside the female reproductive organs of the mate

(Anderson, 1994). Females can usually be fertilized by more than one male (Charnov, 1982, 1987).

Curiously, some isolated individuals with no nearby potential mates, and in species that lack dwarf males, still have fertilized eggs. The ability to self-fertilize could be of great value here, as individuals that settle too far away from potential mates could still reproduce (Furman and Yule, 1990). Nonetheless, so far, self-fertilization has been confirmed only in a few species (Barnes and Crisp, 1956b; Barnes and Barnes, 1958; Furman and Yule, 1990; Klepal, 1990). Therefore, in the absence of self-fertilization, isolated individuals would have to obtain sperm from other sources, perhaps from the water, in a manner similar to spermcasters. However, this pattern has never been reported in barnacles. Presumably barnacles do not normally release sperm into the water, and normally unfertilized eggs only appear in the mantle cavity during or after copulation (Barnes and Crisp, 1956a). If it happens though, spermcast mating would permit larger mating group sizes because individual barnacles would not be restricted to copulating only with immediate neighbors. The main focus of my thesis was to test which mating pattern(s) — self-fertilization or spermcast mating — permit fertilization in isolated barnacles. The answer to this question could also have significant implications for our understanding of barnacle mating behavior in general. In addition to other factors, water currents could determine the success of reproductive behaviors including spermcast mating in intertidal organisms (Bishop, 1998). Depending on sperm features, wave action could either facilitate or hamper efficient dispersal of sperm.

1.3 Adaptive responses to flow regimes in intertidal organisms

Most intertidal organisms live under challenging environmental conditions. They need to resist the dislodging forces of strong waves during high tide and to tolerate desiccation and thermal stress during the low tide (Denny, 2006; Harley and Helmuth, 2003). Therefore, most of them develop different types of adaptations to survive such conditions. Many of these adaptations are plastic. For example, barnacles alter the form and size of their feeding legs and penis accordingly when transplanted to different flow regimes (Marchinko, 2003, 2007; Neufeld and Palmer, 2008; Neufeld and Rankine, 2012). Sea stars also change the length and thickness of their arms in response to differences in wave-exposure (Hayne and Palmer, 2013). Intertidal snails also increase the relative size of their shell apertures when exposed to extreme wave action (Trussell, 1997). Moreover, dispersal, settlement success and recruitment rates of the larvae of intertidal species are influenced by flow conditions (Gaylord, et al., 2013). So, wave action has a significant impact on the vertical distribution of shore organisms (Denny, 1987; Harley and Helmuth, 2003). Similarly, sperm dispersal in spermcasters also depends on wave action (Bishop, 1998). Spermcasting species might develop phenotypic or genetic adaptations in sperm form or reproductive behavior in response to variation in water flow to maximize mating success. Therefore, the study of mating systems in general — and spermcast mating in particular — in any intertidal species requires comparisons between wave-protected and wave-exposed shores.

Barnacles are among the most successful inhabitants of the rocky intertidal shores and they can be found in a broad range of wave conditions, from protected bays to highly wave-exposed shores (Newman and Abbott, 1980). Thus, their mating activities could be greatly affected by differences in water flow.

1.4 Experimental sites and study species

Intertidal shores of the northeastern Pacific are dominated by different barnacle species, including one stalked species, *Pollicipes polymerus* Sowerby 1833, and two acorn barnacles, *Balanus glandula* Darwin, 1854 and *Chthamalus dalli* Pilsbry, 1916 (Kozloff, 1996; Lamb and Hanby, 2005).

P. polymerus is unusual compared to other barnacles. It has a relatively short penis that is smaller than its feeding legs (Barnes, 1992; Darwin, 1851) and copulation between individuals has never been directly observed even after hours of monitoring them under water (Barnes, 1992, 1996). Yet, solitary individuals in the aquarium never contained fertilized eggs, so, self-fertilization has been rejected in *P. polymerus* (Hilgard, 1960; Strathmann, 1987). Therefore, all reports of the copulation incidence in *P. polymerus* are based on the observations of fertilized eggs inside mantle cavities.

B. glandula and *C. dalli*, on the other hand, bear some of the longest known penises among barnacles that can reach up to seven times their body length (Barazandeh, et al., In Press; Neufeld and Palmer, 2008). Copulation commonly happens in both species, however self-fertilization has also been reported in *C. dalli* (Barnes and Barnes, 1958).

Isolated individuals of all three species, which have no neighbors within penis range, sometimes bear fertilized eggs (personal observations, 2009-2013), which is surprising for *P. polymerus* and *B. glandula* that are thought not to self-fertilize. These three species were selected for study because they provide a good range of variation in mating patterns.

All the fieldwork was conducted in Barkley Sound, west coast Vancouver Island, British Columbia, Canada using the facilities and supervision provided by Dr. Rich Palmer at Bamfield Marine Sciences Centre.

1.5 Thesis chapters overview

In **Chapter 2**, I examined the possibility of spermcast mating and self-fertilization in *Pollicipes polymerus* from one highly wave-exposed and one moderately wave-exposed shore. Molecular markers provide a powerful tool for studying mating systems. So, in this study, I developed and used Single Nucleotide Polymorphism (SNP) markers to determine the source of sperm for fertilized embryo-masses of isolated individuals and isolated pairs (two adjacent individuals far away from any other barnacles). The goal here was to test whether isolated individuals obtain sperm from the water or self-fertilize. Also, isolated pairs were examined to test whether they a) mate exclusively with each other, b) self-fertilize, or c) capture sperm from the water. I found that spermcast mating commonly happens in isolated individuals and pairs of *P. polymerus* because many embryo-masses contained alleles that were absent from the genome of the parents and adjacent partners. No individuals exclusively self-fertilized, however I could not reject the possibility of some self-fertilization with the available data.

In **Chapter 3**, I tested whether self-fertilization or spermcast mating occurred in isolated individuals and pairs of *Balanus glandula* and *Chthamalus dalli* from one wave-exposed and one wave-protected shore using a similar method to that used for *P. polymerus*. Using SNP markers, I found that spermcast mating occasionally happens in both species, however to a lesser extent than in *P. polymerus*. Some molecular evidence of self-fertilization was also observed in *C. dalli*.

In **Chapter 4**, I conducted extensive descriptive field studies on the reproductive behavior of *P. polymerus*. The primary goals were to determine: 1) whether, in addition to spermcast mating, they also copulate with neighbors, 2) the frequencies of spermcast mating and copulation in the field, 3) what environmental factors might influence mating behavior. Using a belt transect method, I monitored sperm-leakage at low tide in continuous populations of *P. polymerus* at two highly wave-exposed and two moderately wave-exposed sites. The same populations were also videotaped as the tide was rising to record mating behavior. At all sites monitored and in almost every day, some barnacles were leaking sperm at low tide. I was also able to record the incidence of copulation in *P. polymerus* for the first time. Unlike other barnacles that copulate while completely immersed (Anderson, 1994), *P. polymerus* start copulating with neighbors shortly after their opercular plates are contacted by the first breaking waves as the tide comes in, and they copulate much less frequently when completely immersed. Copulation happened more often than sperm-leakage and I

detected no significant impact of environmental factors (weather, tide time and height, swell heights, etc.) on the incidence of sperm-leakage.

In **Chapter 5**, I tested for potential genetic structure among *P. polymerus* populations. Both larval and sperm dispersal in marine sessile organisms depend on wave action (Bishop, 1998; Gouhier, et al., 2010). Therefore, different mating behaviors might be selected for in different flow regimes, which could yield genetic differentiation among populations. I tested eight barnacle populations from the high shore and low shore of two highly wave-exposed and two moderately wave-protected sites using two mitochondrial genes (COI and D-loop). None of the analyses revealed any significant genetic diversity among local populations.

The findings of these four data chapters have the potential to overturn some long-established and generally accepted beliefs about barnacle reproductive biology and to increase the current knowledge about the population genetic structure of intertidal animals. In **Chapter 6** my findings are placed in a bigger context and I provide suggestions for future research.

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Chapter 2. Something Darwin didn't know about barnacles: Spermcast mating in a common stalked species[1]

2.1 Introduction

Textbooks note two bizarre observations about typical stalked and acorn barnacles (Thoracica). First, unlike nearly all free-living arthropods, adults are sessile and typically glued to hard surfaces (Anderson, 1994). Second, they appear to be constrained by their evolutionary history to exchange sperm either by a form of copulation called pseudo-copulation (release of sperm into a partner's mantle cavity) or, in hermaphrodites, by self-fertilization (Barnes and Crisp, 1956; Barnes, 1992; Charnov, 1987). Ever since Darwin's epic monographs (Darwin, 1851, 1854), these beliefs about possible modes of barnacle mating have been strongly held (Charnov, 1987; Høeg, 1995). Unfortunately, although pseudo-copulation is readily observable in many species (Barnes, 1992; Darwin, 1851), self-fertilization is almost always inferred from observations of isolated individuals bearing fertilized embryos (Barnes and Crisp, 1956; Barnes and Barnes, 1958; Charnov, 1987; Desai, et al., 2006; Elkomi and Kajihara, 1991; Furman and Yule, 1990; Hilgard, 1960; Kent, et al., 2003; Santos and Bueno, 2002).

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Obligate pseudo-copulation imposes two significant constraints on mating success in a sessile species: 1) fertilization is limited to immediate neighbors, and 2) individuals with no immediate neighbors can't reproduce, except by self-fertilization. As a consequence, barnacles are famous for their long penises, including some of the longest in animals, relative to their body size (Hoch and Yuen, 2009; Neufeld and Palmer, 2008).

Not all barnacles have long penises, however. Species of *Pollicipes* are unusual among thoracican barnacles because their penises are shorter than their feeding legs (Figure 2.1a), not very extensible, and vary little in size throughout the breeding season (Barnes, 1992; Darwin, 1851). *Pollicipes polymerus* Sowerby 1833 is a hermaphroditic, stalked barnacle that inhabits wave-exposed intertidal shores of the Northeast Pacific, where it typically occurs in dense clusters or beds (Barnes, 1996; Hoffman, 1989). However, solitary individuals or small groups are not uncommon (personal observations).

Spermcast mating — where sperm released into the water by males fertilize eggs retained in the body of a partner — has been reported in many sessile or sedentary marine invertebrates, including sponges, cnidarians, polychaetes, bivalved molluscs, entoprocts, ectoprocts, brachiopods, pterobranch hemichordates and colonial ascidians (Bishop and Pemberton, 2006). Despite being sessile, however, spermcast mating has never been considered possible in barnacles (Barnes, 1989; Bishop and Pemberton, 2006).

In typical hermaphroditic barnacles, a functional male searches for partners by random penis movements and then deposits sperm into the partner's mantle

cavity. A functional female can probably be fertilized by more than one male (Charnov, 1982, 1987). To copulate, though, a barnacle must be within penis range of at least one neighbor. Nonetheless, isolated individuals too distant to copulate sometimes bear developing embryos in many barnacle species in the field and laboratory, which has led many authors to conclude that self-fertilization must have occurred (Barnes and Crisp, 1956; Barnes and Barnes, 1958; Charnov, 1987; Desai, et al., 2006; Elkomi and Kajihara, 1991; Furman and Yule, 1990; Hilgard, 1960; Kent, et al., 2003; Santos and Bueno, 2002).

All attempts to observe fertilization in solitary *P. polymerus* in the laboratory have failed (Hilgard, 1960; Strathmann, 1987). Even more curiously, although pseudo-copulation has been directly observed in the field or in the laboratory in many other thoracican barnacles, it has never been reported in *P. polymerus* despite multiple attempts to observe it (Barnes, 1992, 1996). Thus, pseudo-copulation is presumed to occur in this species based only on the presence of sperm in the mantle cavity (Barnes, 1996; Hilgard, 1960).

Significantly, we observed occasional *P. polymerus* individuals in the field leaking sperm between their opercular plates during low tide (Figure 2.1b). In addition, the occurrence of isolated yet fertilized individuals (Hilgard, 1960) raises three important questions about this species: 1) Can sperm leaked into the water fertilize eggs in distant individuals? 2) Do fertilized eggs in isolated individuals arise from self-fertilization or from sperm captured from the water? 3) When a barnacle has a single neighbor within copulation range, does it mate only

with that neighbor or are some eggs fertilized by sperm captured from distant individuals?

2.2 Materials and Methods

2.2.1 Barnacles sampling and measurements

Pollicipes polymerus were collected from shores of intermediate (Helby Island, 48.847°N, 125.168°W) and high wave-exposure (Seppings Island, 48.841°N, 125.209°W) near the Bamfield Marine Sciences Centre in Barkley Sound, British Columbia (Canada) in August 2009 and August 2010. Maximum velocities of breaking waves at the exposed site are nearly twice as high as the intermediate site during the calm summer months of July and August (Arsenault, et al., 2001).

We sampled and scored 599 individuals for body length, fertilization status, distance to a nearest neighbor and nearest-neighbor body length. Body length was measured as rostro-carinal opercular length: linear distance between the scutal plate anterior margin and tergal plate posterior margin parallel to the gape. Distances between neighbors were measured from the mid-point of the capitulum of each neighbor (where the scutal-tergal plate suture intersects the gape). Degree of isolation was defined as the ratio of nearest neighbour distance to body (rostro-carinal) length of a given individual. We considered an individual fully isolated if it was more than two body lengths from its nearest neighbor (roughly three times the maximum reach of the extended penis, see below).

We collected 37 isolated individuals bearing embryo masses. To avoid potential problems posed by individuals with supple stalks we only sampled

individuals with short stalks, or wedged tightly into a crevice or between mussels. We did not sample individuals with juveniles attached to their stalks, even when juveniles were well below the minimum size of sexual maturity (approx. 4 mm) (Barnes, 1996). We also collected 34 individuals found as isolated pairs: adjacent individuals where both were more than two body lengths from any other conspecifics where at least one individual carried embryo masses.

We removed the stalks of adult barnacles and the embryo masses, placed them individually into 70%-95% ethanol in labeled 1.5 ml microfuge tubes, and stored them at -20°C until DNA extraction.

2.2.2 Penis measurements

We measured relaxed and extended penis length in *P. polymerus* and in a common, sympatric acorn barnacle, *Balanus glandula* Darwin 1854. Relaxed penis length was measured by freezing barnacles, thawing them in seawater, carefully extending and photographing the penis under a microscope (or the whole animal under water, as for Figure 2.1a), and then digitally measuring length using Image-J (Rasband, 1997–2012). Extended penis length was measured using a gravity-fed pressure system for inflation (Neufeld and Palmer, 2008) and used to calculate extension ratios (extended length / relaxed length).

2.2.3 Marker development and genotyping

Numerous attempts to develop microsatellite markers for *Pollicipes polymerus* were not successful, so we developed Single Nucleotide Polymorphism (SNP) markers to assess genetic relationships between embryo masses and potential parents (Appendix1, Barazandeh and Davis, 2012). We used a pool of

five *P. polymerus* individuals to construct a genomic library, selected 30 contigs with the largest product sizes, and then amplified them in seven individuals. Twenty-two putative SNP loci, each with two alleles, were identified where the rare allele was observed in at least two of seven individuals and at least one heterozygote was observed. Among those 22, 16 SNP markers amplified successfully (Appendix1, Barazandeh and Davis, 2012; Rozen and Skaletsky, 2000). Both adults and embryo masses were genotyped using the ABI PRISM$^{@}$ SNaPshot^{TM} Multiplex Kit and the results were scored using GeneMapper, v4.0 (ABI) (Appendix 1, Barazandeh and Davis, 2012; Filippini, et al., 2007; Kaderali, et al., 2003; Lindblad-Toh, et al., 2000; Rozen and Skaletsky, 2000). Although we were unable to genotype individual embryos, we tested how widespread non-parent alleles were among embryos within an egg mass for six isolated individuals by genotyping eight additional sub-samples from different regions of the embryo lamellae.

An embryonic allele was called a non-parent allele if it was absent from the brooding parent in isolated individuals or absent from both the brooding parent and the adjacent partner in isolated pairs.

2.3 Results

2.3.1 Penis measurements

The penis of *Pollicipes polymerus* was significantly shorter and less extensible than that of *Balanus glandula*. The ratio of relaxed penis length to soft body length was 0.57 ± 0.025 (mean ± SE, N= 10) for *P. polymerus* and 1.39 ± 0.059 (N= 10) for *B. glandula*, and measured extension ratios (extended length

(Neufeld and Palmer, 2008) / relaxed length) were, respectively, 1.21 ± 0.081 (N=3) and 2.26 ± 0.102 (N=10). Therefore, the penis of *P. polymerus* does not even extend to one body length (maximum extension of 0.69 body lengths) whereas a *B. glandula* penis can reach nearly three times its body length (maximum extension of 3.14 body lengths).

2.3.2 Fertilization/isolation measurements

The smallest fertilized *P. polymerus* we observed had a rostro-carinal length of 11 mm and we found no fertilized individuals beyond 7.14 body lengths (approx. 14 cm) from a nearest neighbor (N= 63; Figure 2.2). The proportions of fertilized individuals in clumps of barnacles did not differ significantly between the wave-exposed and intermediate site (X^2 test, p= 0.47) so these data were pooled.

We confirmed results from an earlier study (Hilgard, 1960) that fertilization incidence declines with increasing distance to nearest neighbor in *P. polymerus* in the field (Figure 2.2). Significantly, these results clearly reveal that some fertilized *P. polymerus* were well outside the extended penis range from any neighbor (i.e., beyond two body lengths).

2.3.3 Genotyping results

Embryo masses from all 37 isolated individuals had non-parent alleles at one or more loci (Figure 2.3), and all but one had non-parent alleles at two or more loci (Figure 2.3; Table 2.1). All eight replicate subsamples of egg masses from four of the subsampled barnacles exhibited the same multilocus genotype as the original egg mass sample (Table 2.2). In the two remaining individuals, the

multilocus genotype of all eight subsamples was identical to the original sample at 15 of 16 loci. In both cases the allele that was not present in a subsample (in three of eight or all eight subsamples, respectively) was the rare allele. By re-scoring the genotypes of all individuals and egg masses at all loci, we estimated an average allelic scoring error rate (number of allelic mismatches / total number of alleles scored in all individuals) of 0.26%, which is 100 times lower than the average rate of non-parent alleles observed in egg masses (27.4%; range 6.3-62.5%).

Among egg masses from 34 individuals from isolated pairs, eight (24%) also carried SNP alleles not present in either parent (Figure 2.3). In these eight cases, on average 13.2% of alleles were non-parent alleles (range 6.3-25.0%); roughly 50 times the genotyping error rate.

The average frequency of the dominant allele across all loci was 84% (range 62-97%; Table 2.1). Among 120 pairs of 16 SNP loci, only two exhibited significant linkage disequilibrium (Pp190-1 and 190-2, Pp117-2 and 117-5; $P \leq 0.0004$) (Appendix1, Barazandeh and Davis, 2012).

2.4 Discussion

Although crustaceans are generally thought to copulate somehow (Addison and Hart, 2005) our observations confirm that a significant fraction of eggs of isolated *Pollicipes polymerus* were fertilized by sperm released by individuals beyond copulation range. Water is the most likely source for these non-parent alleles. Although we cannot rule out partial self-fertilization, our results reveal a) that self-fertilization is not necessary for isolated individuals and b) that non-

parent alleles are widespread throughout embryo masses. Even more remarkably, in isolated pairs where both partners were within penis range, many embryos throughout the embryo mass were still fertilized by sperm from the water in 24% of individuals. Therefore — quite contrary to all prior expectations about mating in barnacles — *P. polymerus* appear able to obtain sperm from the water in the field and do so even when an adjacent partner is available.

Our estimates of sperm capture rates are necessarily underestimates, perhaps by a large margin. Many instances of sperm capture would not have been detected because the overall frequency of the dominant allele exceeded 80% at 11 of 16 SNP loci (Table 2.1). Unfortunately, we could not determine the number of males contributing to a given embryo mass because of a) the high cost and difficulty of extracting enough DNA from individual embryos, especially early stage embryos, and b) the limited power of SNP loci to detect paternity. Significantly, however, we did observe the same multilocus genotype among all eight subsamples from four of the six subsampled egg masses. Furthermore, in each of the remaining two subsampled egg masses, only one of 16 SNP loci differed from the original genotype and in both cases the allele missing from some subsamples was the rare SNP allele (Table 2.2). Non-parent alleles were therefore widespread throughout individual embryo masses and were not simply an artifact of PCR amplification of a few stray captured sperm. This observation further reduces the likelihood that these embryo masses arose primarily from self-fertilization. We are also confident in our non-parent embryo counts because our use of multiple loci decreased the chance that parents and egg masses would appear identical when truly different

(Kalinowski, et al., 2006). In addition, our allele-scoring error rate was very low (0.26%), so our counts of non-parent alleles were not inflated by genotyping errors. Finally, because embryos from all isolated individuals had one or more non-parent alleles, *P. polymerus* are clearly able to obtain sperm from the water, even if it is not the primary mode of mating in this species.

Three alternative hypotheses for how these isolated individuals might have been fertilized can be dismissed. First, fertilization by an adjacent individual that subsequently died is highly unlikely. *P. polymerus* eggs require around 25 days to hatch after fertilization (Lewis, 1975). Stalk remnants or footprints of a dead barnacle, which are readily visible, typically require longer than this to degrade and we observed no such remnants near any barnacle we sampled. Second, we took great care to avoid sampling individuals that might have been able to reposition themselves with their flexible stalk. Third, although *P. polymerus* is reportedly a hermaphrodite with no dwarf males (Anderson, 1994), we nonetheless avoided sampling any barnacles with attached 'juveniles' on their stalks, no matter how small.

Pollicipes polymerus therefore appear to be capable of spermcast mating, a type of mating seen in at least nine other phyla of sessile or sedentary marine invertebrates (Bishop and Pemberton, 2006) but never before reported for barnacles. At present, we cannot say how prevalent spermcast mating is compared to pseudo-copulation in this species because we only sampled isolated individuals or isolated pairs — pseudo-copulation may still be an important mode of sperm transfer in dense clumps. Nonetheless, we can conclude that spermcast mating can

and does occur. We also do not yet know whether the primary mode of sperm release is: a) leakage of sperm at low tide (Figure 2.1b), b) active ejection of sperm when immersed, or c) leakage of sperm from mated individuals because pseudo-copulation is "sloppy". For example, in barnacle pseudo-copulation sperm are deposited into the mantle cavity (Anderson, 1994) and subsequent feeding or respiratory movements would likely cause some leakage from a recently mated individual. Given the high overall densities of *P. polymerus* — well over 1000 m^{-2} in suitable habitat (Barnes, 1996; Hilgard, 1960) — free sperm may therefore be widely available in the water. In addition, because *P. polymerus* feed by extending feeding legs into the backwash after waves break (Barnes, 1996), sperm leaked out at low tide (Figure 2.1b) could readily be captured from the backwash of the first waves to reach that height on the shore on an incoming tide.

Even though mating systems vary considerably among stalked barnacles (Anderson, 1994; Yusa, et al., 2012), capture of sperm from the water adds a wholly new potential mode to the array. We do recognize that *Pollicipes polymerus* is somewhat unusual: it has a relatively short and inextensible penis (see (a) *Penis measurements* above) and it lives in an extreme physical environment (wave-swept rocky shores Barnes, 1996; Hoffman, 1989), so sperm capture may be more likely in this species than in others. Nonetheless, our observation of sperm capture is significant for three reasons. First, it challenges the widely held belief that some form of copulation is obligatory when crustaceans mate (Addison and Hart, 2005). Second, it raises doubts about prior reports of self-fertilization in isolated barnacles (Barnes and Crisp, 1956; Barnes

and Barnes, 1958; Desai, et al., 2006; Elkomi and Kajihara, 1991; Furman and Yule, 1990; Hilgard, 1960; Kent, et al., 2003; Santos and Bueno, 2002), because spermcast mating was not considered a possibility. Third, if spermcast mating occurs — even occasionally — in other thoracican barnacle species, some re-thinking of barnacle reproductive biology may be required. Depending on the prevalence of sperm capture in other species, population genetic (Kent, et al., 2003; Véliz, et al., 2006) and sex allocation (Charnov, 1982, 1987) models for barnacles may need to be revised because functional females a) may be fertilized by many more males than previously thought, b) will not be restricted to mating with immediate neighbors, and c) can still mate even if no neighbors are within copulation range. We are currently testing whether spermcast mating occurs in two acorn barnacles (*Balanus glandula* Darwin, 1854 and *Chthamalus dalli* Pilsbry, 1916). In another acorn barnacle, *Tetraclita rubescens* Darwin, 1854, individuals appear to mate reciprocally with their nearest neighbor, occasionally even at distances of multiple body lengths (Kelly, et al., 2012). But only 17 out of 130 broods (13%) were fertilized by more than one father — even when many potential mates were nearby — and 14 of these were fertilized by only two fathers. So capture of sperm from the water may not occur in *T. rubescens*.

Finally, much of interest remains to be learned about spermcast mating in barnacles. Three obvious questions arise for *Pollicipes polymerus*: 1) How prevalent is spermcast mating compared to pseudo-copulation under normal conditions? 2) How are sperm released and captured? 3) Is behavior, limb form, sperm form (Healy and Anderson, 1990) or ejaculate form, modified to increase

the success of sperm capture? Intriguing questions are also raised about how widespread this surprising ability is in other barnacles. For example, several species have relatively short penises (e.g., shorter than the feeding legs), including stalked barnacles like *Scalpellum* (Darwin, 1851) and acorn barnacles like *Pachylasma* and *Octomeris* (Darwin, 1854). Clearly further work is needed to test how often spermcast mating supplements pseudo-copulation in other species.

Table 2.1 Multilocus genotypes of isolated individuals and isolated pairs and their egg masses of *Pollicipes polymerus*. Red circles indicate a locus where the egg mass genotype contained an allele not present in the brooding parent. Blue circles indicate a locus where the egg mass genotype contained an allele not present in either parent of the pair.

Isolated Individuals + Egg Masses*	Individuals†	Next Nearest Neighbor Distance (mm)	Next Nearest Neighbor Body Length	Ratio of Next Nearest Neighbor Distance to Body Length	Locus																Number of Loci with Non-Parental Alleles
					12a-3	11-1	105-1	172-6	190-1	107-2	117-1	117-5	151-2	105-2	107-3	151-3	172-5	190-2	117-2	119-1	
1	Ad 1	31	14	2.21	G G	G G	T T	T T	A A	T T	G G	G A	C C	C C	C C	G G	T T	G G	C C	T T	
	Em 1				G G	G G	T T	C T	A A	C T	G T	G A	C A	C C	C T	G G	C T	G T	C C	C T	8
2	Ad 1	35	10	3.50	G G	G G	T T	T T	A A	C C	T T	G G	A A	C C	C T	G C	T T	G T	C C	T T	
	Em 1				G G	G G	T T	T T	A A	C C	T T	G A	A A	C C	C T	G C	C T	G T	C C	T T	2
3	Ad 1	40	19	2.11	G G	G G	T T	T T	A A	C T	G G	A A	A A	C C	C T	G G	C T	G G	C T	C T	
	Em 1				G G	G G	G T	T T	A A	C T	G T	G A	A A	C C	C T	G C	C T	G G	C T	C T	4
4	Ad 1	40	15	2.67	G A	G G	T T	C C	A A	C C	G T	G A	A A	C C	C C	G G	T T	G G	C C	T T	
	Em 1				G A	G G	T T	C C	A A	C C	G T	G A	A A	C C	C C	G G	C T	G G	C C	T T	1
5	Ad 1	40	18	2.22	G G	G G	T T	T T	A A	C C	G T	A A	A A	C C	C C	G G	T T	G G	C T	C C	
	Em 1				G A	G G	G T	T T	A A	C T	G T	G A	A A	C C	C C	G G	C T	G G	C T	C T	6
6	Ad 1	40	10	4.00	G G	G G	T T	C T	G A	C C	G T	G G	C A	C C	C T	G G	T T	G G	C T	C T	
	Em 1				G G	G A	G T	C T	G A	C C	G T	G G	C A	A C	C T	G G	C T	G T	C T	C T	5
7	Ad 1	45	19	2.37	G A	G G	T T	C T		C C	G G	G A	C A	C C	C C	G G	C T	G T	C T	T T	
	Em 1				G A	G A	G T	C T	G A	C T	G T	G A	C A	C C	C C	G G	C T	G T	C T	T T	4
8	Ad 1	45	20	2.25	G G	G G	G T	T T	A A	C C	G G	G A	C A	C C	C C	G G	T T	G G	C T	T T	
	Em 1				G G	G G	G T	C T	G A	C C	G T	G A	C A	C C	C C	G G	T T	G T	C T	T T	4
9	Ad 1	45	18	2.50	G A	G G	T T	T T	A A	C T	G T	A A	A A	C C	C C	G G	C C	G G	C C	T T	
	Em 1				G A	G A	G T	T T	A A	C T	G T	A A	A A	C C	C C	G G	C C	G T	C C	C T	4
10	Ad 1	45	15	3.00	G G	G G	G T	T T	A A	C C	G G	A A	A A	A C	C T	G G	T T	G G	C T	T T	
	Em 1				G A	G G	G T	T T	G A	C T	G T	G A	A A	A C	C T	G G	T T	G T	C T	T T	6
11	Ad 1	50	23	2.17	G A	G G	G T	T T	G A	C C	G G	A A	A A	C C	C T	G G	C T	-	C C	T T	
	Em 1				G A	G G	G T	C T	G A	C T	G G	G A	A A	C C	C T	G G	C T	G T	C T	T T	4
12	Ad 1	50	22	2.27	G G	G G	T T	C C	A A	C T	G G	G G	A A	C C	C C	G G	T T	G G	T T	T T	
	Em 1				G A	G G	G T	C T	G A	C T	G T	G A	A A	C C	C T	G G	C T	G T	C T	T T	10
13	Ad 1	50	25	2.00	G G	A A	T T	T T	A A	T T	G G	G A	A A	C C	C C	G G	C T	G G			
	Em 1				G G	G A	T T	T T	A A	C T	G T	G A	A A	C C	C C	G G	C T	G T	C C	T T	4
14	Ad 1	50	20	2.50	G A	A A	T T	T T	A A	C T	G G	G A	A A	C C	C C	G G	T T	G G	C T	T T	
	Em 1				G A	G A	G T	C T	G A	C T	G G	G A	A A	C C	C C	G G	C T	G G	C T	C T	7
15	Ad 1	50	9	5.56	G G	G G	T T	T T	A A	C T	T T	G A	C A	C C	C C	G G	C T	G G	C C	T T	
	Em 1				G G	G G	T T	T T	A A	C T	G T	G A	C A	C C	C C	G G	C T	G G	C T	T T	2
16	Ad 1	50	16	3.13	G G	A A	T T	C T	A A	C C	G G	G A	A A	C C	C C	G G	T T	G G	C C	T T	
	Em 1				G G	G A	G T	C T	A A	C T	G G	G A	A A	C C	C C	G C	T T	G T	C C	C T	6
17	Ad 1	55	21	2.62	G G	G G	G C	C A	A C	C G	G A	A A	C C	C T	G G	C T	G G	C C	T T		
	Em 1				G A	G G	G T	C T	A A	C T	G G	A A	A A	C C	C C	G G	C T	G G	C C	T T	4
18	Ad 1	55	20	2.75	G A	G G	T T	C T	A A	C T	G G	A A	A A	C C	C C	G G	T T	G G	C C	T T	
	Em 1				G A	G G	G T	C T	A A	C T	G G	A A	A A	C C	C C	G G	C T	G G	C C	T T	2
19	Ad 1	55	12	4.58	G G	G G	T T	T T	A A	C C	G G	G G	A A	C C	C C	G G	C T	G G	C T	T T	
	Em 1				G G	G G	G T	T T	A A	C T	G G	G A	A A	C C	C T	G G	C C	T T	G G	C T	4
20	Ad 1	60	16	3.75	G G	G G	T T	T T	A A	C C	G G	G G	A A	C C	C C	G G	T T	G G	C T	T T	
	Em 1				G G	G G	G T	C T	A A	C C	G G	G A	A A	C C	C C	G G	C T	G G	C T	C T	5
21	Ad 1	60	16	3.75	G G	G G	G T	C T	A A	C C	G G	A A	-	C C	C C	G G	C T	G G	C C	C T	
	Em 1				G A	G G	G T	C T	A A	C T	G G	G A	C A	C C	C T	G G	C T	G G	C T	C T	5
22	Ad 1	60	10	6.00	G G	G G	T T	T T	A A	C C	G T	G A	A A	C C	C T	G G	T T	G G	C C	T T	
	Em 1				G G	G A	T T	C T	A A	C C	G T	G A	A A	C C	C T	G G	C T	G G	C C	T T	3
23	Ad 1	60	18	3.33	G G	G G	T T	T T	A A	C C	G G	G G	A A	C C	C C	G G	T T	G G	C T	T T	
	Em 1				G G	G G	G T	C T	A A	C C	G T	G G	A A	C C	C C	G G	T T	G G	C T	T T	3
24	Ad 1	60	22	2.73	G G	G G	T T	T T	A A	C C	G G	A A	A A	C C	C T	G G	T T	G G	C C	T T	
	Em 1				G G	G G	T T	T T	A A	C C	G G	G A	A A	C C	C T	G G	T T	G G	C T	T T	2
25	Ad 1	70	20	3.50	G A	G G	G T	C T	A A	C T	G T	G G	C C	C C	C C	G G	C T	G G	C C	T T	
	Em 1				G A	G G	G T	C T	A A	C T	G T	G A	C A	C C	C C	G G	C T	G G	C T	T T	3
26	Ad 1	70	24	2.92	G G	G G	G T	C T	A A	C C	G G	G A	A A	C C	C C	G G	C T	G G	C T	C T	
	Em 1				G G	G G	G T	C T	A A	C T	G G	G A	C A	C C	C T	G G	C T	G G	C T	C T	3
27	Ad 1	70	21	3.33	G G	G G	G T	C T	A A	T T	G G	G A	A A	C C	C C	G G	T T	G G	C C	C T	
	Em 1				G G	G G	G T	C T	A A	C T	G G	G A	A A	A C	C T	G G	C T	G G	C T	C T	5
28	Ad 1	75	18	4.17	G A	G G	G T	T T	A A	C C	G T	G G	A A	C C	C C	G G	C T	G G	C T	T T	
	Em 1				G A	G G	G T	C T	A A	C C	G T	G G	A A	C C	C C	G G	C T	G G	C T	C T	2
29	Ad 1	75	18	4.17	G G	G G	T T	T T	A A	C C	G G	A A	A A	C C	C C	G G	T T	G T	C C	C T	
	Em 1				G G	G G	T T	T T	G A	C T	G G	G A	A A	C C	C C	G G	C T	G T	C C	C T	4
30	Ad 1	77	16	4.81	G G	G G	T T	C C	A A	C T	G T	G G	A A	C C	C C	G G	T T	G G	C C	C T	
	Em 1				G A	G G	T T	C T	A A	C T	G T	G A	C A	C C	C C	G G	C T	G T	C C	C T	6
31	Ad 1	80	20	4.00	G G	G G	T T	C T	G A	C C	G T	G G	A A	C C	C C	G G	C T	T T	C C	T T	
	Em 1				G A	G A	G T	C T	G A	C T	G T	G A	C A	C C	C C	G G	C T	G T	C T	T T	6
32	Ad 1	80	19	4.21	G G	G G	T T	T T	A A	C T	G G	G G	A A	C C	C C	G G	C C	G G	C T	C T	
	Em 1				G G	G G	T T	T T	A A	C T	G T	G A	A A	C C	C C	G G	C T	G G	C T	C T	3
33	Ad 1	80	19	4.21	G A	A A	T T	C T	A A	C C	T T	G G	A A	C C	C C	G G	C T	G G	C C	T T	
	Em 1				G A	G A	G T	C T	A A	C C	G T	G A	A A	C C	C C	G G	C T	G T	C C	C T	6
34	Ad 1	85	19	4.47	G G	G G	T T	T T	A A	C T	G G	G A	C A	C C	C C	G G	T T	G G	C C	T T	
	Em 1				G G	G G	T T	C T	A A	C T	G T	G A	C A	A C	C C	G G	C T	G G	C T	T T	5
35	Ad 1	85	23	3.70	G G	G G	G T	T T	A A	C T	G G	G A	A A	C C	C C	G G	C C	G G	C T	T T	
	Em 1				G A	G A	G T	T T	G A	C T	G T	G A	A A	C C	C C	G G	C T	G T	C T	C T	7
36	Ad 1	95	17	5.59	G G	G G	G T	T T	A A	C T	G G	G A	A A	C C	C C	G G	T T	G G	C C	C T	
	Em 1				G G	G G	G T	C T	A A	C T	G T	G A	A A	C C	C C	G C	T T	G G	C C	C T	3
37	Ad 1	100	14	7.14	G G	G G			C T	A A	C T	G T	A A	A C	C C	G G	T T	G G	C C	T T	
	Em 1				G A	G G	G G	C T	A A	C T	G T	G A	A A	C C	C T	G G	C T	G G	C T	T T	5

Table 2.1. Continued.

Isolated Pairs + Egg Masses**	Individuals†	Partner Distance (mm)	Partner Body Length (mm)	Ratio of Partner Distance to Body Length	Next Nearest Neighbor Distance (mm)	Next Nearest Neighbor Body Length (mm)	Ratio of Next Nearest Neighbor Distance to Body Length	Locus																		Number of Loci with Non-Parental Alleles
								12a-3	11-1	105-1	172-6	190-1	107-2	117-1	117-5	151-2	105-2	107-3	151-3	172-5	190-2	117-2	119-1	
1	Ad 1	10	22	0.45	75	20	3.75	G A	G G	G G	T T	A A	C C	G G	G A	A A	C C	C C	G G	T T	G G	C T	T T	
	Em 1							G A	G A	G T	T T	A A	C C	G T	G A	A A	C C	C C	G G	C T	G G	C T	T T	4
	Ad 2	10	20	0.50				G G	G A	G T	T T	A A	C C	G T	G G	A A	C C	C C	G G	C T	G G	C T	T T	
2	Ad 1	10	18	0.56	110	23	4.78	G A	G G	T T	C C	A A	C T	G G	G A	A A	A C	C C	G G	T T	G G	C C	T T	
	Em 1							G A	G G	G T	C T	A A	C T	G T	G A	A A	A C	C T	G G	C T	G G	C C	C T	6
	Ad 2	10	22	0.45				A A	G G	T T	T T	A A	C T	G G	G A	A A	C C	C T	G G	C T	G G	C C	C T	
	Em 2							G A	G G	T T	C T	A A	C T	G G	G A	A A	A C	C T	G G	C T	G G	C C	C T	3
3	Ad 1	10	21	0.48	115	18	6.39	A A	G G	T T	T T	A A	C C	T T	G A	A A	C C	C C	G G	C C	G G	C C	T T	
	Em 1							G A	G G	T T	T T	G A	C C	G T	G A	C A	C C	C T	G G	C T	G T	C C	T T	7
	Ad 2	10	16	0.63				G A	G G	T T	T T	G A	C C	G G	G G	C A	C C	C T	G G	C T	G T	C C	T T	
4	Ad 1	10	18	0.56	75	16	4.69	G A	G G	T T	T T	A A	C C	G G	G G	C C	C C	C C	G G	C T	G T	T T	T T	
	Em 1							G A	G G	T T	C T	G A	C T	G T	G G	C A	C C	C C	G G	C T	G T	C T	C T	7
	Ad 2	10	16	0.63				G G	G G	T T	C T	G G	C C	G T	G G	A A	C C	C C	G G	T T	G G	C C	T T	
	Em 2							G A	G G	T T	C T	G A	C C	G T	G G	C A	C C	C C	G G	C T	G T	C T	T T	6
5	Ad 1	12	21	0.57	75	12	6.25	G G	G G	T T	T T	A A	C T	G G	G G	A A	C C	C T	G G	C T	G G	C T	T T	
	Em 1							G G	G A	T T	T T	A A	C T	G G	G A	A A	C C	C T	G G	C T	G G	C T	T T	2
	Ad 2	12	20	0.60				G G	G A	T T	T T	A A	C C	G G	G A	A A	C C	C C	G G	C T	G G	C T	T T	
6	Ad 1	12	9	1.33	50	14	3.57	G A	G G	G T	C T	A A	C C	G T	G A	A A	C C	C C	G G	C T	G G	C C	T T	
	Em 1							G A	G G	G T	C T	A A	C C	G T	G G	A A	C C	C C	G G	C T	G G	C C	C T	1
	Ad 2	12	19	0.63				G G	G G	T T	T T	A A	C C	G G	G G	A A	C C	C C	G G	T T	G G	C C	C T	
7	Ad 1	12	17	0.71	45	13	3.46	G G	G G	G T	T T	A A	C C	G G	G A	A A	C C		G G	C C	G G	C T	T T	
	Em 1							G G	G G	G T	T T	G A	C T	G G	G G	A A	C C	C T	G G	C C	G T	C T	T T	3
	Ad 2	12	14	0.86				G G	G G	G G	T T	A A	C C	G G	G G	A A	C C	C C	G G	C C	C G	C C	T T	
	Em 2							G G	G G	G T	T T	A A	C C	G G	G A	A A	C C	C T	G G	C C	G G	C T	T T	4
8§	Ad 1	15	21	0.71	>200	NA	>8	G G	G G	G T	C T	G A	C C	G G	G A	A A	C C	C C	G G	T T	G G	C C	T T	
	Em 1							G G	G G	G T	C T	G A	C C	G T	G A	A A	C C	C C	G G	T T	G T	C C	T T	2
9	Ad 1	6	10	0.60	85	NA	>3.4	G A	G G	G T	T T	A A	C C	G T	G G	A A	C C	C C	G G	C T	G G	C C	T T	
	Em 1							G A	G G	G T	T T	A A	C C	G T	G A	A A	C C	C T	G G	C T	G G	C C	T T	2
	Ad 2	6	16	0.38				G G	G G	G G	T T	A A	C C	G G	G G	A A	C C	C T	G G	T T	G G	C C	T T	
10	Ad 1	15	25	0.60	105	20	5.25	G A	G G	G T	T T	A A	C C	G T	G G	A A	C C	C C	G G	T T	G G	C C	T T	
	Em 1							G A	G G	G T	T T	A A	C C	G T	G G	A A	C C	C C	G G	T T	G G	C T	C T	1
	Ad 2	15	25	0.60				G G	G G	G G	T T	A A	C C	G G	G G	A A	C C	C C	G G	C C	C C	C T		
	Em 2							G A	G G	G T	T T	A A	C C	G T	G G	A A	C C	C C	G G	T T	G G	C T	C T	4
11	Ad 1	15	10	1.50	65	20	3.25	G G	G G	T T	C T	A A	C C	G G	G A	A A	C C	C C	G G	T T	G G	C T	T T	
	Em 1							G G	G G	T T	C T	A A	C C	G G	G A	A A	C C	C C	G G	T T	G T	C T	T T	2
	Ad 2	15	19	0.79				G G	G G	T T	T T	A A	C C	G T	G G	C C	C C	C C	G G	T T	G G	C C	T T	
12	Ad 1	15	19	0.79	35	13	2.69	G G	G G	T T	T T	G A	C C	G G	G G	A A	C C	C T	G G	C T	G T	T T	T T	
	Em 1							G G	G G	T T	C T	G A	C C	G G	G G	C A	C C	C T	G C	C T	G T	T T	T T	3
	Ad 2	15	17	0.88				G G	G G	T T	C T	A A	C C	G G	G G	C A	C C	C C	G G	C T	G T	T T	T T	
13	Ad 1	16	15	1.07	85	10	8.50	G A	G G	T T	C T	A A	C C	G T	G A	A A	C C	C T	G G	T T	G G	C C	T T	
	Em 1							G A	G G	T T	C T	A A	C T	G T	G A	A A	C C	C T	G G	C T	G G	C T	C T	4
	Ad 2	16	15	1.07				G G	G G	T T	T T	A A		G T	G G	A A	C C	C C	G G	C C	G G	C T	C T	
	Em 2							G A	G G	T T	C T	A A	C T	G T	G A	A A	C C	C T	G G	C T	G G	C T	C T	5
14	Ad 1	20	21	0.95	50	20	2.50	G G	G G	T T	T T	G A	C T	G T	G G	A A	C C	C C	G G	C T	G T	C C	T T	
	Em 1							G G	G A	T T	T T	G A	C T	G T	G G	A A	C C	C C	G G	C T	G T	C C	T T	1
	Ad 2	20	19	1.05				G G	G G	T T	T T	G A	C C	G G	G A	A A	C C	C C	G G	C T	G G	C C	T T	
15	Ad 1	20	25	0.80	85	21	4.05	G G	G A	T T	T T	A A	C C	G T	G G	A A	C C	C C	G G	T T	G G	C T	T T	
	Em 1							G A	G A	T T	C T	A A	C C	G T	G G	C A	C C	C C	G G	C T	G T	C T	T T	5
	Ad 2	20	22	0.91				G A	G G	T T	C T	A A	C C	G G	G G	C C	C C	C C	G G	C T	G T	C C	T T	
16	Ad 1	20	12	1.67	65	16	4.06	G G	G G	T T	C C	A A	C T	G T	G G	C A	C C	C T	G G	C T	G G	C T	C T	
	Em 1							G A	G G	T T	C T	A A	C T	G T	G A	C A	C C	C T	G G	C T	G G	C T	C T	3
	Ad 2	20	12	1.67				G A	G G	T T	C T	A A	C C	G G	G A	C A	C C	C C	G G	C C	T T	G G	C C	
17	Ad 1	20	17	1.18	75	20	3.75	G G	G G	T T	T T	G A	C T	G G	G A	A A	C C	C C	G G	C C	C C	C C		
	Em 1							G G	G G	T T	C T	G A	C T	G G	G A	A A	C C	C C	G G	C T	G T	C C	C T	3
	Ad 2	20	19	1.05				G A	A A	G T	C T	A A	C C	G G	G A	A A	C C	C C	G G	C T	G G	T T	T T	
	Ad 3	20	19	1.05				G G	G G	T T	C T	A A	C C	G G	G G	A A	C C	C C	G G	T T	G G	C C	T T	
18	Ad 1	20	13	1.54	65	13	5.00	G G	G G	G T	C T	A A	C T	G G	G A	C C	C C	C T	G G	T T	G G	C C	T T	
	Em 1							G G	G G	G T	C T	A A	C T	G G	G A	C A	C C	C T	G G	T T	G G	C T	C T	3
	Ad 2	20	16	1.25				G G	G G	G T	T T	A A	C T	G G	G G	A A	C C	C C	G G	T T	G G	C C	T T	
19	Ad 1	20	17	1.18	100	16	6.25	G A	G A	T T	T T	G A	C C	G G	G A	A A	C C	C C	G G	C C	G T	C T	T T	
	Em 1							G A	G A	T T	T T	G A	C C	G G	G A	A A	C C	C C	G G	C T	G T	C T	T T	1
	Ad 2	20	16	1.25				G G	G G	T T	T T	A A	C C	G G	G G	A A	C C	C C	G G	T T	G G	C T	T T	
20	Ad 1	25	23	1.09	105	NA	>4.2	G A	G A	T T	T T	A A	C C	G G	G A	A A	C C	C C	G G	T T	G G	C C	T T	
	Em 1							G A	G A	G T	T T	G A	C C	G T	G A	A A	C C	C C	G G	C T	G T	C C	T T	6
	Ad 2	25	24	1.04				G G		G T	T T	G A	C C	G T	G A	A A	C C	C C	G G	C T	G T	C C	T T	
21	Ad 1	25	19	1.32	70	NA	>2.8	G G	G G	T T	C C	A A	C C	G T	G A	A A	C C	C C	G G	T T	G G	C C	T T	
	Em 1							G A	G G	G T	C T	A A	C C	G T	G A	A A	C C	C T	G G	C T	G G	C T	T T	6
	Ad 2	25	18	1.39				G G	G G	T T	T T	A A	C C	G T	G A	A A	C C	C C	G G	C C	G G	C C	T T	
22	Ad 1	25	19	1.32	85	18	4.72	G A	G G	G T	T T	A A	C C	G G	G A	A A	C C	C C	G G	C C	T T	G G	T T	
	Em 1							G A	G G	G T	T T	A A	C C	G G	G A	C A	C C	C C	G G	C T	G G	C T	T T	3
	Ad 2	25	21	1.19				G G	G G	T T	T T	A A	C C	G G	G G	C A	C C	C C	G G	C C	T T	G G	T T	
23	Ad 1	25	17	1.47	75	22	3.41	G G	G G	G T	T T	A A	C T	G G	G A	C A	C C	C C	G G	T T	G G	C C	T T	
	Em 1							G G	G G	G T	C T	A A	C T	G G	G A	C A	C C	C T	G G	C T	G G	C C	C T	4
	Ad 2	25	17	1.47				G G	G G	T T	C T	A A	C C	G G	G G	A A	C C	C T	G G	C T	G G	C C	C C	
24	Ad 1	25	21	1.19	80	20	4.00		G G	T T	T T	A A	C T	G G	G A	A A	C C	C C	G G	C C	G G	C C	C C	
	Em 1							G A	G G	G T	C T	A A	C T	G G	G A	A A	C C	C C	G G	C T	G G	C T	C T	5
	Ad 2	25	15	1.67				G G	G G	G T	C T	A A	C C	G G	G G	A A	C C	C C	G G	T T	G G	C T	C T	
25	Ad 1	30	18	1.67	120	NA	>4.8	G G	G G	G T	T T	A A	C C	G G	A A	A A	C C	C C	G G	T T	G G	C C	T T	
	Em 1							G G	G G	G T	C T	A A	C C	G T	G A	A A	C C	C T	G G	C T	G G	C T	C T	7
	Ad 2	30	22	1.36				G G	G G	G G	C C	A A	C C	G T	G G	A A	C C	C T	G G	C T	G G	C T	C T	
26	Ad 1	30	19	1.58	40	16	2.50	G G	G G	T T	T T	G G	C T	G G	G G	A A	C C	C C	G C	T T	G T	C C	T T	
	Em 1							G G	G G	G T	C T	G A	C T	G T	G A	A A	A C	C C	G C	C T	G T	C C	C T	8
	Ad 2	30	18	1.67				G G	G G	G G	C T	A A	C T	G T	G A	A A	A A	C C	G C	C C	G T	C C	C C	
	Em 2							G G	G G	G T	C T	G A	C T	G T	G A	A A	A C	C C	G C	C T	G T	C C	C T	7
27	Ad 1	30	20	1.50	45	18	2.50	G A	G G	G T	C T	A A	C T	G G	A A		C C	C C	G G	C C	C C	C C		
	Em 1							G A	G G	G T	C T	G A	C T	G T	G A	A A	C C	C C	G G	C T	G T	C T	C T	6
	Ad 2	30	18	1.67				G G	G G	T T	T T	A A	C T	G G	G G	A A	C C	C C	G G	T T	G G	C C	T T	
	Em 2							G A	G G	G T	C T	A A	C T	G G	G A	A A	C C	C C	G G	T T	G G	C C	C T	5
Frequency of the most common allele (of two)								0.83	0.91	0.8	0.8	0.9	0.8	0.8	0.62	0.9	1	0.89	0.97	0.7	0.91	0.8	0.84	average= 0.84

* Each colored row includes one isolated adult individual (and its egg mass) that was more than two body lengths away from any other Pollicipes (Next Nearest Neighbor Distance).

** Each colored row includes a pair of adults (and their egg masses) adjacent to each other (Partner Distance) but more than two body lengths away from any other Pollicipes (Next Nearest Neighbor Distance). At least one adult barnacle in each pair was fertilized.

† Ad 1: Adult barnacle 1; Em 1: Egg mass belonging to the Ad 1; Ad 2: Adult barnacle 2; Em 2: Egg mass belonging to the Ad 2.

§ No DNA was available for Ad 2 of this pair.

NA: Not Available- multiple adults were within a similar distance so no one individual was measured; the ratio of next nearest neighbor distances to body length are minimum estimates, assuming the body length of that neighbor was 25mm-- the largest we observed.

Table 2.2 Multilocus genotypes of eight replicate subsamples taken from embryo masses of each of six isolated individuals confirm that non-parent alleles were widespread throughout each egg mass tested.

Egg Masses of Isolated Individuals	Original Genotypes and Combined Genotypes of 8 Replicate Subsamples of 6 Isolated Individuals' Egg Masses	Locus																						Notes
		12a-3		11-1		105-1		172-6		190-1		107-2		117-1		117-5		151-2		105-2		107-3		151-3		172-5		190-2		117-2		119-1		
8 - Em 1	Original multilocus genotype	G	G	G	G	G	T	C	T	G	A	C	C	G	T	G	A	C	A	C	C	C	C	G	G	T	T	G	T	C	T	T	T	
	Genotypes of 8 subsamples	G	G	G	G	G	T	C	T	G	A	C	C	G	T	G	A	C	A	C	C	C	C	G	G	T	T	G	T	C	T	T	T	
14 - Em 1	Original multilocus genotype	G	A	G	A	G	T	C	T	G	A	C	T	G	G	G	A	A	A	C	C	C	C	G	G	C	T	G	G	C	T	C	T	All eight subsamples of locus 190-1 differed from the original genotype and the absent allele in these subsamples was the rare allele.
	Genotypes of 8 subsamples	G	A	G	A	G	T	C	T	A	A	C	T	G	G	G	A	A	A	C	C	C	C	G	G	C	T	G	G	C	T	C	T	
16 - Em 1	Original multilocus genotype	G	G	G	A	G	T	C	T	A	A	C	T	G	G	G	A	A	A	C	C	C	C	G	C	T	T	G	T	C	C	C	T	
	Genotypes of 8 subsamples	G	G	G	A	G	T	C	T	A	A	C	T	G	G	G	A	A	A	C	C	C	C	G	C	T	T	G	T	C	C	C	T	
27 - Em 1	Original multilocus genotype	G	G	G	G	G	T	C	T	A	A	C	T	G	G	G	A	A	A	A	C	C	T	G	G	C	T	G	G	C	T	C	T	
	Genotypes of 8 subsamples	G	G	G	G	G	T	C	T	A	A	C	T	G	G	G	A	A	A	A	C	C	T	G	G	C	T	G	G	C	T	C	T	
35 - Em 1	Original multilocus genotype	G	A	G	A	G	T	T	T	G	A	C	T	G	T	G	A	A	A	C	C	C	C	G	G	C	T	G	T	C	T	C	T	Three subsamples of locus 190-2 differed from the original genotype and the absent allele in these three subsamples was the rare allele.
	Genotypes of 8 subsamples	G	A	G	A	G	T	T	T	G	A	C	T	G	T	G	A	A	A	C	C	C	C	G	G	C	T	G	T/G	C	T	C	T	
37 - Em 1	Original multilocus genotype	G	A	G	G	G	G	C	T	A	A	C	T	G	T	G	A	A	A	C	C	C	T	G	G	C	T	G	G	C	T	T	T	
	Genotypes of 8 subsamples	G	A	G	G	G	G	C	T	A	A	C	T	G	T	G	A	A	A	C	C	C	T	G	G	C	T	G	G	C	T	T	T	

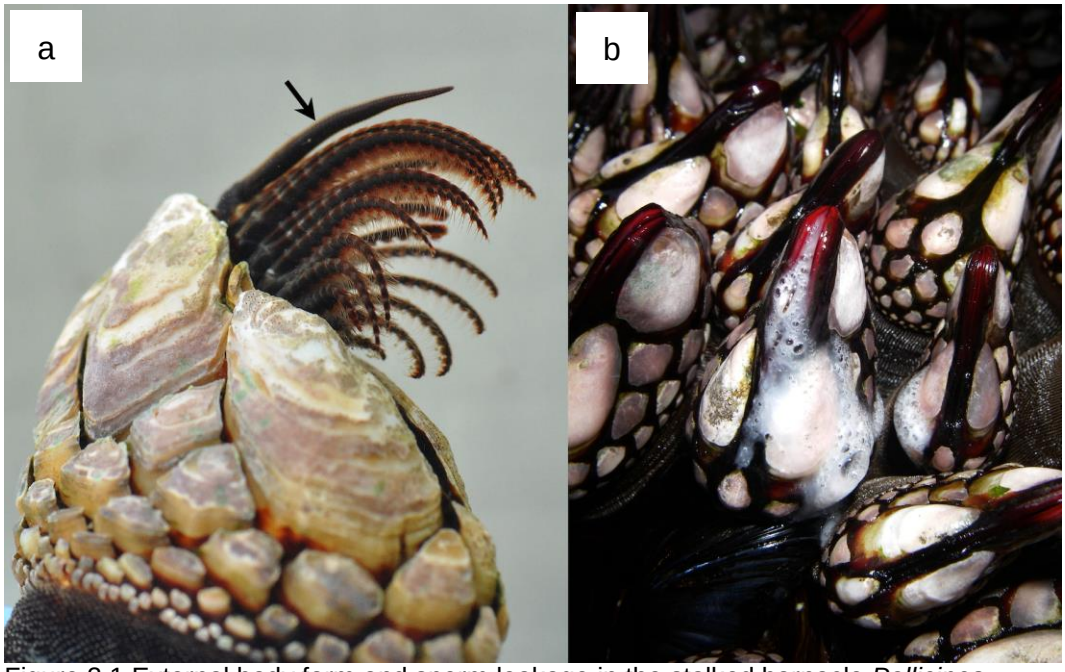

Figure 2.1 External body form and sperm leakage in the stalked barnacle *Pollicipes polymerus*. (a) Relaxed penis (arrow) and feeding legs of *P. polymerus* (soma wet mass= 0.785 g, rostro-carinal length= 19 mm) from a moderately wave-exposed shore near Bamfield, British Columbia, Canada, (b) *P. polymerus* leaking sperm in the field at low tide on Tatoosh Island, Washington, USA.

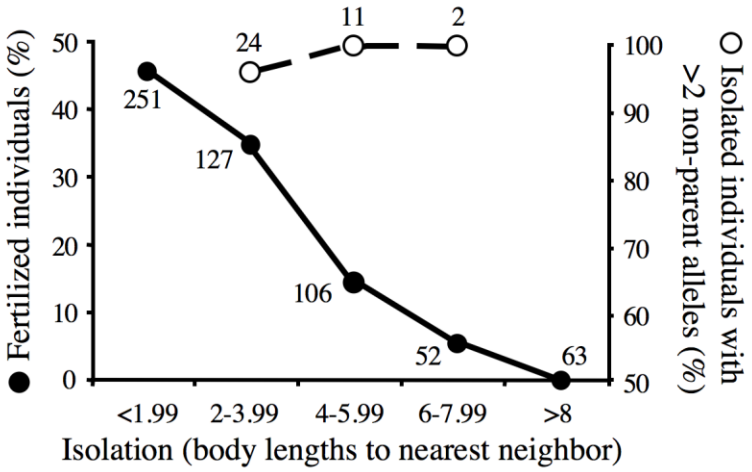

Figure 2.2 Percentage of *Pollicipes polymerus* individuals fertilized (solid symbols) or bearing non-parent alleles at two or more SNP loci (open symbols), as a function of isolation (number of body lengths to nearest neighbor). Numbers adjacent to points indicate sample sizes. Counts were pooled from the two sites sampled. The presence of non-parent SNP alleles indicates sperm capture.

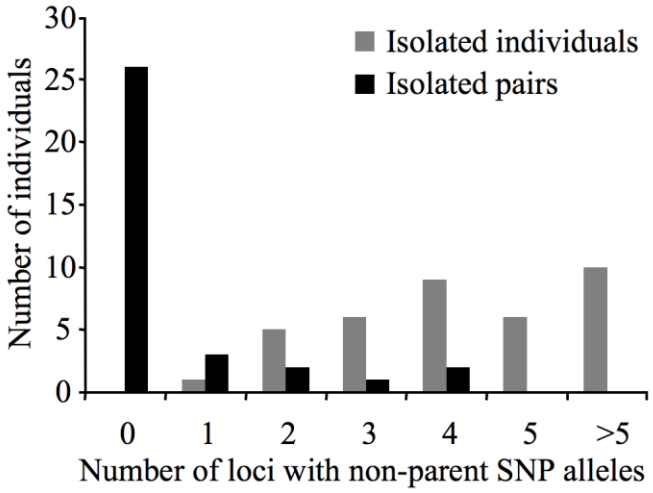

Figure 2.3 Numbers of embryo masses with non-parent SNP alleles from 37 isolated individuals and 34 isolated pairs. A total of 16 SNP loci were scored per individual. The presence of non-parent SNP alleles indicates sperm capture.

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Chapter 3. Where even a long penis can't help: Evidence of long-distance spermcast mating in two acorn barnacles[2]

3.1 Introduction

Barnacles are famous for their exceptionally long penises that, in extreme cases, can extend up to eight times their body length (e.g., *Cryptophialus minutus* Darwin, 1854). Such long intromittent organs are crucial for barnacle reproduction because: 1) unlike other arthropods, they are sessile as adults, and 2) despite being typically hermaphroditic, self-fertilization rarely happens in barnacles. Most species are presumed to transfer sperm only by pseudo-copulation (Darwin, 1851, 1854), where a functional male extends its penis into a partner's mantle cavity to release sperm that induce the release of eggs (Anderson, 1994; Buhl-Mortensen and Høeg, 2006). Such long penises also imply that within a clump of barnacles, a functional female could potentially be fertilized by more than one functional male (Charnov, 1982, 1987).

However, even a long penis cannot ensure mating success in isolated individuals that lie outside the penis range of any neighbors. A few species are thought to overcome this problem by self-fertilization, which is usually assumed when fertilized embryo masses are found inside the mantle cavities of isolated

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barnacles (Barnes and Crisp, 1956; Barnes and Barnes, 1958; Furman and Yule, 1990). But the recent discovery of spermcast mating in a stalked barnacle, *Pollicipes polymerus* (Barazandeh, et al., 2013), raises the possibility that eggs in the mantle cavity of isolated acorn barnacles could also be fertilized by sperm released by distant individuals.

Spermcast mating occurs in many sessile marine invertebrates including sponges, hydroids, bryozoans, pterobranch hemichordates and ascidians (Bishop and Pemberton, 2006), but has not been reported in any barnacles other than *P. polymerus* (Barazandeh, et al., 2013). In the acorn barnacle, *Tetraclita rubescens* Darwin, 1854, although some remote individuals were fertilized, 87% of the many broods studied were nonetheless fertilized by only one father, even when multiple mates were nearby (Kelly, et al., 2012). Sperm capture may therefore occur only rarely, if it occurs at all, in this species. However, individuals of other acorn barnacles, *Amphibalanus amphitrite* and *Chirona hameri* (formerly *Balanus hameri*), sometimes eject sperm into the water when a copulation attempt fails (Walker, 1980) W.A. Newman, personal communication, 2013), and "alternative mechanisms of outcrossing" other than pseudo-copulation have been suggested as a source of sperm in isolated fertilized *Chthamalus montagui* (Pannacciulli and Bishop, 2003). Therefore, whether spermcast mating occurs at all in acorn barnacles remains an open question.

Among barnacles, spermcast mating might have evolved only in species like *Pollicipes polymerus* that have relatively short penises (e.g., maximum extension up to 0.7 body lengths), and live in extreme environments (highly wave-exposed

shores) that could facilitate the process. If so, the presence of fertilized eggs in isolated individuals of other species that have longer penises and hence have many more potential mates within penis range must be due to self-fertilization if spermcast mating is not a possibility. We therefore tested for spermcast mating in two acorn barnacles, *Balanus glandula* Darwin, 1854 and *Chthamalus dalli* Pilsbry, 1916 that possess relatively long penises and that live in a wide range of wave exposures (calm to highly exposed shores). These barnacles are common on the rocks and mussels of northeast Pacific intertidal shores. Mating occurs mostly in winter and spring in *B. glandula* and from spring through fall in *C. dalli* (Newman and Abbott, 1980; Strathmann, 1987). The penis length of many barnacles depends on their reproductive status. Indeed, some species lose their penis altogether at the end of the mating season and grow a new one as the next breeding season begins, with the maximum size occurring at the peak of the mating season (Barnes, 1992). In *B. glandula*, relaxed and artificially extended penis lengths are around 1.4 and 3 times the body length, respectively (Barazandeh, et al., 2013) and the maximum penis length is achieved from December to June (Barnes, 1992). The average penis length for *C. dalli* may depend on the number of fertile individuals nearby, among other factors. For instance, the relaxed penis is as long as the feeding legs when only 25% of the population is fertilized but twice that long when 75% of the population is fertilized (Barnes, 1992). *B. glandula* is believed to mate only by pseudo-copulation with neighbors (Kado, 2003; Neufeld and Palmer, 2008). *C. dalli*,

however, is thought to self-fertilize when neighbors are out of reach (Barnes and Barnes, 1958; Newman and Abbott, 1980).

A number of interesting questions remain to be addressed here. Are species with long penises also capable of spermcast mating? How valid are reports of self-fertilization in *C. dalli* — and therefore possibly other acorn barnacles — where spermcast mating is a possibility? Do individuals with an immediate neighbor but far away from other barnacles (isolated pairs) copulate only with each other or is neighbor-mating supplemented with captured sperm as well?

3.2 Materials and Methods

3.2.1 Barnacle sampling and measurements

To test whether spermcast mating, if it happens, is not only limited to shores with high wave actions (Barazandeh, et al., 2013), barnacles were sampled from sites with different flow regimes. *Balanus glandula* were collected from rocks, mussels (*Mytilus* sp.) and *Pollicipes* plates of high (Seppings Island, 48.841° N, 125.209° W), intermediate (Wizard Islet, 48.866° N, 125.169° W) and low (Grappler Inlet, 48.833° N, 125.119° W) wave-exposed shores (Arsenault, et al., 2001) in Barkley Sound (near Bamfield, British Columbia, Canada) in August 2010 and May 2012. *Chthamalus dalli* were sampled from rocks and mussels on shores of high (Seppings Island) and intermediate (Helby Island, 48.847° N, 125.168° W) wave-exposure (Arsenault, et al., 2001) in August 2010.

A total of 430 *B. glandula* and 110 *C. dalli* were scored for fertilization incidence, body length (= opercular length: length of the aperture parallel to the gape) of the focal individual and its nearest neighbor, and the distance to the

nearest neighbor (shortest possible distance between the nearest margins of two barnacles' opercula). Isolation was defined as the ratio of nearest-neighbor distance to opercular length of the nearest neighbor.

Isolated individuals in both species were identified as individuals separated by more than seven body lengths from their nearest neighbor. Isolated pairs consisted of two adjacent barnacles, each of which was more than seven body lengths away from any conspecifics (see *2.2 Penis measurements* below).

A total of nine and 23 isolated individuals bearing fertilized eggs were collected for *B. glandula* and *C. dalli*, respectively. In addition, 26 fertilized *B. glandula* and 22 fertilized *C. dalli* from isolated pairs were also collected. For barnacles on mussels, we avoided sampling those close to the shell margin to reduce the possibility that barnacles might have mated with individuals on adjacent shells that had shifted position. We also did not collect any barnacles close to the remnant basal plates (*B. glandula*) or attachment halos (*C. dalli*) of recently dead barnacles to ensure that sampled individuals had not been near another individual that had recently died.

The whole body of each adult and whole embryo mass were removed from the shells and put separately in labeled 1.5 ml microfuge tubes containing 70% ethanol and stored at -20°C until DNA extraction.

3.2.2 Penis measurements

To estimate the length of fully extended penises in actively mating barnacles, and to assess the true extent of erect penis-length variation among populations from different wave-exposure regimes, small rocks and mussels

carrying *Balanus glandula* and *Chthamalus dalli* were collected from two highly wave-exposed (Seppings Island, Helby Island), two moderately exposed (Dixon Island, Brady's Beach) and three very calm sites (Grappler Inlet, Bamfield Inlet, Ross Islets) in Barkley Sound in May 2013 and taken to the laboratory. We kept them in running-seawater tanks with aeration under a 18:6 hr light:dark regime and watched them for mating activity each day following 2 - 3 hrs exposure to air. Any penis movement or extension, regardless of success, was videotaped. Full penis extension of spontaneously mating individuals was recorded for 123 *B. glandula* and 186 *C. dalli*. The opercular length and the total curved length of the extended penis of each target individual was measured on screen from different angles and the highest ratio of total penis length to opercular length was recorded for each individual to estimate the maximum possible reach.

3.2.3 Marker development, genotyping and simulation

Attempts to cross-amplify existing microsatellite markers developed for *Balanus amphitrite* and *Chthamalus montagui* (Pannacciulli, et al., 2005; Robson, et al., 2009) in *Balanus glandula* and *Chthamalus dalli* were unsuccessful. Consequently, we developed Single Nucleotide Polymorphism (SNP) markers.

Library construction involved total genomic DNA extraction from the whole body of five *B. glandula* and five *C. dalli* using a DNeasy® Blood and Tissue Kit (QIAGEN). For each species, we digested 5 µl of pooled DNA with XbaI, extracted 400-650 bp fragments from an agarose gel and ligated them to SuperSNX linkers (Glenn and Schable, 2005). Inserts were amplified by PCR and cloned using the TOPO TA cloning kit (Invitrogen). A total of 63 *B. glandula* and

15 *C. dalli* clones were amplified and sequenced using BigDye® Terminator v3.1 (Applied Biosystems, ABI) and resolved on a 3730 ABI DNA analyzer. By aligning and trimming the resulting sequences using SeqMan® (DNASTAR), we obtained 28 *B. glandula* and 11 *C. dalli* contigs, for which primers were designed using Primer3 (Version 0.4.0) (Rozen and Skaletsky, 2000). Seven individuals of each species were amplified and those primer sets that resulted in expected product sizes and amplified in all individuals (13 primer sets in *B. glandula* and 8 primer sets in *C. dalli*) were re-sequenced. Five *B. glandula* and four *C. dalli* contigs contained a total of nine putative SNP markers in each species. Amplification and SNaPshot interrogation primers were designed for the SNP loci using Primer3 (Version 0.4.0) (Rozen and Skaletsky, 2000, Table 3.1, Table 3.2) and seven individuals were amplified using the ABI PRISM® SNaPshot™ Multiplex kit. Seven polymorphic SNP loci were identified for each species, where each had two alleles, the rare allele was observed at least twice among seven individuals, and there was at least one heterozygote individual (Appendix 1, Barazandeh and Davis, 2012; Filippini, et al., 2007; Kaderali, et al., 2003; Lindblad-Toh, et al., 2000). Because we were only using SNPs as markers to test for the presence of non-self sperm, and not to assign paternity, the use of SNPs from the same genomic fragment was not a concern because this would only tend to underestimate the incidence of sperm capture (Barazandeh, et al., 2013).

Total genomic DNA was extracted from the whole body of each sampled adult and from each whole embryo mass using DNeasy® Blood and Tissue Kit (QIAGEN). All samples were genotyped at seven SNP markers using ABI

PRISM® SNaPshot™ Multiplex kit and the results were scored using GeneMapper, v4.0 (ABI) (Appendix 1, Barazandeh and Davis, 2012; Filippini, et al., 2007; Kaderali, et al., 2003; Lindblad-Toh, et al., 2000). An embryo-mass allele was recognized as a non-maternal allele if it was absent from the brood-parent's DNA in isolated individuals and was called a non-maternal/partner allele if it was absent from both the brooding parent and the adjacent individual in isolated pairs.

Population-level tests for deviations from Hardy-Weinberg and linkage disequilibrium of adult genotypes were performed using GENEPOP (v4.1) (Raymond and Rousset, 1995).

To assess the potential underestimation of the spermcast mating rate in acorn barnacles (due to the lower number of loci) compared to the stalked barnacle, *Pollicipes polymerus* (Barazandeh, et al., 2013), we simulated this rate in *P. polymerus* by 40 random draws of seven SNP loci from the original pool of sixteen loci. For each trial of seven subsampled loci, we calculated the percentage of isolated individuals with non-maternal alleles and isolated pairs with non-maternal/partner alleles and then used the average of the 40 simulations as the final spermcasting rates and used the variation among these random subsamples to estimate the standard error of this average.

3.3 Results

3.3.1 Penis measurements

The average penis length relative to opercular length was 3.2 in *Balanus glandula* and 2.8 in *Chthamalus dalli*, with a maximum ratio of 6.7 in both

species. Both species were in the middle of their mating season when monitored in May 2013 (Newman and Abbott, 1980) and they were actively mating when multiple partners were available within penis range (M. Barazandeh 2013, personal observations). So, 6.7 is probably the maximum length a penis could reach. However, to be conservative, we used seven body lengths as the maximum penis reach for both species and defined all individuals beyond this distance as isolated. Isolated pairs were similarly defined as two adjacent individuals both of whom were more than seven body lengths from another conspecific.

3.3.2 Fertilization as a function of distance

The opercular length of the smallest fertilized individuals was 1.5 mm in *Balanus glandula* and 0.4 mm in *Chthamalus dalli*. As sample sizes of fertilized individuals from different sites were small, the data from all sites and years were pooled. In *B. glandula*, as the distance to nearest neighbor increased, the percent of fertilized individuals decreased and no fertilized individuals were detected beyond an isolation distance of 10 (n = 183; Figure 3.1). *C. dalli* exhibited a similar pattern, but had a higher percentage of fertilized individuals within seven body lengths (74% vs 45% in *B. glandula*) and the number of individuals bearing embryo masses never reached zero: even at isolation distances of 11 - 70 body lengths, 36% of individuals carried fertilized eggs (Figure 3.2).

3.3.3 Genotyping and simulation results

3.3.3.1 Balanus glandula

Embryo masses of 91% of all genotyped individuals and 100% of all nine isolated individuals had at least one non-maternal allele and 70% of all and 78% of isolated individuals had two or more non-maternal alleles (Figure 3.1, Figure 3.3). Among 26 individuals in isolated pairs, only two had one non-maternal/partner allele (Figure 3.3; Table 3.3).

The average frequency of the dominant allele was 0.73 (range 0.59 to 0.95). None of the loci deviated significantly from Hardy-Weinberg equilibrium after sequential Bonferroni correction for multiple comparisons (i.e., $p \leq 0.007143$ for N= 7 loci; Table 3.1). Among 21 comparisons of seven loci, only one pair indicated significant linkage disequilibrium (Bg117-2 and Bg117-3; i.e., $p \leq 0.0024$ for N= 21 tests).

3.3.3.2 Chthamalus dalli

Embryo masses of 74% of all genotyped individuals and 70% of 23 isolated individuals had at least one non-maternal allele and 37% of all and 35% of isolated individuals had non-maternal alleles at two to five loci (Figure 3.2, Figure 3.4). Among 22 individuals in isolated pairs, only two had one non-maternal/partner allele (Figure 3.4; Table 3.4).

The average frequency of the dominant allele was 0.71 (range 0.55 to 0.83). Three loci significantly deviated from Hardy-Weinberg equilibrium after sequential Bonferroni correction for multiple comparisons (N= 7; Chd103-1 and

Chd103-2 [heterozygote excess], Chd131-3 [heterozygote deficit]; $p \leq 0.007143$ for N= 7 loci; Table 3.2). Among 21 comparisons of seven loci, four pairs indicated significant linkage disequilibrium (Chd131-1 and Chd131-2, Chd131-1 and Chd131-3, Chd131-2 and Chd131-3, Chd131-2 and Chd131-4; i.e., $p \leq 0.0024$ for N= 21 tests).

3.3.3.3 Pollicipes polymerus

Based on 40 trials of seven subsampled SNP loci, an average of 90% and 60% of 37 isolated individuals had at least one and more than one non-maternal alleles, respectively. Among 34 individuals in isolated pairs, an average of 15% and 5% had at least one and more than one non-maternal/ partner alleles, respectively (Table 3.5).

3.4 Discussion

As in other crustaceans, copulation is the preferred way to mate in most barnacles including *Balanus glandula* and *Chthamalus dalli* (Addison and Hart, 2005; Anderson, 1994). Our results do not suggest otherwise, but they do provide strong evidence that eggs of acorn barnacles can also be fertilized by waterborne sperm when no neighbor is within copulation range (Figures 3.1, 3.2). More surprisingly — as observed in the stalked barnacle *Pollicipes polymerus* (Barazandeh, et al., 2013) — individual acorn barnacles, at least occasionally, capture sperm even if they have an immediate neighbor (Figures 3.3, 3.4). Nonetheless, for all species examined so far, the percentage of fertilized individuals decays with distance (Figures 3.1, 3.2, 3.5 and Barazandeh, et al.,

2013), and in both *B. glandula* and *P. polymerus* few if any isolated individuals beyond 10 body lengths from a neighbor were fertilized. So, the spatial scale over which spermcast mating can occur in barnacles appears to be limited.

The evidence of spermcast mating reported here for acorn barnacles is not a result of genotyping errors. Based on *P. polymerus* SNP data, the average allelic error rate is 0.26 percent (obtained by genotyping all SNPs for all individuals twice and counting the number of allelic mismatches between two sets of genotypes per total scored alleles in all individuals; Barazandeh, et al., 2013). This error rate is 116 and 74 times less than the average rate of non-maternal alleles in *B. glandula* and *C. dalli*, respectively (30.23%, range 0 - 85.71 and 19.34%, range 0 - 57.14). So, spermcast mating does appear to occur in these species.

Nonetheless, spermcast fertilization appears to be less common in isolated individuals of *C. dalli* and isolated pairs of both *C. dalli* and *B. glandula* than in the stalked barnacle, *Pollicipes polymerus* (Table 3.5). Non-maternal alleles were around one-third less common in isolated individuals of *C. dalli* (70%) than *B. glandula* (100%) and *P. polymerus* (100%). In addition, non-maternal/partner alleles were between one-third and one-half as common in isolated pairs of both acorn barnacles (7.7% and 9.1%) compared to the stalked barnacle (23.5%; Table 3.5). These values and the number of individuals with more than one non-maternal or non-maternal/partner alleles in the acorn barnacles might be low partly due to the fewer SNP markers we used compared to *P. polymerus* (seven versus sixteen). The simulation of spermcasting rate in *P. polymerus* using seven

loci resulted in lower number of non-maternal and non-maternal/partner alleles compared to sixteen loci, which confirms some underestimation of spermcasting in acorn barnacles due to low number of loci. However, this rate in the stalked barnacle was still higher than the similar rates in isolated individuals of *C. dalli* and isolated pairs of both acorn barnacles (Table 3.5), which is consistent with lower rates of spermcast mating in acorn barnacles.

Despite similar rates of fertilization among individuals within penis range (approx. 50%; Figure 3.1, and Figure 2.2) isolated individuals of *B. glandula* were less than one-third as likely to be fertilized than isolated individuals of *P. polymerus* (3.6% compared to 10.6%). At least in part this may be due to the longer penis reach of *B. glandula* (4 - 7 body lengths) compared to *P. polymerus* (less than 1 body length): proportionally fewer acorn barnacles would be outside of penis range but still within the effective range of spermcast fertilization (less than 10 body lengths). Furthermore, *P. polymerus* can spermcast over much a larger spatial scale than *B. glandula*: nearly 100 mm for *P. polymerus* compared less than 30 mm for most *B. glandula* (Figure 3.5), likely because *P. polymerus* are much larger bodied. This will result in a higher rate of spermcast mating in isolated *P. polymerus* than *B. glandula.* In addition, at low tide we have observed small but consistent numbers of adult *P. polymerus* leaking sperm from their opercular plates in what appears to be an active behavior (Barazandeh & Palmer, in review), yet we observed no such leakage in *B. glandula* or *C. dalli*.

Our results suggest that mating in the acorn barnacle *Chthamalus dalli* differs considerably from the other two species. In particular, our results strongly

suggest that *C. dalli* do self-fertilize for three reasons: 1) significantly more isolated individuals were fertilized (52.1%, $P < 0.001$) compared to *P. polymerus* and *B. glandula* (Table 3.5), 2) unlike *P. polymerus* and *B. glandula,* some fertilized *C. dalli* were found even at isolation distances of more than 10 body lengths (Figure 3.2), and 3) only 70% of isolated fertilized individuals bore non-maternal alleles, compared to 100% in *P. polymerus* and *B. glandula* (90% in simulated *P. polymerus*; Table 3.5). These results support prior claims of self-fertilization in *C. dalli* based solely on the presence of fertilized isolated individuals in the field (Barnes and Barnes, 1958). Unfortunately, we cannot reliably estimate the incidence of self-fertilization in this species because of the limited number of SNP markers we used. *C. dalli* might be better sperm-casters than *P. polymerus* and *B. glandula*, i.e. their sperm can survive for a longer time in the water or can swim longer distances. Alternatively, because *C. dalli* lack calcareous basal plates, there is a greater risk we may not have recognized cases where a neighbor barnacle had recently died. Nonetheless, we think these do not likely account for the differences.

Therefore, self-fertilization seems likely in *C. dalli*. In addition, we cannot reject the possibility that *B. glandula* also self-fertilize because we did not genotype single embryos, so the source of sperm in single fertilized eggs was unknown. Deviations from Hardy-Weinberg equilibrium can sometimes help explain different observed mating patterns (Addison and Hart, 2005). Heterozygote deficiency in sessile organisms is a deviation that could be caused by several factors including inbreeding (Furman and Yule, 1990). On the other

hand, outbreeding and mixing could yield heterozygote excess. In the end, because of the limited number of SNP markers — especially those that deviated from Hardy-Weinberg equilibrium — we are not able to make any conclusions about self-fertilization.

Our observations of linkage disequilibrium among some loci are not surprising since all those loci were on the same genomic fragments. This does not affect our conclusions because we simply compared the genotypes of adults and offspring, and each locus is independently informative in this regard.

Regardless of the rates of self-fertilization and pseudo-copulation, we have strong evidence that spermcast mating does happen, at least occasionally, in isolated individuals of *Balanus glandula* and *Chthamalus dalli* even though these acorn barnacles have quite long penises. The occurrence of spermcast mating in three different species, which represent two orders and three families of the subclass Cirripedia (Newman and Abbott, 1980), suggests that occasional spermcast mating may be widespread in thoracican barnacles. However, the long penises of *B. glandula* and *C. dalli* — up to seven body lengths — strongly suggest that copulation is the preferred mode of mating when another barnacle is nearby. So, occasional spermcast mating — if not accidental — is most likely only a supplementary method.

The mechanisms of spermcast mating in barnacles remain unknown. Sperm longevity in water ranges from a few seconds to hours depending on the species (Bishop, 1998). The high densities of *B. glandula* and *C. dalli* on many rocky shores (Newman and Abbott, 1980) certainly raises the possibility that sperm may

be locally abundant in the sea. Like other sessile marine suspension-feeders, the feeding activities of barnacles may help them capture sperm from the water (Bishop, 1998). Depending on the lifespan of sperm, barnacles might gather sperm to a certain concentration and then release the eggs for fertilization inside the mantle cavity. Sperm form (Healy and Anderson, 1990) might also have an impact on the success of spermcast mating in different barnacle species. These, and many other questions, remain unanswered.

Our results provide strong evidence that spermcast mating does occur, at least occasionally, even in acorn barnacles with very long penises. Significantly, our observations reveal that spermcasting may be widespread as a supplementary mating method in barnacles, a possibility overlooked in crustacean mating systems for over a century by many scientists including Darwin (Darwin, 1851, 1854). Representatives of at least nine phyla commonly spermcast (Bishop and Pemberton, 2006). Given that supplemental spermcast mating occurs in three distantly related cirripedes (Barazandeh, et al., 2013), Arthropoda should now be included in the list. Moreover, both population genetic (Kent, et al., 2003; Véliz, et al., 2006) and sex allocation (Charnov, 1982, 1987) models for barnacles may need to be reassessed because mating is not restricted to copulation with immediate neighbors.

Several fascinating questions still remained to be answered: i) How widespread is spermcast mating in other barnacles? ii) What are the mechanisms by which sperm are released and captured? iii) Do any morphological or behavioral modifications facilitate sperm capture? iv) Under normal densities,

where multiple potential mates are nearby, what proportion of eggs is fertilized by waterborne sperm? v) Which claims of self-fertilization in other barnacles may actually be cases of spermcast mating?

Table 3.1 Characteristics of seven SNP loci isolated from 55 *Balanus glandula*.

Locus	Allele Frequency	Amplification primers (5'-3')	Tm	Interrogation primer (5'-3')	Tm	Observed Heterozygosity	Expected Heterozygosity	*P-value*
119	A/T 0.75/0.25	F:AACGAATCGATTGAAACAG R:TGCACCCAATTGTTATTCTA	53.66 54.23	**(GACT)$_5$G**GCTAATCTGTCATTAATTTGTTA	51.13	0.26	0.37	0.037
174	A/G 0.89/0.11	F:AATTTTGTTTAATTGTCTCTATGTG R:TTGAGATAAGATGAAAAAGATAGTT	53.91 52.94	**(GACT)$_8$GA**GTGTATATTTATATGTTTTTATTTGT	48.72	0.22	0.2	0.358
109-1	G/A 0.65/0.35	F:ATGGTTCATATCGTCTACCG R:CGGTCCCTGTATCAAAATAA	55.04 55.20	**GA**GGGATCTCTCCTTCCA	50.83	0.42	0.46	0.522
109-3	T/A 0.63/0.37	F:CCCTGTATCAAAATAAACTGC R:ATATCGTCTACCGGCAAG	54.16 53.63	**(GACT)$_2$G**GAAGGCAGCTGTGGT	50.16	0.42	0.47	0.433
117-1	T/C 0.95/0.05	F:CAGATGACTCGTGTGAAAAT R:TGTTTGATGACGACATAGC	53.52 52.78	**(GACT)$_4$GA**TTGTCATCCAAGCTGA	48.74	0.11	0.1	0.669
117-2	A/G 0.59/0.41	F:GTTGGATTCGGTCTCATAAT R:TATTTTGTCATCCAAGCTGA	54.04 54.32	**(GACT)$_3$G**TGATGACGACATAGCCT	49.92	0.42	0.48	0.316
117-3	A/G 0.67/0.33	F:TATTTTGTCATCCAAGCTGA R:GTTGGATTCGGTCTCATAAT	54.32 54.04	**(GACT)$_5$G**GTCGTCATCAAACATGC	50.74	0.47	0.44	0.585
None of *P-values* deviated significantly from Hardy-Weinberg equilibrium after sequential Bonferroni correction (i.e., $p \leq 0.0071$ for N= 7 tests).								
Tm (Melting Temperature): the temperature at which 50% of the DNA molecules are double stranded.								

Table 3.2 Characteristics of seven SNP loci isolated from 75 *Chthamalus dalli*.

Locus	Allele Frequency	Amplification primers (5'-3')	Tm	Interrogation primer (5'-3')	Tm	Observed Heterozygosity	Expected Heterozygosity	*P-value*
103-1	C/T 0.55/0.45	F:TTTATGATCACGCCTTACAG R:TCTGCTGATCCAAATTGTTA	53.97 54.32	**(GACT)$_7$GAA**GCGTTTGCTTCTGCT	51.06	0.81	0.49	**0.0000000**
103-2	C/G 0.73/0.27	F:GTCACAGTTTCCAAGAACAA R:TTTATGATCACGCCTTACAG	53.59 53.97	**GACTGAC**TTTCAGTTTAGTGTGATGAAA	51.00	0.55	0.40	**0.0011240**
127-3	T/A 0.80/0.20	F:CAGACGGACAGGTGTGA R:GATGACGAGTTTACCCTACG	54.34 54.83	**(GACT)$_4$**ACGTCACGGTTAGAGC	49.47	0.23	0.32	0.0217900
131-1	A/C 0.83/0.17	F:GATACCTGCTTAGCCAATTC R:ATCAGACAACCTAGCAGACA	54.16 53.24	**(GACT)$_4$G**GATAATCGTCTGGCATATC	50.31	0.23	0.28	0.1180000
131-2	A/G 0.63/0.37	F:GTGTTACTGCAGGGGAGA R:GCCCTAAGGTCACACAGTA	54.38 53.56	**(GACT)$_8$T**GACTCCCAGGAATGTAG	47.64	0.44	0.46	0.6480000
131-3	G/A 0.82/0.18	F:GTGTTACTGCAGGGGAGA R:GCCCTAAGGTCACACAGTA	54.38 53.56	**GACT**GTCCAGACTCCGCC	50.10	0.16	0.29	**0.0001800**
131-4	G/C 0.60/0.40	F:TCTCCCCTGCAGTAACAC R:GAATAGAAGCTCCCACTGTC	54.38 53.95	**(GACT)$_9$**GCTGTCTCTCAGAAGGAG	50.30	0.56	0.48	0.1490000

P-values **in bold red indicate significant deviation from Hardy-Weinberg equilibrium even after sequential Bonferroni correction (i.e., $p \leq 0.0071$ for N= 7 tests).**

Tm (Melting Temperature): the temperature at which 50% of the DNA molecules are double stranded.

Table 3.3 Multilocus genotypes of isolated individuals and isolated pairs of *Balanus glandula* and their egg masses. Red circles indicate a locus where the egg mass genotype contained an allele not present in the brooding parent. Blue circles indicate a locus where the egg mass genotype contained an allele not present in either parent of the pair.

Isolated Individuals + Egg Masses*	Individuals†	Next Nearest Neighbor Distance (mm)	Next Nearest Neighbor Body Length (mm)	Ratio of Next Nearest Neighbor Distance to Body Length	Locus 109-1	109-3	117-2	117-1	117-3	119	174	Number of Loci with Non-Parental Alleles
1	Ad 1	21.7	2.6	8.35	G G	A T	G G	T T	A A	A A	A A	
	Em 1				G G	A T	G A	T T	G A	A T	G A	4
2	Ad 1	18.2	2.4	7.58	G G	T T	G A	T T	G A	A A	A A	
	Em 1				G G	T T	G A	T T	G A	A T	A A	1
3	Ad 1	30.0	3.1	9.68	G G	A A	A A	C T	A A	A A	A A	
	Em 1				G G	A T	G A	C T	A A	A T	A A	3
4	Ad 1	28.6	3.4	8.41	G A	T T	G G	T T	A A	A A	A A	
	Em 1				G A	A T	G G	T T	A A	A A	A A	1
5	Ad 1	30.8	4.4	7.00	G G	A A	A A	T T	G A	-- --	A A	
	Em 1				G G	A T	G A	T T	G A	-- --	A A	2
6	Ad 1	12.8	1.4	9.14	A A	A A	G A	C T	A A	A T	A A	
	Em 1				G A	A T	G A	C T	G A	A T	A A	3
7	Ad 1	15.5	2.2	7.05	G G	T T	A A	C T	A A	A A	A A	
	Em 1				G G	A T	A A	C T	G A	A T	A A	3
8	Ad 1	19.4	2.5	7.76	G A	A A	A A	T T	G G	T T	A A	
	Em 1				G A	A T	G A	C T	G A	A T	G A	6
9	Ad 1	16.1	1.8	8.94	G A	A A	A A	C T	G A	A T	A A	
	Em 1				G A	A T	A A	C T	G A	A T	G A	2

Isolated Pairs + Egg Masses**	Individuals†	Partner Distance (mm)	Partner Body Length (mm)	Ratio of Partner Distance to Body Length	Next Nearest Neighbor Distance (mm)	Next Nearest Neighbor Body Length (mm)	Ratio of Next Nearest Neighbor Distance to Body Length	Locus 109-1	109-3	117-2	117-1	117-3	119	174	Number of Loci with Non-Parental Alleles
1	Ad 1	6.0	2.9	2.07	130.0	3.9	33.33	G G	A T	G A	T T	G A	T T	A A	
	Em 1							G A	A T	G A	T T	G A	A T	A A	2
	Ad 2	6.0	3.0	2.00	130.0	3.9	33.33	A A	A T	G G	T T	A A	A A	A A	
	Em 2							G A	A T	G A	T T	G A	A T	A A	4
2	Ad 1	4.0	2.8	1.43	55.0	2.2	25.00	G A	A A	G A	T T	G A	A T	G A	
	Em 1							G A	A T	G A	T T	G A	A T	G A	1
	Ad 2	4.0	3.1	1.29	55.0	2.2	25.00	G A	T T	G A	T T	A A	A A	A A	
3	Ad 1	4.5	3.8	1.18	45.0	2.9	15.52	G A	A T	G A	T T	G A	A A	G A	
	Em 1							G A	A T	G A	T T	G A	A A	G A	0
	Ad 2	4.5	4.5	1.00	45.0	2.9	15.52	G A	A A	A A	T T	G A	A A	A A	
4	Ad 1	4.2	2.7	1.56	35.0	2.8	12.50	G G	A T	G A	T T	G A	A A	A A	
	Em 1							G A	A T	G A	T T	G A	A A	A A	1
	Ad 2	4.2	3.6	1.17	35.0	2.8	12.50	G A	A T	A A	T T	G A	A A	A A	
5	Ad 1	5.8	2.8	2.07	35.0	2.1	16.67	G G	T T	A A	T T	G A	A A	A A	
	Em 1							G G	A T	G A	T T	G A	A A	A A	2
	Ad 2	5.8	2.3	2.52	35.0	2.1	16.67	G G	A T	G G	T T	A A	A A	A A	
	Em 2							G G	A T	G A	T T	G A	A A	A A	2
6	Ad 1	4.5	3.2	1.41	110.0	2.6	42.31	A A	T T	G G	T T	A A	A T	A A	
	Em 1							G A	T T	G G	T T	A A	A T	G A	2
	Ad 2	4.5	2.9	1.55	110.0	2.6	42.31	G A	T T	G G	T T	A A	A A	G A	
7§	Ad 1	25.0	3.6	6.94	NA	NA	NA	G A	A A	A A	T T	A A	T T	A A	
	Em 1							G A	A T	G A	T T	G A	A T	A A	4
8	Ad 1	9.7	1.8	5.39	120.0	1.7	70.59	G G	T T	A A	T T	G A	A A	A A	
	Em 1							G A	T T	A A	C T	G A	A T	A A	3
	Ad 2	9.7	2.0	4.85	120.0	1.7	70.59	A A	T T	A A	C T	A A	A T	A A	
9	Ad 1	6.5	2.7	2.41	70.0	1.5	46.67	G A	T T	G A	T T	A A	A A	G A	
	Em 1							G A	A T	G A	T T	G A	A A	G A	2
	Ad 2	6.5	4.0	1.63	70.0	1.5	46.67	G A	A T	G A	T T	G A	A A	A A	
10	Ad 1	8.0	4.0	2.00	70.0	3.5	20.00	G G	T T	G A	T T	G A	A A	A A	
	Em 1							G G	T T	G A	T T	G A	A A	G A	1
	Ad 2	8.0	3.7	2.16	70.0	3.5	20.00	G G	T T	G A	T T	G A	A A	G A	
	Em 2							G G	T T	G A	T T	G A	A A	G A	0
11	Ad 1	6.0	3.5	1.71	50.0	1.7	29.41	G A	T T	G A	C T	A A	A A	A A	
	Ad 2	6.0	2.9	2.07	50.0	1.7	29.41	G G	T T	G A	T T	G A	A A	A A	
	Em 2							G A	T T	G A	C T	G A	A A	A A	2
12	Ad 1	5.0	2.1	2.38	21.0	1.6	13.13	G A	A T	A A	T T	G A	A T	G A	
	Em 1							G A	A T	G A	T T	G A	A T	G A	2
	Ad 2	5.0	2.0	2.50	21.0	1.6	13.13	G A	A T	G A	T T	G G	A T	G A	
13	Ad 1	10.0	1.6	6.25	60.0	3.4	17.65	G G	A T	A A	T T	A A	A T	A A	
	Em 1							G A	A T	G A	T T	G A	A T	A A	3
	Ad 2	10.0	2.3	4.35	60.0	3.4	17.65	G A	A T	G A	T T	G A	A T	A A	
14	Ad 1	6.0	2.8	2.14	80.0	2.0	40.00	G G	A T	A A	T T	G A	A T	G A	
	Em 1							G G	A T	A A	T T	G A	A T	G A	0
	Ad 2	6.0	2.2	2.73	80.0	2.0	40.00	G G	T T	A A	T T	G A	A T	A A	
	Em 2							G G	A T	A A	T T	G A	A T	G A	2
15	Ad 1	12.0	2.0	6.00	50.0	4.2	11.90	G G	T T	G A	T T	G A	A A	A A	
	Em 1							G A	T T	G A	T T	G A	A A	A A	1
	Ad 2	12.0	2.8	4.29	50.0	4.2	11.90	G A	T T	A A	T T	G G	A A	A A	
	Em 2							G A	T T	G A	T T	G A	A A	A A	2
16	Ad 1	11.3	2.8	4.04	30.0	3.2	9.38	G G	T T	A A	T T	G A	A A	G A	
	Em 1							G G	A T	G A	T T	G A	A A	G A	2
	Ad 2	11.3	3.8	2.97	30.0	3.2	9.38	G G	A T	A A	T T	A A	A A	A A	
	Em 2							G G	A T	A A	T T	G A	A A	G A	2
17	Ad 1	3.0	2.4	1.25	18.2	2.5	7.28	G G	T T	G A	T T	A A	T T	A A	
	Em 1							G A	A T	G A	T T	G A	A T	A A	4
	Ad 2	3.0	2.4	1.25	NA	NA	NA	A A	A T	G G	T T	A A	A A	A A	
	Em 2							G A	A T	G A	T T	A A	A T	A A	3
18§	Ad 1	16.4	2.5	6.56	NA	NA	NA	G A	A T	A A	T T	G G	T T	A A	
	Em 1							G A	A T	G A	T T	G A	A T	A A	3
19§	Ad 1	10.0	1.6	6.25	NA	NA	NA	G G	T T	G G	T T	A A	A A	A A	
	Em 1							G A	T T	G A	T T	A A	A A	A A	2

* Each colored row includes one isolated adult individual (and its egg mass) that was more than seven body lengths away from any other Balanus (Next Nearest Neighbor Distance).

** Each colored row includes a pair of adults (and their egg masses) adjacent to each other (Partner Distance) but more than seven body lengths away from any other Balanus (Next Nearest Neighbor Distance). At least one adult barnacle in each pair was fertilized.

† Ad 1: Adult barnacle 1; Em 1: Egg mass belonging to the Ad 1; Ad 2: Adult barnacle 2; Em 2: Egg mass belonging to the Ad 2.

§ No DNA was available for Ad 2 of this pair.

NA: Not Available- multiple adults were within a similar distance so no one individual was measured; the ratio of next nearest neighbor distances to body lengths were more than seven.

Table 3.4 Multilocus genotypes of isolated individuals and isolated pairs of *Chthamalus dalli* and their egg masses. Red circles indicate a locus where the egg mass genotype contained an allele not present in the brooding parent. Blue circles indicate a locus where the egg mass genotype contained an allele not present in either parent of the pair.

Isolated Individuals + Egg Masses*	Individuals†	Next Nearest Neighbor Distance (mm)	Next Nearest Neighbor Body Length (mm)	Ratio of Next Nearest Neighbor Distance to Body Length	Locus							Number of Loci with Non-Parental Alleles
					131-3	103-2	127-3	131-1	103-1	131-2	131-4	
1	Ad 1	7.2	0.7	10.29	G G	G C	T T	A A	C T	A A	G G	
	Em 1				G G	G C	T T	A A	C T	A A	G G	0
2	Ad 1	11.8	0.7	16.86	G A	G C	A T	A C	C G	G G	G G	
	Em 1				G A	G C	A T	A C	G T	G G	G G	1
3	Ad 2	8.0	0.5	16.00	G G	G C	A T	A A	C T	G A	G C	
	Em 2				G G	G C	A T	A A	C T	G A	G C	0
4	Ad 2	14.0	1.4	10.00	G G	C C	-- --	A A	C T	A A	G C	
	Em 2				G G	C C	A T	A A	C T	A A	G C	0
5	Ad 3	10.0	1.3	7.69	G G	C C	T T	A C	C T	G A	G C	
	Em 3				G A	G C	T T	A C	C T	G A	G C	2
6	Ad 3	12.8	1.4	9.14	A A	C C	A A	C C	C T	G G	G G	
	Em 3				G A	G C	A T	A C	C T	G A	G G	5
7	Ad 4	12.8	1.4	9.14	G G	C C	T T	A A	C T	G A	G G	
	Em 4				G A	G C	T T	A C	C T	G A	G C	4
8	Ad 4	5.2	0.6	8.67	G G	G C	T T	C C	C T	G A	G G	
	Em 4				G A	G C	A T	A C	C T	G A	G C	4
9	Ad 5	7.8	1.1	7.09	G G	G C	T T	A A	C T	A A	G C	
	Em 5				G G	G C	T T	A A	C T	A A	G C	0
10	Ad 5	6.4	0.7	9.14	G G	C C	T T	A A	C T	A A	G G	
	Em 5				G G	G C	T T	A A	C T	A A	G C	2
11	Ad 6	8.4	1.1	7.64	G A	G C	A T	A C	T T	G A	G C	
	Em 6				G A	G C	A T	A C	T T	G A	G C	0
12	Ad 6	9.6	0.9	10.67	A A	G C	T T	A C	C T	G A	G C	
	Em 6				A A	G C	T T	A C	C T	G A	G C	1
13	Ad 7	8.4	1.0	8.40	G G	C C	T T	A C	C T	G G	G G	
	Em 7				G G	G C	-- --	-- --	-- --	-- --	G G	1
14	Ad 7	10.9	0.8	13.63	A A	G C	T T	A C	C C	G A	G C	
	Em 7				G A	G C	T T	A C	C T	G A	G C	2
15	Ad 8	100.0	1.4	71.43	G G	C C	-- --	A A	C T	A A	G C	
	Em 8				G G	C C	T T	A A	C T	G A	G C	1
16	Ad 8	9.4	1.3	7.23	G G	C C	T T	A A	C T	G A	G G	
	Em 8				G G	C C	A T	A A	C T	G A	G G	1
17	Ad 9	11.4	1.3	8.77	G G	G C	A T	A A	T T	G A	G C	
	Em 9				G G	G C	A T	A A	T T	G A	G C	0
18	Ad 9	11.4	1.3	8.77	G G	G C	T T	A A	C T	G A	G C	
	Em 9				G G	G C	A T	A A	C T	G A	G C	1
19	Ad 10	15.0	1.0	15.00	G G	C C	A T	A A	C T	G A	G C	
	Em 10				G A	C C	A T	A A	C T	G A	G C	1
20	Ad 10	30.0	0.5	60.00	-- --	G C	T T	A A	C T	A A	C C	
	Em 10				G G	G C	T T	A A	C T	A A	G C	1
21	Ad 11	9.9	1.3	7.62	G A	G C	T T	A C	C T	G G	G C	
	Em 11				G A	G C	T T	A C	C T	G A	G C	1
22	Ad 11	100.0	1.4	71.43	A A	G C	T T	A C	C T	G G	G C	
	Em 11				G A	G C	T T	A C	C T	G A	G C	2
23	Ad 1	18.1	1.2	15.08	G G	C C	T T	A A	C T	A A	G G	
	Em 1				G G	G C	A T	A A	C T	A A	G C	3

Table 3.4. Continued.

Isolated Pairs + Egg Masses**	Individuals†	Partner Distance (mm)	Partner Body Length (mm)	Ratio of Partner Distance to Body Length	Next Nearest Neighbor Distance (mm)	Next Nearest Neighbor Body Length (mm)	Ratio of Next Nearest Neighbor Distance to Body Length	Locus							Number of Loci with Non-Parental Alleles
								131-3	103-2	127-3	131-1	103-1	131-2	131-4	
1	Ad 1	4.5	1.1	4.09	11.7	0.9	13.00	G G	G C	T T	A A	C T	A A	G C	
	Em 1							G G	G C	T T	A A	C T	A A	G C	0
	Ad 2	4.5	1.7	2.65	11.7	0.9	13.00	G G	G C	T T	A A	C T	A A	G G	
	Em 2							G G	G C	T T	A A	C T	A A	G C	1
2	Ad 1	2.8	2	1.40	9.4	0.9	10.44	G G	G C	A T	A A	C T	A A	G G	
	Em 1							G G	G C	A T	A A	C T	G A	G C	2
	Ad 2	2.8	2	1.40	9.4	0.9	10.44	G G	G C	T T	A A	-- --	G A	G C	
	Em 2							G G	G C	T T	A A	C T	G A	G C	0
3	Ad 1	2.4	0.5	4.80	21	0.7	30.00	G G	C C	T T	A A	C T	G A	G C	
	Em 1							G G	G C	T T	A A	C T	G A	G C	1
	Ad 2	2.4	0.5	4.80	21	0.7	30.00	G G	C C	T T	A A	C T	G A	G C	
4§	Ad 1	7.8	1.2	6.50	NA	NA	NA	G G	C C	A T	A A	C T	A A	G C	
	Em 1							G G	C C	A T	A A	C T	A A	G C	0
5	Ad 1	2.9	0.8	3.63	9.8	0.5	19.60	G G	G C	T T	A A	C T	A A	G C	
	Em 1							G G	G C	T T	A A	C T	A A	G C	0
	Ad 2	2.9	0.7	4.14	9.8	0.5	19.60	G G	C C	T T	A A	C T	A A	G C	
	Em 2							G G	G C	A T	A A	C T	A A	G C	2
6§	Ad 1	9.4	1.5	6.27	100	1.4	71.43	G G	C C	A T	A A	C C	G G	G G	
	Em 1							G G	C C	A T	A A	C T	G A	G G	2
7	Ad 1	5.1	1.1	4.64	40	1.4	28.57	G A	G C	T T	A C	C T	G A	G C	
	Em 1							G A	G C	T T	A C	C T	G A	G C	0
	Ad 2	5.1	1.8	2.83	40	1.4	28.57	G A	C C	T T	A C	C T	G A	G G	
8	Ad 1	4.1	1.5	2.73	10.1	1.2	8.42	G G	C C	T T	A A	C T	A A	G C	
	Em 1							G A	C C	T T	A C	C T	G A	G C	3
	Ad 2	4.1	1.3	3.15	10.1	1.2	8.42	G A	C C	T T	C C	C T	G G	G G	
9§	Ad 1	10.1	1.5	6.73	NA	NA	NA	G G	C C	T T	A A	C T	A A	G C	
	Em 1							G G	G C	T T	A A	C T	A A	G C	1
10	Ad 1	4.4	1.4	3.14	9.6	1.2	8.00	G G	C C	A T	A A	C T	A A	C C	
	Ad 2	4.4	1.3	3.38	9.6	1.2	8.00	G G	G C	A A	A A	C T	G A	G C	
	Em 2							G G	G C	A T	A A	C T	G A	G C	1
11§	Ad 1	8.9	1.3	6.85	NA	NA	NA	G A	G C	T T	A A	C T	G A	G C	
	Em 1							G A	G C	T T	A A	C T	G A	G C	0
12	Ad 1	4.7	1.8	2.61	13.2	0.5	26.40	G G	C C	T T	A A	C T	G A	G C	
	Em 1							G G	G C	T T	A A	C T	G A	G C	1
	Ad 2	4.7	1.8	2.61	13.2	0.5	26.40		G C	T T	A A	C C	A A	C C	
	Em 2							G G	G C	T T	A A	C T	G A	G G	3
13	Ad 1	3.7	1.4	2.64	100	1.4	71.43	G G	C C	A T	A A	C T	G A	G G	
	Em 1							G G	G C	A T	A A	C T	G A	G G	1
	Ad 2	3.7	1.3	2.85	100	1.4	71.43	G G	G C	T T	A A	C T	G A	G G	
	Em 2							G G	G C	A T	A A	C T	G A	G G	1
14	Ad 1	5.8	1.5	3.87	11.2	1.4	8.00	G A	G C	A A	C C	C T	G A	G G	
	Em 1							G A	G C	A T	-- --	C T	G A	G G	1
	Ad 2	5.8	1.5	3.87	11.2	1.4	8.00	G G	C C	T T	-- --	C T	A A	G G	
	Em 2							G A	G C	A T	A C	C T	G A	G G	4
15	Ad 1	2.4	1	2.40	100	1.4	71.43	G G	C C	T T	A A	C T	G G	G C	
	Ad 2	2.4	1.3	1.85	100	1.4	71.43	G A	G C	A T	A A	C T	G A	G C	
	Em 2							G A	G C	A T	A A	C T	G A	G C	0
16	Ad 1	2.3	1	2.30	10.4	1	10.40	G G	C C	T T	A A	C T	A A	G G	
	Em 1							G A	C C	A T	A A	C T	G A	G C	4
	Ad 2	2.3	1.2	1.92	10.4	1	10.40	A A	C C	A T	A A	C T	G G	C C	

* Each colored row includes one isolated adult individual (and its egg mass) that was more than seven body lengths away from any other Chthamalus (Next Nearest Neighbor Distance).
** Each colored row includes a pair of adults (and their egg masses) adjacent to each other (Partner Distance) but more than seven body lengths away from any other Chthamalus (Next Nearest Neighbor Distance). At least one adult barnacle in each pair was fertilized.
† Ad 1: Adult barnacle 1; Em 1: Egg mass belonging to the Ad 1; Ad 2: Adult barnacle 2; Em 2: Egg mass belonging to the Ad 2
§ No DNA was available for Ad 2 of this pair
NA: Not Available- multiple adults were within a similar distance so no one individual was measured; the ratio of next nearest neighbor distances to body lengths were more than seven.

Table 3.5 Incidence of spermcast mating in one stalked barnacle (a) *Pollicipes polymerus*, Thoracica, Pedunculata) (Barazandeh, et al., 2013) and two acorn barnacles (c) *Balanus glandula*, Thoracica, Balanomorpha, Balanoidea; d) *Chthamalus dalli*, Thoracica, Balanomorpha, Chthamaloidea) (present study). The average (± SE) spermcasting rate estimated from seven loci was simulated in *P. polymerus* (b) by 40 random subsamples of seven loci from the full set of 16.

		Isolated individuals			Isolated pairs		
Species	Number of loci	N (% fertilized)	1 non-maternal SNP allele*	2 non-maternal SNP alleles*	N (% fertilized)	1 non-maternal/partner SNP allele†	2 non-maternal/partner SNP alleles†
a) *Pollicipes polymerus*	16 (actual)	348 (10.6%)	100%	97.3%	158 (43.7%)	23.5%	14.7%
b) *P. polymerus (simulated)*	7 (simulated)	na	89.3 ± 0.84	60.2 ± 1.45	na	14.8 ± 0.63	5.2 ± 0.47
c) *Balanus glandula*	7 (actual)	249 (3.6%)	100%	77.7%	126 (36.5%)	7.7%	0%
d) *Chthamalus dalli*	7 (actual)	48 (52.1%)	69.6%	34.8%	37 (67.6%)	9.1%	0%

* percent of fertilized individuals bearing at least that many non-maternal alleles

† percent of fertilized individuals bearing at least that many non-maternal/partner alleles

na- not applicable to simulated results.

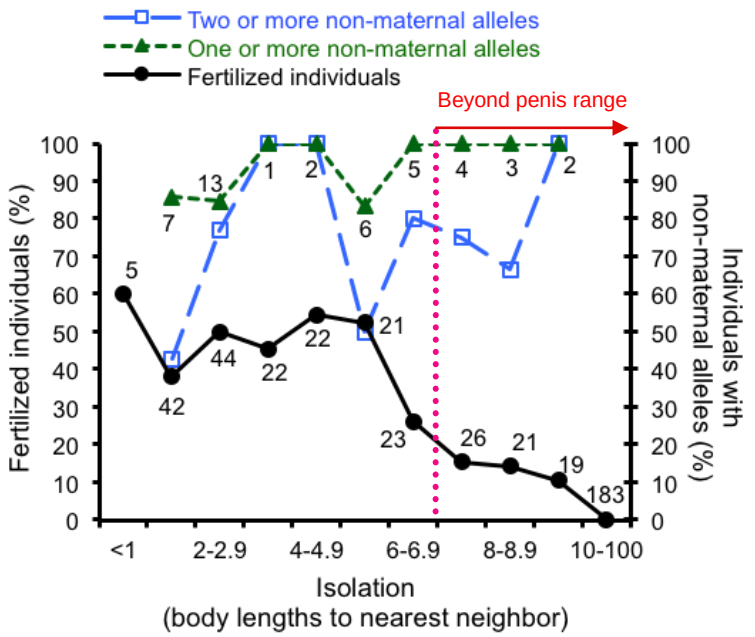

Figure 3.1 Percentage of *Balanus glandula* individuals fertilized (solid curve) or bearing non-maternal alleles at one or more (short dashes), or two or more (long dashes), SNP loci, as a function of isolation (number of body lengths to nearest neighbor). Numbers adjacent to points indicate sample sizes; sample sizes are the same for both dashed curves. Counts were pooled from two sites sampled in two years. The presence of non-maternal SNP alleles in individuals beyond penis range indicates sperm capture.

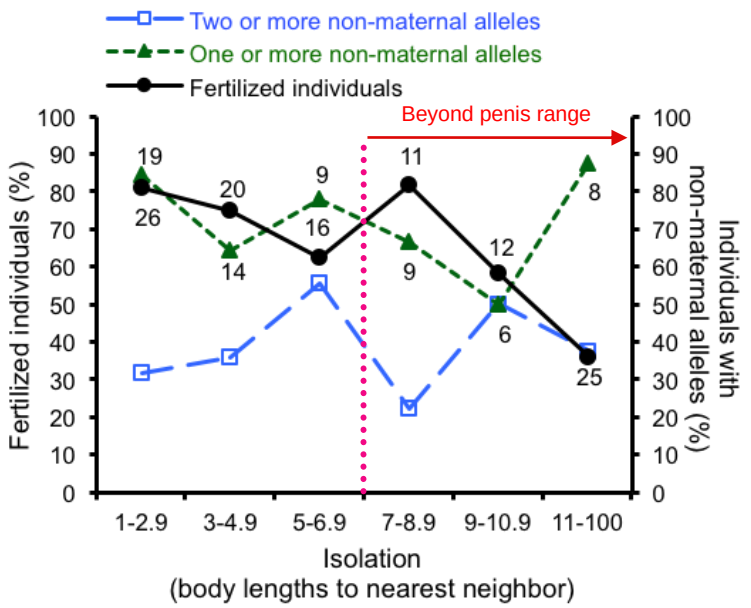

Figure 3.2 Percentage of *Chthamalus dalli* individuals fertilized (solid curve) or bearing non-maternal alleles at one or more (short dashes), or two or more (long dashes), SNP loci, as a function of isolation (number of body lengths to nearest neighbor). Numbers adjacent to points indicate sample sizes; sample sizes are the same for both of the dashed curves. Counts were pooled from two sites sampled in two years. The presence of non-maternal SNP alleles in individuals beyond penis range indicates sperm capture.

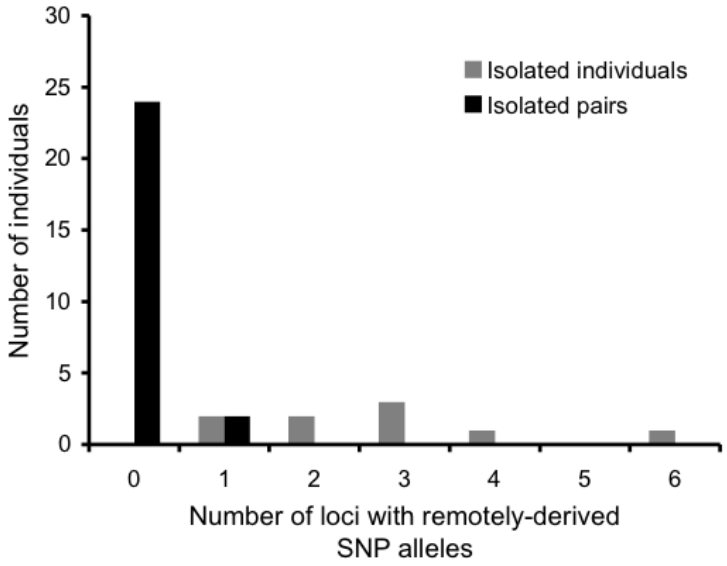

Figure 3.3 Numbers of *Balanus glandula* embryo masses with remotely-derived SNP alleles (non-maternal and non-maternal/partner alleles) from nine isolated individuals and 26 isolated pairs. A total of seven SNP loci were scored per individual. The presence of remotely-derived SNP alleles indicates sperm capture.

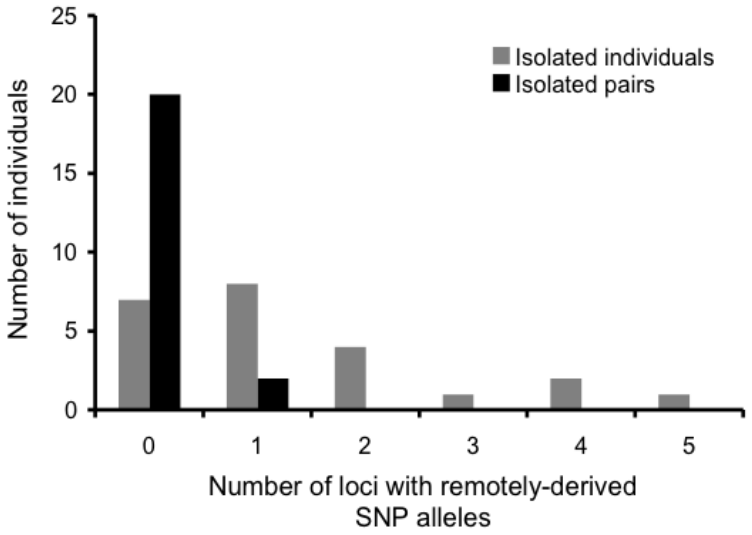

Figure 3.4 Numbers of *Chthamalus dalli* embryo masses with remotely-derived SNP alleles (non-maternal and non-maternal/partner alleles) from 23 isolated individuals and 22 isolated pairs. A total of 7 SNP loci were scored per individual. The presence of remotely-derived SNP alleles indicates sperm capture.

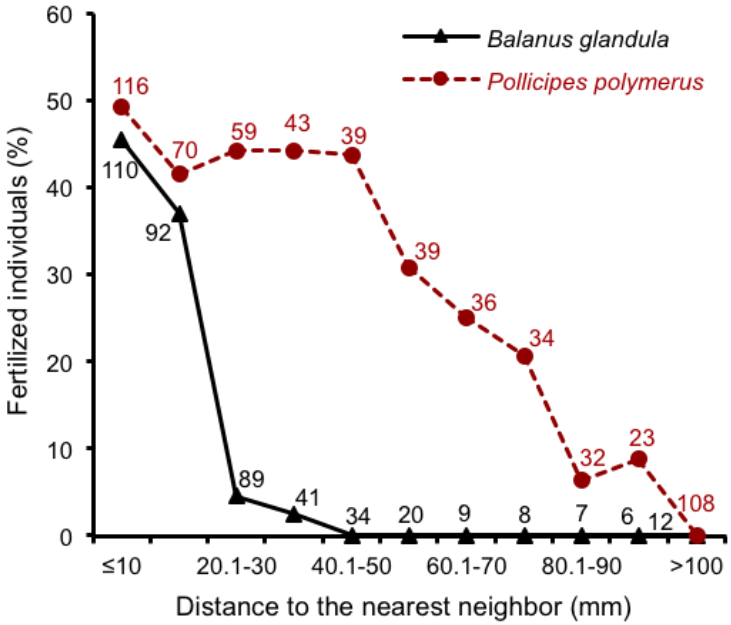

Figure 3.5 Percentage of *Balanus glandula* fertilized individuals (solid curve) and *Pollicipes polymerus* fertilized individuals (short dashes) as a function of distance to the nearest neighbor (mm). Numbers adjacent to points indicate sample sizes. Counts were pooled from two sites sampled in two years.

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Chapter 4. Novel fertilization modes on wave-swept shores: Aerial copulation and sperm release in a stalked barnacle[3]

4.1 Introduction

Barnacles exhibit some decidedly unusual adaptations for mating. Adults in most free-living species are sessile and yet — like other crustaceans — they are presumed to mate primarily by pseudo-copulation (Addison and Hart, 2005; Anderson, 1994; Darwin, 1851, 1854). Therefore, because barnacles are sessile most have a long penis that can extend multiple body lengths to reach nearby partners (Hoch, 2009; Neufeld and Palmer, 2008). Functional males search for functional females using their penises and deposit sperm into their partner's mantle cavity. Presumably, in intertidal barnacles, copulation occurs when they are completely immersed, but few observations have actually been made of barnacle behavior as the tide comes in. To avoid losing sperm to the waves, oviposition and fertilization happens quickly after sperm release (Anderson, 1994; Barnes, et al., 1977). Despite being mostly hermaphroditic, copulation is believed to be non-reciprocal (i.e. at any one time an individual functions as either male or female; Anderson, 1994; Barnes, et al., 1977) and self-fertilization is limited to

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only a few thoracican species (Barnes and Crisp, 1956; Barnes and Barnes, 1958; Furman and Yule, 1990).

Nonetheless, molecular evidence confirms that a third mode of sperm transfer — spermcast mating (Bishop and Pemberton, 2006) — occurs commonly in one intertidal stalked barnacle with a relatively short penis, *Pollicipes polymerus* Sowerby 1833 (Barazandeh, et al., 2013), and occasionally in two acorn barnacles with long penises, *Balanus glandula* and *Chthamalus dalli* (Barazandeh, et al., Submitted). Similar to many other sedentary marine invertebrates (Bishop and Pemberton, 2006), sperm released into the water can fertilize distant individuals. Field observations of occasional *P. polymerus* leaking a foamy white liquid at low tide (Figure 4.1) suggest that sperm release may be active (Barazandeh, et al., 2013). However, this foamy white liquid has not been confirmed to contain sperm. In addition, subsequent to fertilization, barnacles sometimes expel excess sperm out of their mantle cavity in a gel-like "sperm pool" (Barnes, et al., 1977), which could potentially travel through water and fertilize other barnacles. Sperm of barnacles are fully motile and highly active after deposition into the mantle cavity (Barnes, et al., 1977; Healy and Anderson, 1990). These features all increase the likelihood that spermcast mating might occur.

A key question remains unanswered for the first barnacle species in which spermcast mating was detected (Barazandeh, et al., 2013): Is spermcast mating in the stalked barnacle *Pollicipes polymerus* the primary mode of mating in this species with an unusually short penis, or does it also copulate like other

barnacles? Curiously, prior efforts to observe copulation in *P. polymerus* failed, even after hours of observation in aquaria (Barnes, 1992; Hilgard, 1960). In addition, little is known about the actual process of sperm release and capture, and its frequency, in *P. polymerus.*

Pollicipes polymerus occur commonly on intertidal shores of the northeast Pacific, usually in rosette-shaped clusters of tens to hundreds of barnacles in close proximity (Barnes, 1996; Hoffman, 1989; Newman and Abbott, 1980). They live under a wide range of wave-exposures, from moderate to exceedingly high, and range vertically from the low-shore among mussels to the high-shore on bare rock (Hoffman, 1989). In acorn barnacles with long penises, the length and thickness of the penis change significantly between sites that experience different wave action to maximize mating opportunities (Hoch, 2009; Neufeld and Palmer, 2008). But we do not know how the unusually short penis of *P. polymerus* is used in mating.

We therefore 1) tested whether the foamy white material leaked by *P. polymerus* at low tide actually contains sperm, 2) quantified sperm-leaking rates of *P. polymerus* in the field, 3) assessed the impact of environmental factors (e.g. wave-exposure, shore height, tide and swell heights, weather) and the density of barnacles on the frequency of sperm leaking by *P. polymerus*, and 4) tested whether *P. polymerus* copulate with neighbors to exchange the sperm, and, if so, under what conditions.

4.2 Materials and Methods

4.2.1 Sperm samples and imaging

Each day, samples of the foamy white liquid released by some *Pollicipes polymerus* at low tide were placed into 1.5 ml microfuge tubes containing half filtered seawater and half 4% formaldehyde. To confirm that the samples contained sperm, we observed them under an Olympus Fluoview-FV300 microscope and captured images at 400X and 1000X magnifications. We also prepared images of individual sperm using a Philips / FEI (XL30) Scanning Electron Microscope (SEM). The whole microfuge tube containing a sperm sample was sonicated for 5 seconds at 130W with an ultrasonic cleaner (Branson Ultrasonics Corporation, model 2510) to untangle the sperm that were twisted together and to remove debris attached to the sperm. One or two drops of the sonicated sample were put on a Millipore 0.2 μm GTTP filter and placed in a histology cassette. The filter was washed in 0.1M phosphate buffer for 10 minutes followed by washing once in 30%, 50%, 70% and 90% ethanol and twice in 100% ethanol, each for 10 minutes. Filters were then placed into ethanol:HMDS (Hexamethyldisilazane; 75:25, 50:50, 25:75, respectively) and 100% HMDS, each for 15 minutes and left to air dry overnight. Samples were mounted on SEM stubs, sputter-coated with gold/palladium, and examined with SEM (Watson, et al., 1980).

4.2.2 Spatial and temporal variation in sperm-leaking

Using a 1-m wide belt transect deployed along the shore at a given tidal height, 6-10 m^2 of continuous populations of *Pollicipes polymerus* were sampled

in the high shore and low shore of two highly wave-exposed sites (west side Helby Is., 48.847°N, 125.168°W and north side Seppings Is., 48.841°N, 125.209°W) and the high shore of two moderately wave-exposed sites (Brady's Beach, 48.331°N, 125.173°W and southwest side of Dixon Is., 48.519°N, 125.710°W) in Barkley Sound (near Bamfield, British Columbia, Canada). The vertical range of *P. polymerus* was much more restricted on the less wave-exposed shores (Figures 4.2-4.7). Surveys were done during low tides in August-September 2012 (near the end of *P. polymerus* mating season) and April-May 2013 (near the beginning of the mating season). Data from these two years were then combined. Each transect was searched multiple times during one low tide to find individuals leaking foamy white liquid (presumed sperm) from their opercular plates. The location of each leaking barnacle in the transect, the rostro-carinal opercular length (the maximum distance between the posterior margin of the scutum and the anterior margin of the turgum, parallel to the gape), and the time of leaking was recorded. Each individual was then labeled and photographed. The times and heights of the high tides (higher high water tide and lower high water tide), the wave heights and the weather (sunny, cloudy, semi-cloudy) of each day were also recorded. To assess the effect of shore height on sperm-leakage in *P. polymerus*, we conducted equal numbers of scans of transects on the high shore and the low shore of Seppings Is. and Helby Is. on each day in summer 2013.

The total number of barnacles in each — or every other — m^2 of each transect (covering equal ranges in the two halves of the transect) was counted and

the percent of sperm-leakers relative to the total number of individuals was calculated.

4.2.3 Filming of barnacle behavior and video analysis

To record mating activities of *P. polymerus* — to test for active ejection of sperm or copulation — we used an Olympus TG-320 Tough digital compact waterproof camera firmly mounted to the end of a 3 m-long aluminum pole with feet at the end to position the camera and hold it stable in moving water. We started filming *P. polymerus* when the first waves hit the barnacles as the tide came in. Some videos were done during waxing higher high-tides but most were done during waning higher high-tides. We videotaped the barnacles labeled as leakers before the tide came in, as well as random clumps in the transects after we finished searching for sperm-leakers, at all sites in August-September 2012 and April-May 2013. Each video recording was approx. three minutes. Barnacles were covered by water for as short as a few seconds —when waves first started to wash over them — to as long as the whole duration of the video.

All barnacles within the frame of each video were counted, whether copulating or not, and all copulations or copulation attempts were recorded. We measured the time that barnacles were underwater and the total duration of each video for the videos from summer 2013 and categorized the videos into three groups: 0-33%, 33.1-66%, and 66.1-100% of time immersed under breaking waves.

4.3 Results

4.3.1 Sperm leakage and imaging

Foamy white liquid, if observed, was usually seen leaking from near the middle of the gape at the junction between the scutal and tergal plates (Figure 4.1a-d), and rarely from the anterior margin of the gape (Figure 4.1d). Even when sperm was released in still water in a tidepool, it was released from the same spot, without any evident protrusion of the penis (Figure 4.8).

Using the light and electron microscopy, we confirmed that this white foamy liquid contained sperm, usually twisted together (Figure 4.9a). Similar to other thoracicans (Healy and Anderson, 1990), *P. polymerus* bears a filiform spermatozoon, which is approximately 70 μm long (Figure 4.9b). Unfortunately, because samples were fixed right after collection, we could not verify movement or survival of the sperm outside of the barnacle's body.

4.3.2 Patterns of sperm-leaking in Pollicipes polymerus

The rostro-carinal size of sperm-leakers ranged from 9-27 mm, with about 30% of them being 10-15 mm and 50% of them being 15-20 mm (Figure 4.10). The sizes of sperm-leaking barnacles differed somewhat between years, but the difference was not significant overall (Chi-square test, $X^2 = 8.6$, $P = 0.07$). Isolated sperm-leakers were mostly randomly distributed along the transects >20 cm from other leakers, however on 15 occasions, three to six sperm-leaking individuals were observed within the same cluster in an area smaller than 100 cm^2 and >20 cm away from other leakers.

We compared the percentage of the sperm-leakers relative to the total number of barnacles between the two halves of each transect (Figure 4.11). The average of sperm-leaking barnacles per day at each site did not differ significantly between years (Chi-square test, $X^2 = 10.8$, $P = 0.46$), so we pooled the data from 2012 and 2013. Surprisingly, in most transects, the averages of both the actual numbers and the percentages of sperm-leakers were higher in the half of the transect that had fewer barnacles. The only exception was Brady's Beach, where more leakers were found in the half that had more barnacles (Figure 4.11).

We detected no obvious association between the incidence of sperm leaking and the timing of the tides, wave height, time of day, or weather at any site studied (Figures 4.12-4.17). However, in 2013 there were more sperm-leakers on the low shore than on the high shore of Seppings Is. and Helby Is. on almost every day studied. Density of the barnacles on the low shore of Seppings Is. and Helby Is. was also higher than on the high shore: The average number of barnacles per m^2 in the low shore transects were 2.2 times and four times more than the high shores in Seppings and Helby, respectively (Figure 4.11).

4.3.3 Video analysis of mating behavior

We observed — for the first time — *Pollicipes polymerus* copulating in the field. Unlike other intertidal barnacles, many *P. polymerus* started searching for potential mates and copulating with other individuals shortly after contact by the first waves on an incoming tide. Remarkably, copulation continued while the individuals were exposed to air.

Overall, approximately 0.43% of individual *Pollicipes polymerus* observed in the 2013 videos were copulating, ranging from 0.23% at Brady's to 0.65% at Dixon. At the two less-exposed sites (Brady's Beach and Dixon Is.), barnacles were sexually active only when they were underwater 0-33% and 0-66% of the time, respectively. However, in the two highly wave-exposed sites (Helby and Seppings), mating activities were mostly observed when the barnacles were underwater more than 33% of the time (Figure 4.18). In only one case was copulation observed when the barnacles were continuously underwater.

Out of 106 sexually active barnacles captured on video in the summers of 2012 and 2013, six pairs (12 individuals; 11%) were copulating simultaneously with each other (i.e. reciprocal copulation).

4.4 Discussion

Our results provide some valuable new insights into the reproductive biology of an unusual but ecologically significant intertidal stalked barnacle, *Pollicipes polymerus*: the first barnacle species known to exhibit spermcast mating (Barazandeh, et al., 2013). First, we confirmed that the milky liquid leaked by some individuals at low tide (Figures 4.1, 4.8) contained sperm (Figure 4.9). These observations help explain how isolated individuals too far from a neighbor to copulate can bear eggs fertilized by other individuals (Barazandeh, et al., 2013). Second, we observed a consistent low level of sperm-leaking in field populations (Figure 4.11), so sperm leakage appears to be a normal, if infrequent, component of *P. polymerus* reproductive biology. Most importantly, we confirmed that, even though they have an unusually short penis (Barazandeh, et

al., 2013) *P. polymerus* also copulate, and they appear to do so preferentially as the tide comes in, while they are still partly exposed to air, rather than when they are continuously immersed under breaking waves.

Although only a small percentage of *Pollicipes polymerus* leaked sperm at low tide in the field each day — approx. 0.1% overall — a few leaking individuals were observed on all days in all transects. Sperm leakage therefore appears to be a normal feature of their reproductive biology. The incidence of sperm leaking did not vary in any obvious way with weather, height of the high tide, or wave action (Figures 4.12-4.17). However the occasional observation of several leakers in the same cluster suggests that leakage by one barnacle may stimulate the sperm release in neighbors during a subsequent low tide. In barnacles, when functional females are ready to mate, they send chemical signals around to make themselves detectable by functional males (Anderson, 1994). Similar chemical cues could be involved in distributing the signals of "suitable time for sperm release" among the other barnacles nearby.

Sperm leakage rates appeared to vary with local population density. In all sites except for Brady's Beach, more leakers, and a higher percentage of leakers, were found in the half of the transect that contained fewer *P. polymerus* (Figure 4.19). The environmental conditions and scanning criteria were the same for all sections of the same transects on each day, but differed between transects, so the comparison was done only between the two halves of the same transect.

Releasing sperm into the water could potentially increase fitness when barnacles have fewer mates nearby. Theory predicts that as mating group size

(MGS- the number of individuals in a mating group) gets smaller, simultaneous hermaphroditism with less allocation to male function (sperm production) should evolve (Charnov, 1982, 1987). For instance, in barnacles with only 1 neighbor (MGS= 2), sperm production should just be enough to fertilize one partner's eggs. Similarly, when individuals are totally isolated, with no partners around, sperm production should not happen and they should become females instead, as seen in species of *Catomerus polymerus* and *Arcoscalpellum michelottianum* (Raimondi and Martin, 1991; Yamaguchi, et al., 2007; Yamaguchi, et al., 2008; Yusa, et al., 2012). In fully hermaphroditic species such as *P. polymerus*, the ability both to release sperm, and to capture it from the water, has obvious advantages in low-density conditions. But even in high density conditions, this ability could potentially greatly increase effective MGS and therefore favor increased male function (Charnov, 1982, 1987). The presence of more barnacles and still more leakers on the low shore of Helby Is. and Seppings Is. than the high shore is surprising, though. Low shores of both sites are subjected to more wave action and submergence than the high shore, which could facilitate the spermcast mating process for barnacles in those areas. Sperm leaking may therefore depend on the density of barnacles only under similar physical conditions.

Although we expected that barnacles might modify their behavior in response to persistent environmental conditions over several days, we observed no evidence of this. Sperm leaking in *P. polymerus* did not vary in response to tidal height, or weather, or sea conditions (Figures 4.12-4.17). However, more

extensive studies would need to be done throughout the mating season to be confident in this conclusion.

Most spermcasting species actively eject sperm to fertilize conspecific eggs (Bishop, 1998; Bishop and Pemberton, 2006). Our results suggest that sperm release is active for *P. polymerus* as well, even though they normally release sperm while exposed to the air (Figure 4.1). The relaxed penis of *P. polymerus* lies parallel to the bases of feeding legs (cirri I-VI) with its tip located ventrally near the aperture at the joint of the tergum and scutum (Hilgard, 1960). 90% of the time, *P. polymerus* released sperm from the same site on the opercular plates (Figure 4.1a-c; Figure 4.8), which strongly suggests active release of sperm. The sperm seen at the anterior margin of the scutum in a few cases (Figure 4.1d) could be due to the bleeding down of the excess sperm.

Significantly, occasional individuals were observed to release sperm in an extended stream when under water in a tidepool (Figure 4.8), which further suggests that sperm release is active. This observation also raises the possibility that *P. polymerus* may release sperm under water in breaking waves, but we were unable to observe this in any videos taken to document copulating behavior.

The general morphology of the sperm in *P. polymerus* (Figure 4.9) resembles that of other thoracican barnacles. The sperm of almost all barnacles possess an accessory droplet, which is removed before the ejection to give the sperm more motility (Anderson, 1994; Healy and Anderson, 1990). In spermcasting species, sperm usually remain inactive in the water immediately after release, which may conserve energy needed for fertilization to occur after

sperm are captured (Bishop, 1998). Their lifespan in the water varies from a few seconds to hours (Bishop, 1998; Bishop and Pemberton, 2006). We suspect that, similar to other spermcasting species, functional females of *P. polymerus* must gradually gather the diluted sperm to a certain concentration that is high enough to trigger oviposition (Anderson, 1994; Bishop, 1998; Bishop and Pemberton, 2006).

The overall percent of individuals we observed copulating (0.43%) was nearly four times higher than the overall percent of individuals leaking sperm during the same time periods (0.10%) (Figures 4.18, 4.19). So, regardless of the prevalence and mechanisms of spermcast mating, *P. polymerus* clearly uses pseudo-copulation as the predominant mode of sperm transfer when potential mates are nearby even though it has a relatively short penis that barely reaches twice its body length after full extension (Barazandeh, et al., 2013).

Although we confirmed that *P. polymerus* do copulate like other barnacles, they do so with a fascinating twist: copulation is sometimes initiated, and often continued, in air as the waves first start to break over them on an incoming tide. The past inability to observe copulation in *P. polymerus* — despite extensive effort — has always been puzzling (Barazandeh, et al., 2013; Barnes, 1992; Hilgard, 1960), particularly given the ease with which copulation has been observed in other species (Anderson, 1994; Barnes, et al., 1977). In previous studies, *P. polymerus* mating activity was monitored while they were held underwater (Barnes, 1992; Hilgard, 1960). But we discovered that they start copulating as the tide comes in and before becoming fully submerged. In fact, at all sites we studied, barnacles were exposed to air at some point during copulation

(Figure 4.18). This contrasts with all previous reports on barnacle mating, where copulation follows feeding activities and thus happens underwater (Anderson, 1994).

Aerial mating appears to be another adaptation of *P. polymerus* for life on wave-swept shores. Rocky intertidal shores, where barnacles live, are exposed to remarkably different levels of wave motions, which impact behavior and survival (Denny, 1987; Helmuth and Denny, 2003). At Brady's Beach, the least wave-exposed site among the four sites we studied, copulation by *P. polymerus* occurred mostly when they were exposed to air between waves more than 67% of the time (Figure 4.18). Similarly, at the other moderately wave-exposed site, Dixon Is., we observed no further copulations when barnacles were underwater more than 66% of the time (Figure 4.18). In contrast, at the two more wave-exposed sites, Seppings Is. and Helby Is., copulation occurred mainly when barnacles were immersed more than 33% of the time (Figure 4.18). At the less-exposed sites, the high shores may not be fully submerged even at high tides on many days throughout the year, so there may be strong selection for *P. polymerus* to be able to copulate in air, during the short periods when they are only partially awash in waves. Having said this, mating in air may be directly advantageous on wave-swept shores in general as penis mobility may be better controlled in the upper margins of subsiding wave wash than when barnacles are continuously immersed in the surge of breaking waves.

The generally shorter penis of *P. polymerus* compared to other barnacles (Barazandeh et al. 2013) is not unexpected given its many other specializations

for life on wave-swept shores (Barnes, 1996) and given what is known about other barnacles. Acorn barnacles produce shorter and stouter penises in response to higher wave action (Hoch, 2008, 2009; Neufeld and Palmer, 2008; Neufeld and Rankine, 2012), which presumably allows them to mate more effectively in turbulent flow. In addition, *P. polymerus* extend the capitulum away from the substratum as the tide comes in, which would also increase access to potential mates despite having a relatively short penis.

Contrary to prior claims for all other barnacles (Anderson, 1994; Barnes, et al., 1977), reciprocal copulation does happen in *Pollicipes polymerus*. Normally, hermaphrodite barnacles function alternately as either males or females frequently during the mating season (Anderson, 1994). Nonetheless, we observed several *P. polymerus* individuals (11%) copulating with each other concurrently for several minutes, which is enough time for sperm release into the partner's mantle cavity. Such reciprocal copulation implies that resources for gametes may not be as limiting in this stalked barnacle as they are in other barnacles. Alternatively, space in the mantle cavity for egg lamellae may be more limiting in *P. polymerus* so that diversion of some resources to sperm production doesn't reduce the ability to fill the mantle cavity with fertilized eggs after mating. However, as we were unable to examine those barnacles for the presence of the fertilized eggs after copulation, we cannot reject the possibility that both barnacles were acting as functional males (i.e. egg release did not happen in either). If neither bore fertilized eggs, this would either be an example of erroneous mating, or the first example of functionally homosexual behavior in a barnacle.

Our observations revealed some unique mating behaviors in *Pollicipes polymerus*. However, several interesting questions remain unanswered: i) What is the longevity and motility rate of the sperm of *P. polymerus* in sea water and in the females' mantle cavity? ii) What is the effective concentration of the sperm that leads to egg release in the mantle cavity? iii) How are sperm released in other barnacles that sometimes spermcast (Barazandeh, et al., Submitted)? iv) Do any other barnacle species copulate out of water? v) What is the advantage to spermcast mating in *P. polymerus*, given that copulation is still more common than spermcasting?

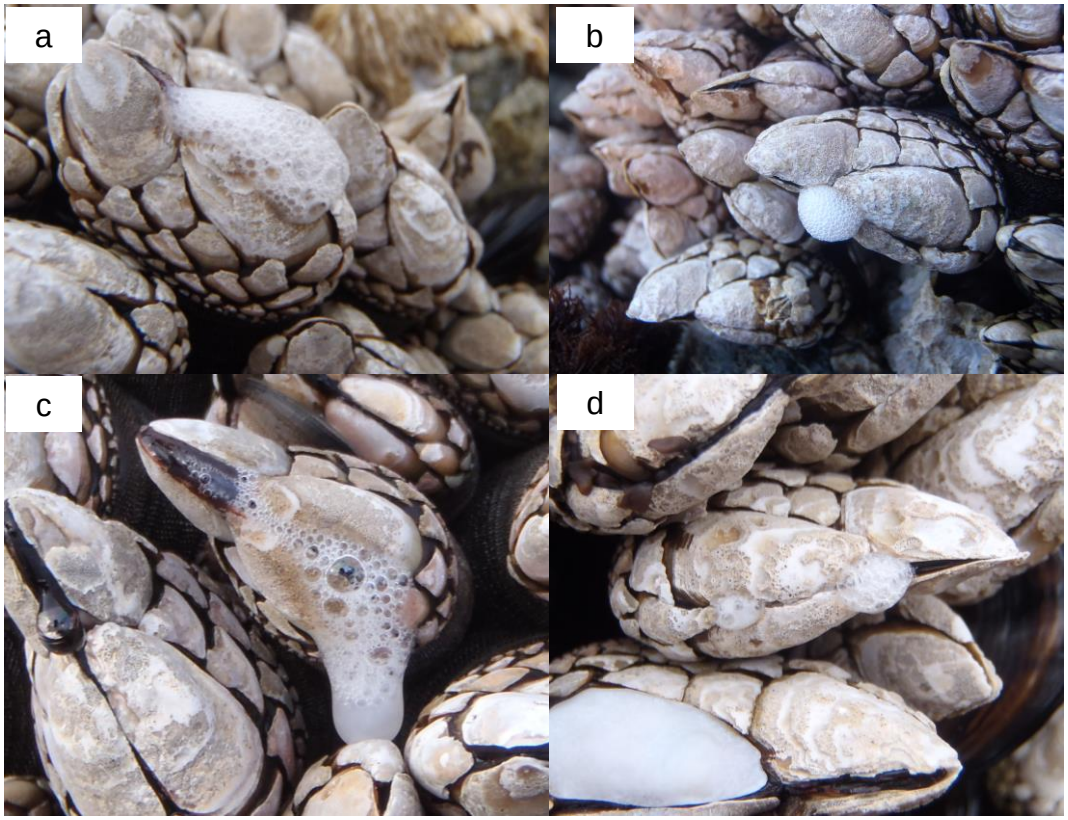

Figure 4.1 *Pollicipes polymerus* leaking sperm at low tide. Sperm leakage typically occurred at the junction between the tergal and scutal plates (a, b and c), but occasionally occurred at the anterior margin of the gape (d).

Figure 4.2 10 m^2-belt transect at Brady's Beach (moderately wave-exposed) for monitoring sperm-leaking in *Pollicipes polymerus.*

Figure 4.3 9 m^2-belt transect at Dixon Island (moderately wave-exposed) for monitoring sperm-leaking in *Pollicipes polymerus.*

Figure 4.4 10 m^2-belt transect at Helby Island (highly wave-exposed) high shore for monitoring sperm-leaking in *Pollicipes polymerus*.

Figure 4.5 6 m^2-belt transect at Helby Island (highly wave-exposed) low shore for monitoring sperm-leaking in *Pollicipes polymerus*.

Figure 4.6 10 m^2-belt transect at Seppings Island (highly wave-exposed) high shore for monitoring sperm-leaking in *Pollicipes polymerus.*

Figure 4.7 7 m^2-belt transect at Seppings Island (highly wave-exposed) low shore for monitoring sperm-leaking in *Pollicipes polymerus.*

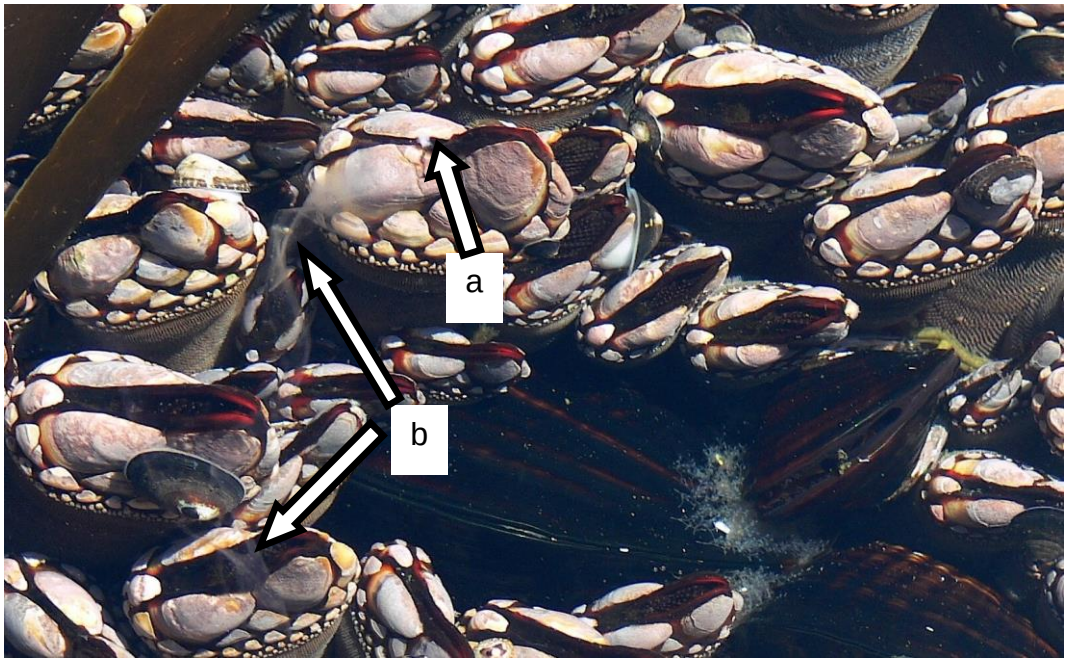

Figure 4.8 An individual *Pollicipes polymerus* leaking sperm into the water of a tidepool at low tide, Seppings Is. May 26, 2012. (a) Point of sperm release. (b) Sperm stream drifting through the water. Note that the point of sperm release in water is the same as that in air (Figure 4.1a-c).

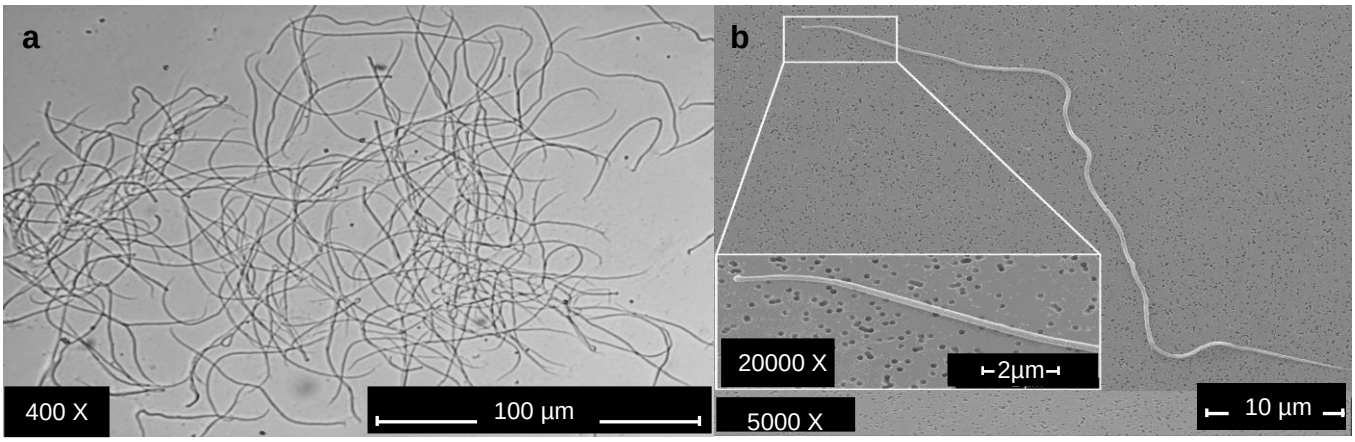

Figure 4.9 *Pollicipes polymerus* sperm collected in the field from leaking barnacles (a) light microscope image of several sperm tangled together; 400 X magnification, (b) Scanning Electron Microscope image of a single sperm (5000X magnification) and its head (20000X magnification).

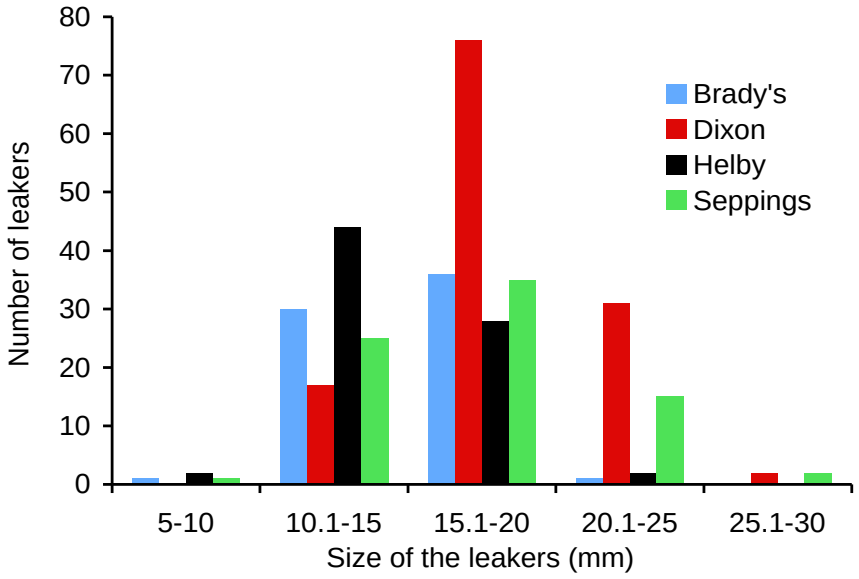

Figure 4.10 Size-frequency distribution of *Pollicipes polymerus* leaking sperm at four field sites, two highly wave-exposed (Seppings Is., Helby Is.) and two moderately wave-exposed (Brady's Beach, Dixon Is.). Data from two years (2012, 2013) were pooled.

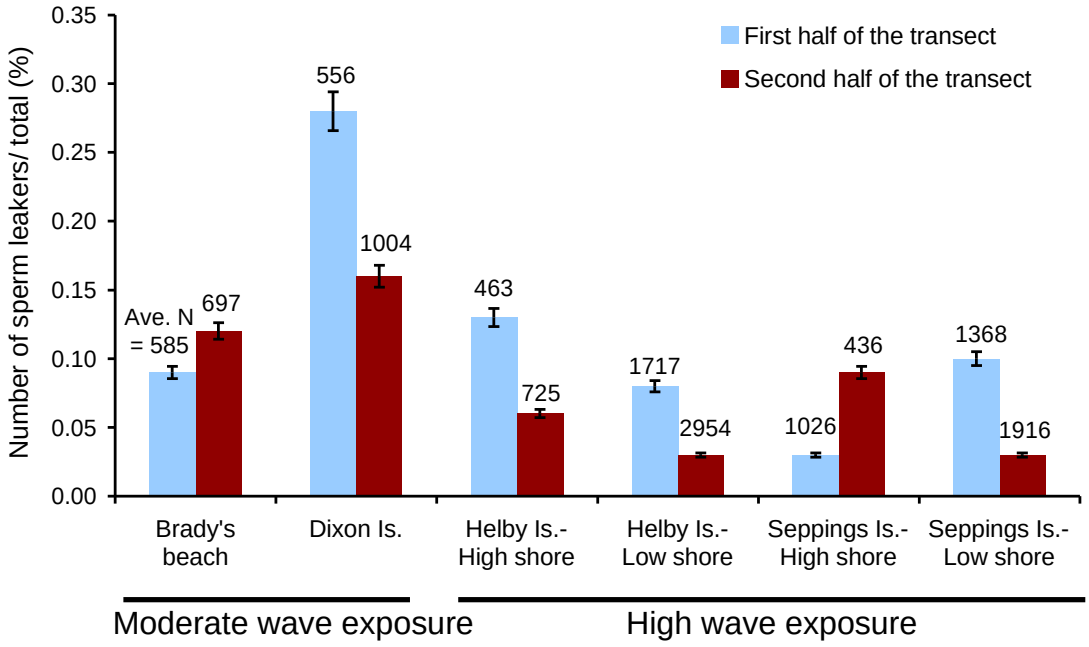

Figure 4.11 The average percentage of individuals leaking sperm per day relative to the total number of individuals in each half of the belt transects at six sites. Numbers above the bars are the total number of individuals per m^2 in each half of the transect. Data from two years (2012, 2013) were pooled.

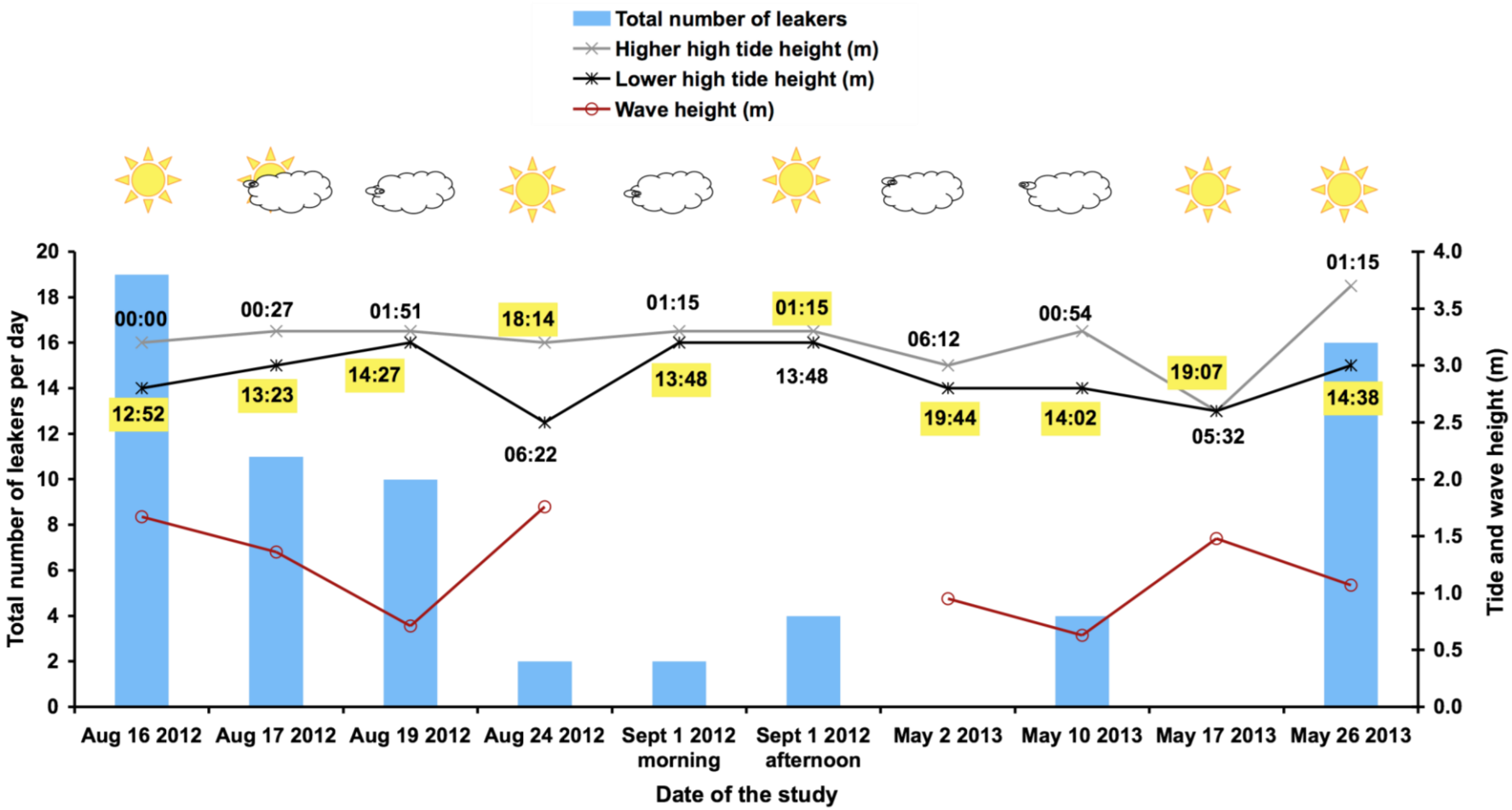

Figure 4.12 Total number of sperm-leaking individuals per day at Brady's Beach (moderately wave-exposed) in relation to weather, wave and tide conditions. The black curve corresponds to the height of the lower high tide and the grey curve corresponds to the height of the higher high tide. Numbers adjacent to points indicate the time of the high tides. Yellow boxes represent the high tides in which the study was done. The red curve shows the average height of the waves in each day. Wave height data were unavailable for September 1, 2012. The images at the top reveal the weather conditions (sun: sunny, cloud: cloudy, sun+cloud: partly-cloudy).

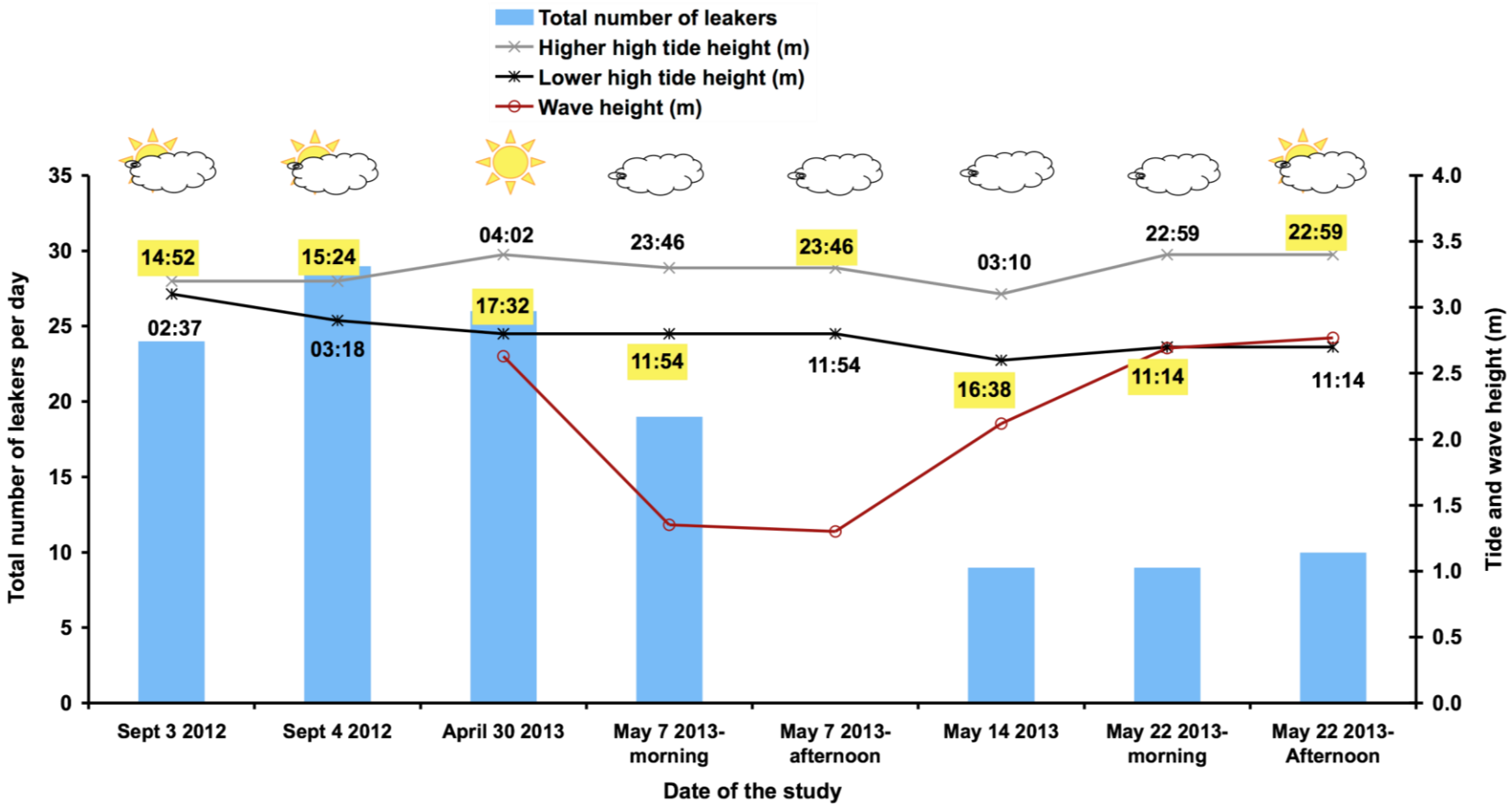

Figure 4.13 Total number of sperm-leaking individuals per day at Dixon Island (moderately wave-exposed) in relation to weather, wave and tide conditions. Labeling as in Figure 4.12 legend. Wave height data were unavailable for September 3-4, 2012.

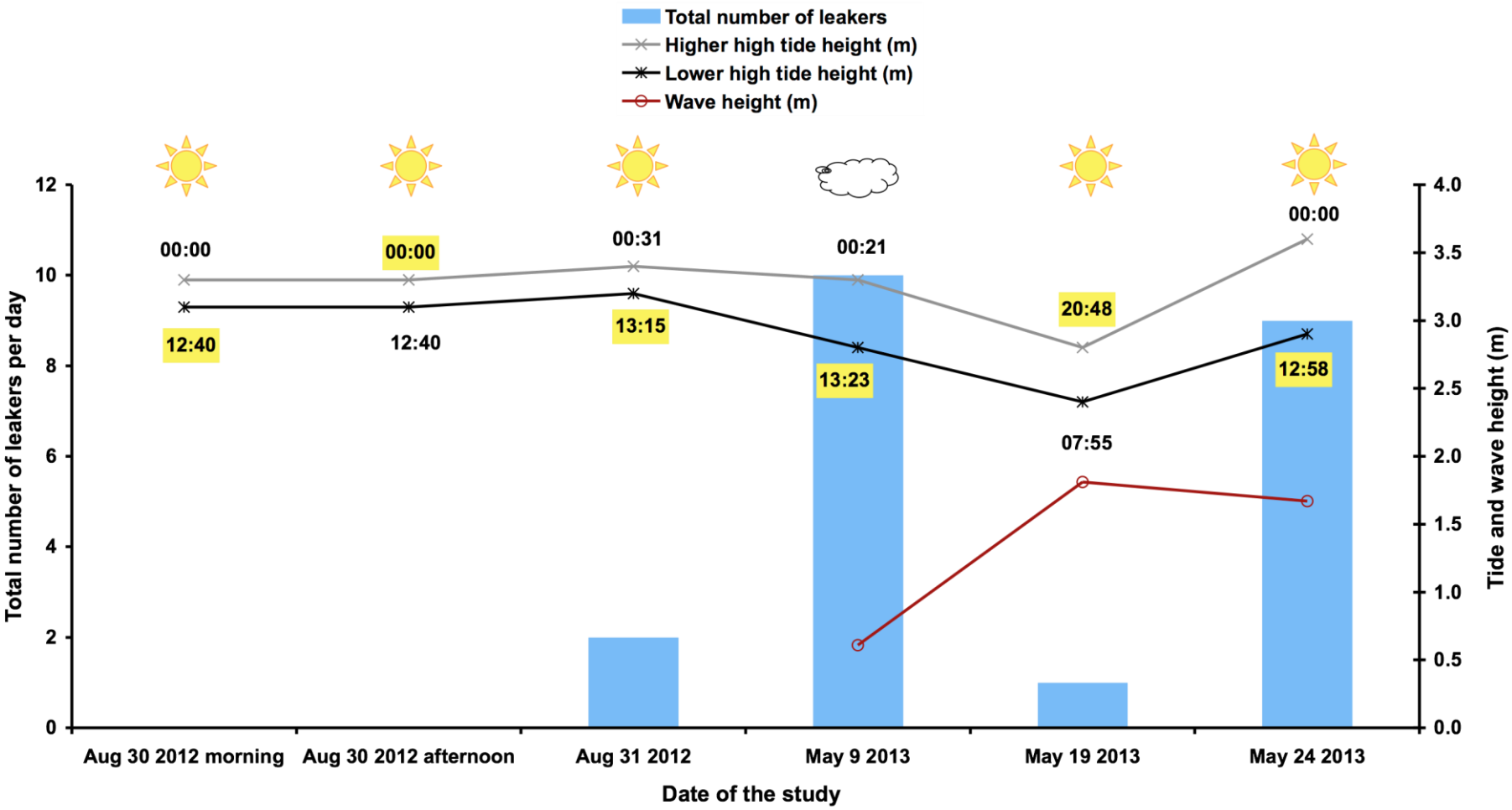

Figure 4.14 Total number of sperm-leaking individuals per day at Helby Island, high shore (highly wave-exposed) in relation to weather, wave and tide conditions. Labeling as in Figure 4.12 legend. Wave height data were unavailable for August 30-31, 2012.

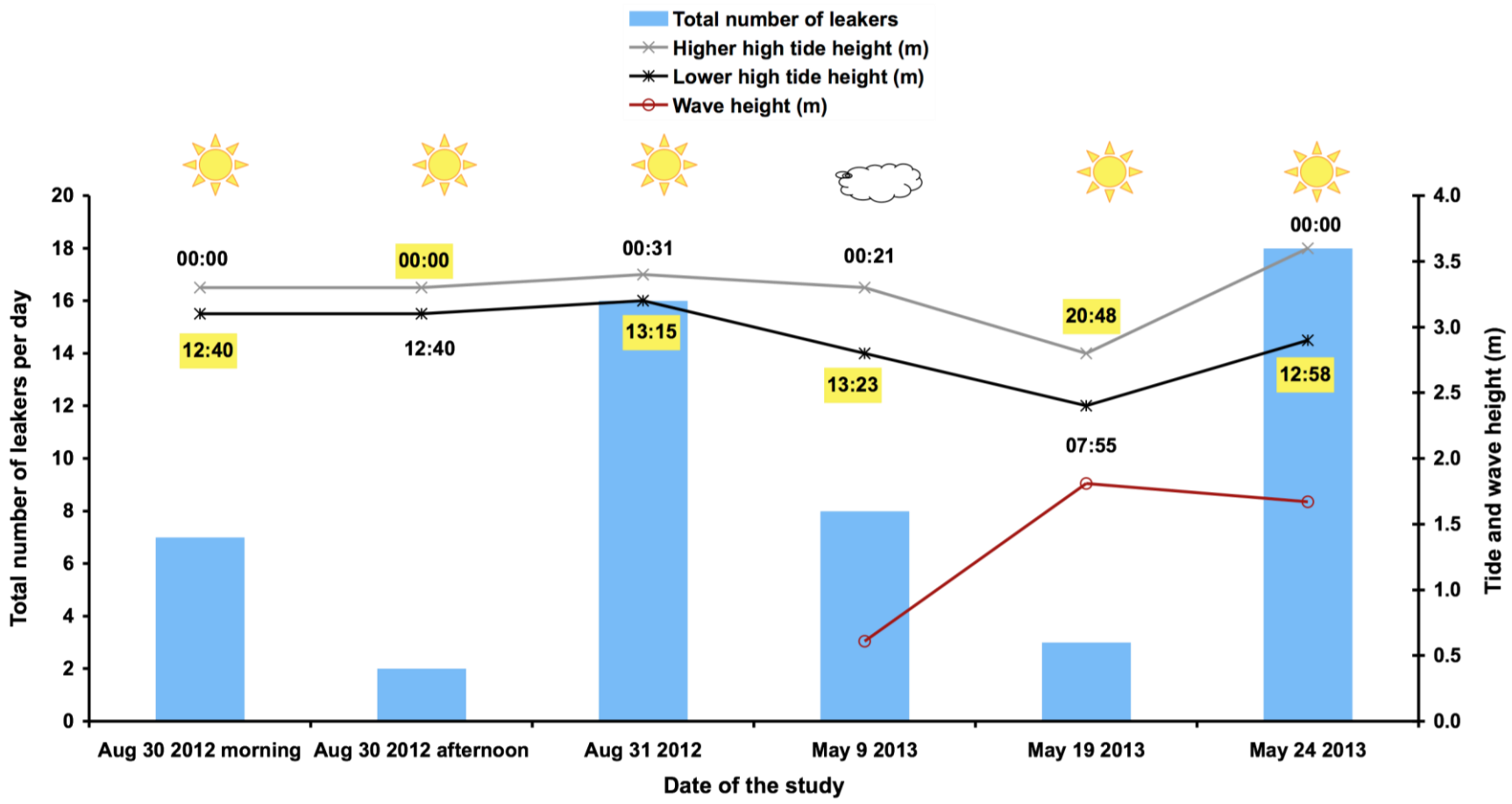

Figure 4.15 Total number of sperm-leaking individuals per day at Helby Island, low shore (highly wave-exposed) in relation to weather, wave and tide conditions. Labeling as in Figure 4.12 legend. Wave height data were unavailable for August 30-31, 2012.

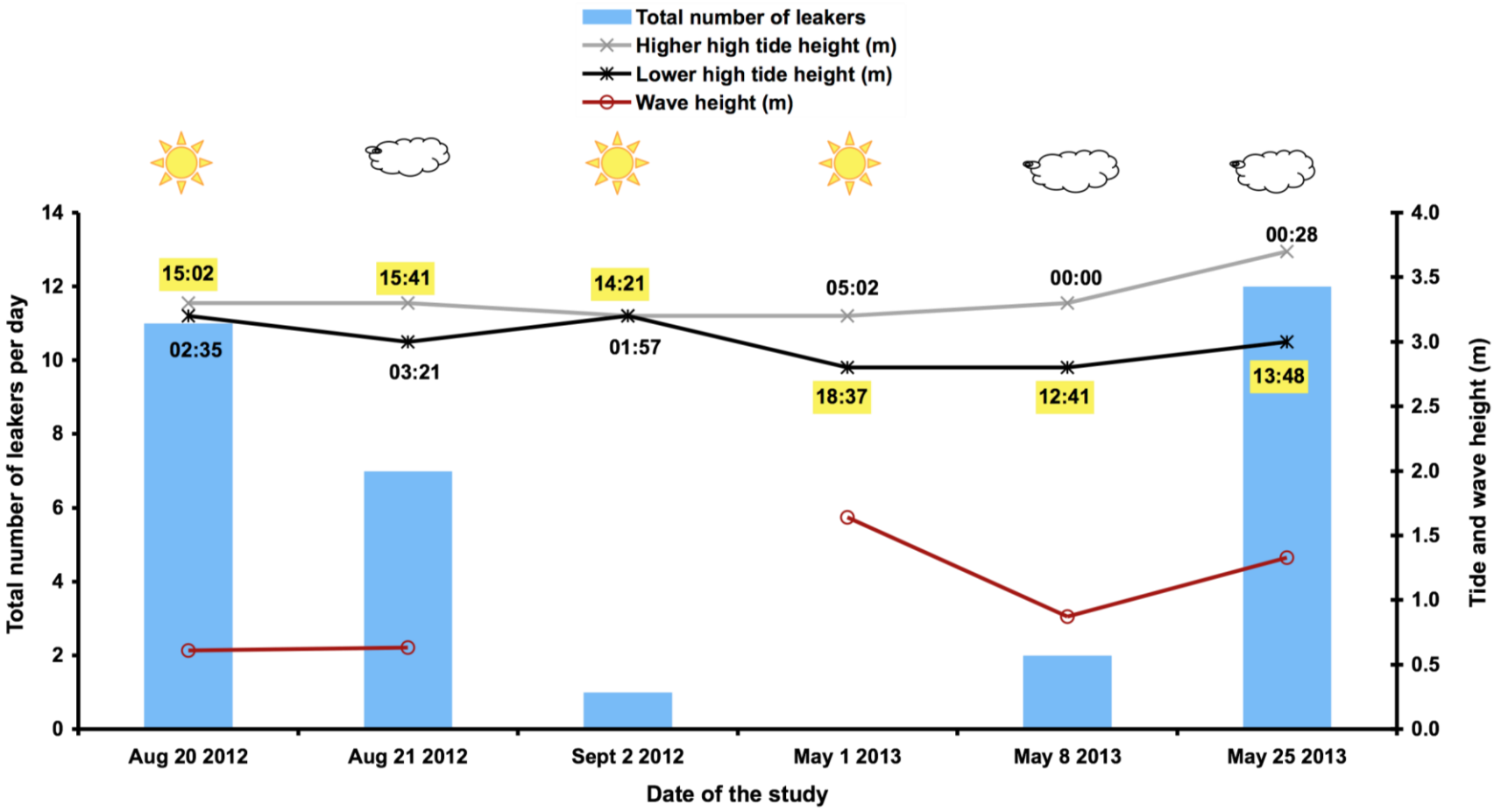

Figure 4.16 Total number of sperm-leaking individuals per day at Seppings Island, high shore (highly wave-exposed) in relation to weather, wave and tide conditions. Labeling as in Figure 4.12 legend. Wave height data was unavailable for September 2, 2012.

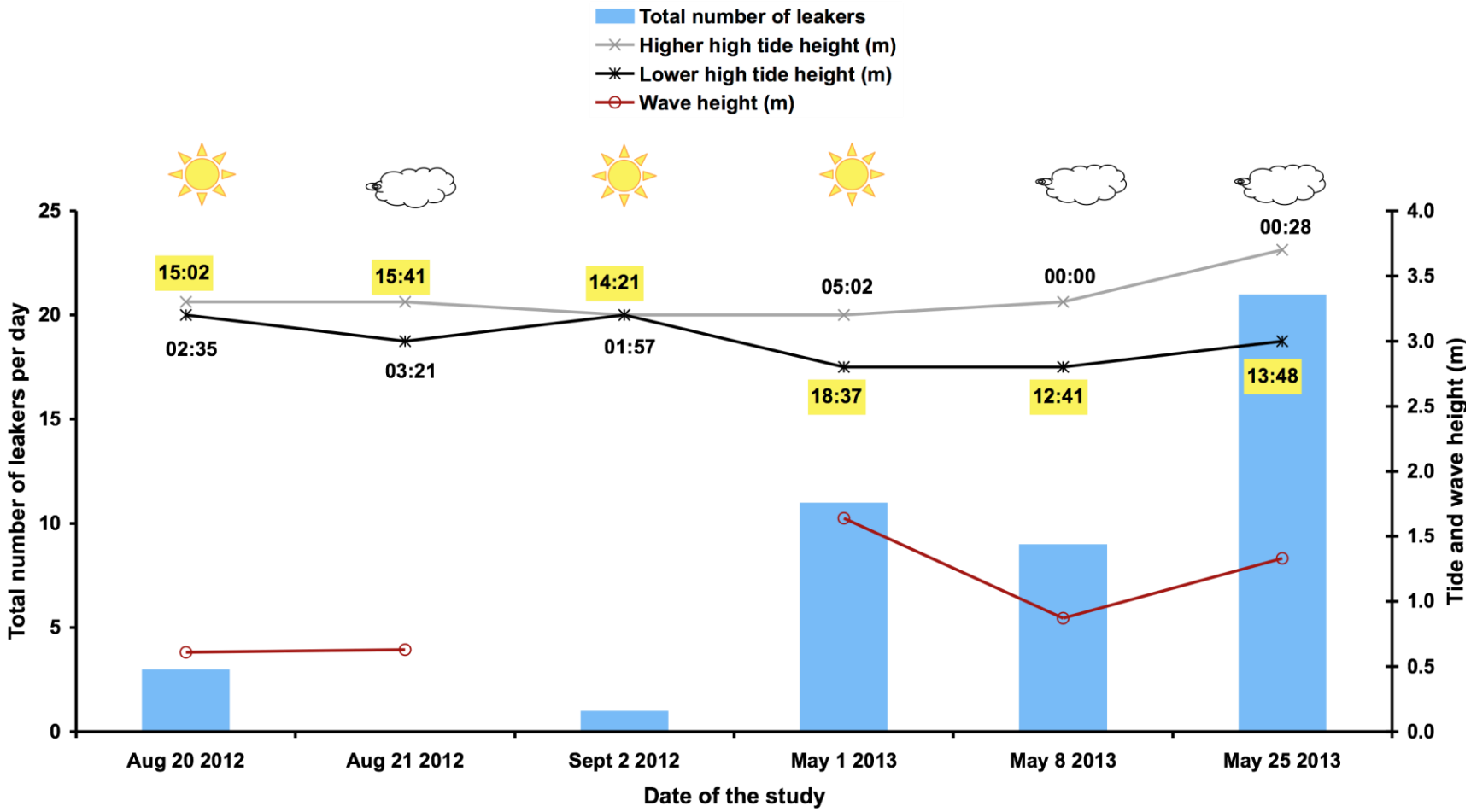

Figure 4.17 Total number of sperm-leaking individuals per day at Seppings Island, low shore (highly wave-exposed) in relation to weather, wave and tide conditions. Labeling as in Figure 4.12 legend. Wave height data was unavailable for September 2, 2012.

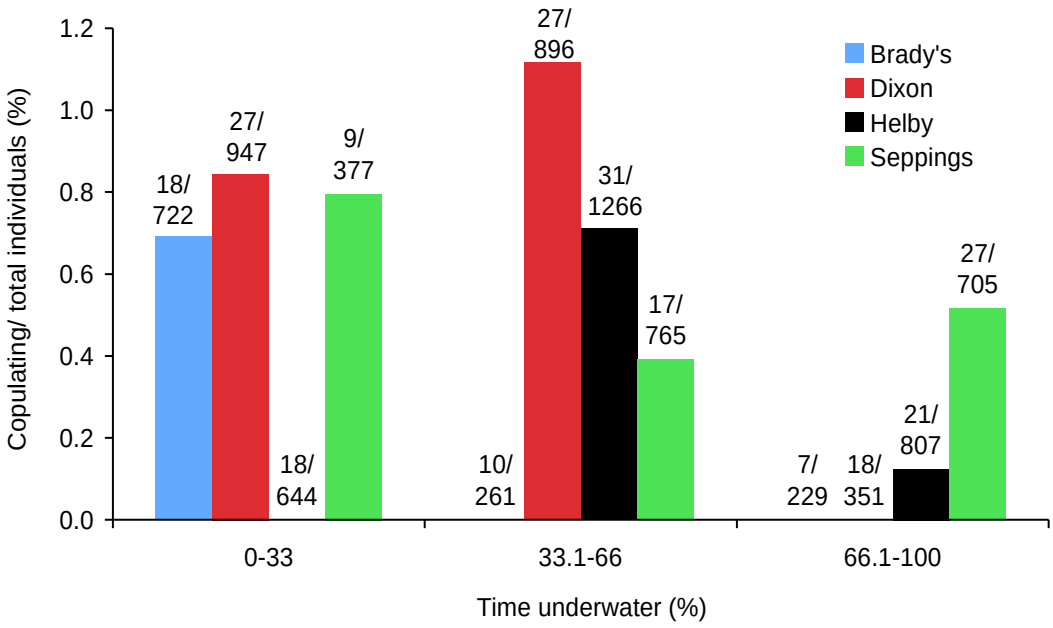

Figure 4.18 Percentage of individuals exhibiting copulating activity relative to the total number of individuals in all videos as a function of the percentage of time the barnacles were underwater. The videos were taken at two moderately wave-exposed sites (Brady's Beach and Dixon Is.) and two highly wave-exposed sites (Helby Is. and Seppings Is.) in May 2013. Upper numbers above each bar represent the total number of videos taken at each category and the lower numbers indicate the total number of individuals recorded in all videos.

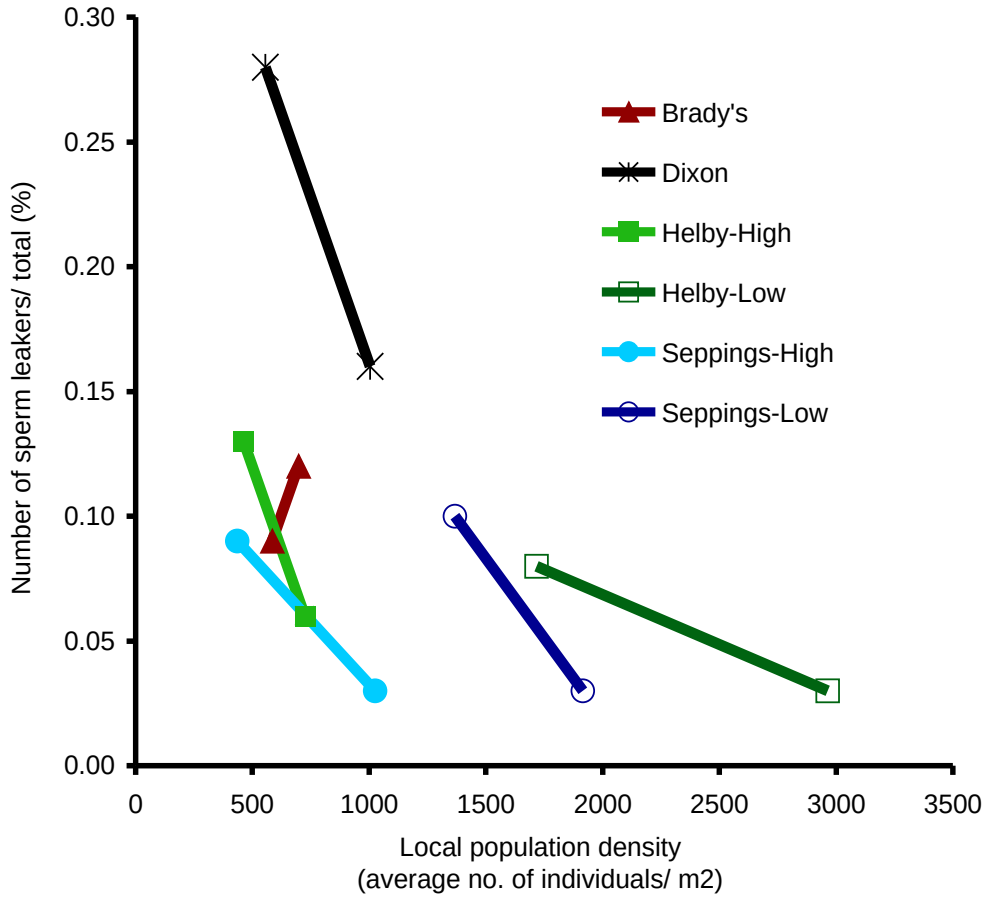

Figure 4.19 The average percentage of individuals leaking sperm per day as compared to the total number of individuals per square meter in each transect at six sites. In all sites, except for Brady's beach, the percentage of sperm-leakers decreased as the local population density increased.

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Chapter 5. The influence of wave exposure and shore height on genetic structure of the stalked barnacle, *Pollicipes polymerus*, in the northeastern Pacific[4]

5.1 Introduction

Most sessile marine organisms have planktonic larvae, whose dispersal is greatly influenced by water currents (Menge, et al., 2003). Depending on the duration of the larval stage, larvae can potentially move kilometres before settling on suitable substrata. In the absence of physical barriers, this results in gene flow and great homogeneity among different populations of the same species on different coasts.

However, several factors impact the dispersal and settlement of larvae, which may lead to variable recruitment in space and time. Tidal and flow regimes, and other hydrodynamic features of the coasts like stratification of the water column (Ladah, et al., 2005), are among the most important physical factors determining larval settlement success (Gaylord, et al., 2013; Gouhier, et al., 2010). For instance, larvae of the purple sea urchin use turbulent shear on wave-exposed shores as a general cue to recognize suitable habitats for settlement (Gaylord, et al., 2013). Interannual variation in ocean upwelling can result in

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dramatic variation in nutrients levels and hence, the recruitment of sessile intertidal invertebrates, including mussels and barnacles (Barth, et al., 2007; Menge, et al., 2003). The strength of coastal upwelling is another important factor. Weaker upwelling can reduce offshore transport of larvae and increase the chance of settlement on their original coasts, close to their parents, which can yield greater population-genetic structure, as seen in the intertidal acorn barnacle *Balanus glandula* (Barshis, et al., 2011). Reproduction and recruitment rates are also influenced by environmental factors such as temperature, salinity, food availability and substratum texture to a significant extent (Menge, et al., 1997; Quinteiro, et al., 2007; Véliz, et al., 2006). For example, larval production in *B. glandula*, is higher in northern California near-shore areas with higher phytoplankton levels (Leslie, et al., 2005). Finally, sometimes the vertical zonation of cyprid larvae in the water column influences the height on the shore at which they settle (Grosberg, 1982) whereas at other times, despite similar distribution of cyprid larvae in the water, different barnacles settle at different shore heights and wave exposures (Jenkins, 2005). Clearly, many factors may yield variable recruitment in space and time that might impact genetic structure.

Genetic differences among local populations may also be enhanced by differential survival of recruits in response to differences in the physical environment, such as wave exposure and shore height, as well as differences in the biotic environment, such as predation and food supply (Brind'Amour, et al., 2002; Connell, 1985; Delany, et al., 2003; Gosselin and Qian, 1997; Gosselin and Jones, 2010; Lathlean, et al., 2013; Morse and Hunt, 2013). In addition, post-

settlement survival may vary greatly from day to day, depending on prevailing weather conditions (Jarrett, 2000).

Finally, the shore height at which sessile adults currently live may not be the same as that at which they settled if shores are rising due to isostatic rebound or tectonic uplift. In the northeast Pacific, the remnant Juan de Fuca plate is subducting under the North American plate (Ostanciaux, et al., 2012). This subduction, combined with the effects of isostatic rebound following the Pleistocene glaciation (Clague and James, 2002), has yielded a surprisingly high rate of coastal elevation: approx. 2 mm/yr vertical rise across the Cascadia subduction zone (Ostanciaux, et al., 2012) and nearly 3-4 mm/yr vertical rise on the southwest coast of Vancouver Island (Hyndman, et al., 1996). For animals that live 10 - 20 years, this could yield an effective shore-height increase of 30-80 mm over their lifetime.

Barnacles are among the most abundant faunal components of rocky intertidal shores of the northeast Pacific (Newman and Abbott, 1980). They are sessile as adults, yet they usually develop through four to six free-swimming naupliar stages and one non-feeding cyprid stage. The larvae typically travel several weeks before settlement (Anderson, 1994). The length of the larval period and the geographical distribution of species can determine their genetic structure. *Tesseropora atlantica*, which has a short pelagic larval period of 24h, shows high genetic diversity among populations, while *Chthamalus stellatus*, whose larval period lasts for 22 days, shows much lower interpopulation diversity (Pannacciulli, et al., 2009). Strong genetic structure has been observed among

populations of *Semibalanus balanoides* from North America and Europe separated by hundreds of kilometers (Flight, et al., 2011). *Pollicipes pollicipes* also show significant isolation-by-distance among northeast Atlantic populations (Quinteiro, et al., 2007).

Nevertheless, not all phenotypic differences among populations have a genetic basis. Morphological characteristics of barnacles such as the length and thickness of feeding cirri and penises are affected by wave action (Hoch, 2009; Marchinko and Palmer, 2003; Neufeld and Palmer, 2008). *Balanus glandula* develops shorter and stouter feeding legs when exposed to stronger wave-action (Marchinko, 2007; Marchinko and Palmer, 2003). Similarly, penises of two acorn barnacles, *B. glandula* and *Semibalanus balanoides*, from wave-exposed sites are shorter and thicker than those from wave-protected sites (Hoch, 2008; Neufeld and Palmer, 2008). Reciprocal transplant experiments suggest that these morphological differences arise predominantly due to developmentally plastic responses to local conditions (Marchinko, 2003; Neufeld and Palmer, 2008). However, barnacles were not tested for genetic diversity among the populations experiencing different wave exposures and shore heights.

Pollicipes polymerus is an intertidal stalked barnacle that lives and breeds on shores of different wave action (moderate to highly wave-exposed) and are vertically distributed from the lower shore, where they are intermingled in mussel beds, to the high shore on bare rock (Hoffman, 1989). Similar to other barnacles, *P. polymerus* copulate with neighbors to transfer sperm (Barazandeh and Palmer, Submitted), however, they also commonly spermcast (Barazandeh, et al., 2013).

So gene flow in this species could arise from two sources: sperm dispersal and larval dispersal. At 12° C, *P. polymerus* nauplii take 42 days to metamorphose into a cyprid after hatching, which could result in extensive transport, potentially between 185 and 930 km before settling (Lewis, 1975). The lifespan and motility of their sperm is not known, though (Barazandeh, et al., 2013). Greater wave action could result in higher dispersal of both larvae and sperm and therefore create more genetically homogeneous populations (Barshis, et al., 2011). Previous population-genetic studies on *P. polymerus* did not show any latitudinal gradient among populations along the northeastern Pacific coast (Miner, 2002; Van Syoc, 1994). However, comparisons were not made among populations from different shore heights or wave-exposure regimes in the same region of coast.

Tectonic processes and post-glacial rebound might also affect genetic structure. The estimated uplift rate of southwest Vancouver Island is up to 4 mm/year (Hyndman, et al., 1996). Consequently, *P. polymerus* on the high shores of Barkley Sound in Vancouver Island could have settled more than 20 years ago, when the shores were less elevated whereas the barnacles located lower on the shore likely include more recent recruits. Annual changes in coastal upwelling strength could result in genetically different larval pools in barnacles (Barshis, et al., 2011). Therefore, the genetic diversity among high-shore (older) *P. polymerus* could be different from low-shore (younger) barnacles.

We therefore analyzed genetic population structure among *Pollicipes polymerus* populations located on the low shore and high shore of moderate- and highly wave-exposed sites of southwestern Vancouver Island. These analyses

allowed us to test whether selective settlement, differential survival, or temporal variation in the planktonic larval pool, had any impact on genetic population structure.

5.2 Materials and Methods

5.2.1 Barnacle sampling

In August 2009 and August 2010 (sample set 1), adult *Pollicipes polymerus* (48 and 50 barnacles, respectively) were collected from one highly wave-exposed shore (Seppings Island, 48.841° N, 125.209° W) and moderately wave-exposed shore (Helby Island, 48.847° N, 125.168° W) in Barkley Sound (Bamfield, British Columbia, Canada; Figure 5.1). In July 2011 (sample set 2), juvenile and adult *Pollicipes polymerus* individuals (24 individuals per site) were also collected from high-shore and mid-shore regions of two highly wave-exposed sites (Seppings Island and Bordelais Islet, 48.818° N 125.229° W) and two moderately wave-exposed sites (Helby Island, and Kelp Bay, 48.866° N, 125.116° W) in Barkley Sound (Figure 5.1). Average late summer maximum water velocities varies considerably among these sites (most wave-exposed to least exposed): Seppings Is.- 4.4 m/s, Bordelais Is.- 4.3 m/s, Kelp Bay- 3.2 m/s, Helby Is.- 2.7 m/s (Arsenault, et al., 2001).

The stalks of all barnacles were preserved in 70 percent ethanol in 2 ml microfuge tubes and stored at -20° C before DNA extraction.

5.2.2 DNA extraction, sequencing and statistical analysis

Nuclear and mitochondrial DNA of all collected barnacles were extracted using a DNeasy® Blood and Tissue Kit (QIAGEN).

Sample set 1 were genotyped for 16 existing Single Nucleotide Polymorphism (SNP) markers (Barazandeh and Davis, 2012; Barazandeh, et al., 2013). Single locus and multilocus F_{st} values were calculated using GENEPOP (v4.2) (Raymond and Rousset, 1995; Rousset, 2008). STRUCTURE v2.3.4. (burn-in period of 5000 and Markov Chain Monte Carlo (MCMC) generations of 50000, admixture model) was used to determine the potential population structure among samples from the two populations. The number of hypothetical subpopulations (*K*) was set between 1 (all individuals from single population) and 98 (every individual forming its own population).

Two mitochondrial genes (Cytochrome C Oxidase subunit 1 (COI) and D-loop) of barnacles of sample set 2 were sequenced. Partial sequences of COI were amplified using pre-designed primers (Ppol_CO1_F: 5'-GGT CAA CCC GGA AGA TTA ATT GG-3'; Ppol_CO1_R: 5'-CTT TAA TAC CTG TAG GGA CAG CA-3') (Van Syoc, et al., 2010). The D-loop (control region) of mitochondrial DNA (mtDNA), which is a non-coding highly variable DNA in barnacles (Brown, et al., 2001), was sequenced for the same individuals. By comparing mtDNAs of *P. polymerus* and *P. pollicipes* and using *P. pollicipes*' pre-designed primers, amplification primers for *P. polymerus* D-loop were designed (Ppol_Dloop_F: 5'-GGC ACG CTA TTT TCC AAC AC-3'; Ppol_Dloop_R: 5'-TCC TCC ATC GGC TAC AAC TT-3') (Quinteiro, et al., 2007).

Both mtDNA genes were amplified in 10 µl PCR reactions containing 1 µl of 10× PCR buffer [500 mM Tris–Cl (pH 9.2), 18 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, 1 mg/ml BSA, 0.025% (v/v) B-mercaptoethanol], 0.2 mM dNTPs, 0.2 µM of each primer and 0.2 U of Taq DNA polymerase. The following thermal cycling program was used for each PCR reaction: 4 min at 94° C, 30 cycles of 15 s at 94° C, 30 s at 54° C for COI and 52° C for D-loop and 30 s at 72° C, and a final extension of 5 min at 72° C. The PCR products were purified using 0.5 µl ExoSAP (0.125 U each of Exonuclease I and Shrimp Alkaline Phosphotase; USB corporation) per 1 µl product incubated for 30 min at 37° C and 15 min at 80° C. The purified products were then sequenced using BigDye® Terminator v3.1 chemistry (Applied Biosystems, ABI) and resolved on a 3730 DNA analyzer (ABI). The sequences were aligned and trimmed using BioEdit Sequence Alignment Editor, v7.0.9.0 (Hall, 1999). The barnacles sampled from low shore and high shore of each site were treated as both different populations and as a single population (low shore and high shore pooled together), ultimately resulting in 12 potential samples for analysis (Seppings total, high, and low; Bordelais total, high, and low; Helby total, high, and low; Kelp Bay total, high, and low). COI and D-loop genes were linked together and treated as a single gene thereafter (COI+D-loop). The number of haplotypes, number of polymorphic sites, nucleotide diversity (π) and pairwise genetic distances between populations (F_{st}; 58 pairwise comparisons) were calculated using DnaSP v5.0 (Librado and Rozas, 2009) for COI, D-loop and COI+D-loop. The isolation-by-distance measurements (pairwise correlations between genetic (F_{st} values of COI+D-loop) and

geographical distances of four main populations; six pairwise comparisons) were done using IBD Web Service v3.23 (Bohonak, 2002). Bootstrapped maximum parsimony (1000 replications) phylogenetic trees were constructed for sequences of COI+D-loop and COI (which was less polymorphic than D-loop and COI+D-loop) using MEGA4 (Tamura, et al., 2007). A statistical parsimony haplotype network was built for COI sequences using TCS v1.21 (Clement, et al., 2000).

5.3 Results

5.3.1 Population structure of sample set 1

Single locus and multilocus F_{st} values of SNPs were less than 0.003 between two barnacle populations (in most cases indistinguishable from 0) of Seppings Is. and Helby Is. in sample set 1, which implies that these two populations are not genetically distinct. Similarly, SNP data analysis using STRUCTURE, regardless of the runs criteria (K = 1 to K = 98), did not indicate any cryptic subpopulations in sample set 1, i.e. all individuals could be equally assigned to any hypothetical subpopulations (example given for 2 subpopulations (K = 2) in Figure 5.2).

5.3.2 Population structure of sample set 2

COI amplification failed for one individual from Bordelais Is. high shore, so it was removed from the analysis. The total number of base pairs, excluding sites with gaps or missing data, was 773 for COI and 601 for the D-loop, among which 106 and 220 sites were polymorphic and 60 and 152 sites were parsimony informative, respectively. 111 and 190 haplotypes (out of 191 sequences) were obtained for COI and D-loop/ COI+D-loop, respectively. The average nucleotide

diversity of COI+D-loop across all populations was 0.021 (ranging from 0.018 to 0.023 in single populations). Pairwise comparisons of F_{st} values using COI+D-loop and D-loop (island-island, shore-shore) did not show any significant genetic differentiation among populations (all $F_{st} \leq 0.047$; $p \geq 0.05$). However, for COI at one site (Kelp Bay) there appeared to be a slight difference between the high-shore and low-shore samples ($F_{st} = 0.060$, $p = 0.04$). Despite low F_{st} values of COI+D-loop, a significant, albeit weak, pairwise isolation-by-distance trend was observed among the four sites sampled (Figure 5.3), but this association was heavily influenced by one sample pair (Bordelais Is. - Kelp Bay).

Furthermore, none of the constructed trees (maximum parsimony or haplotype network) revealed any genetic structure among populations (between and within islands). All individuals were randomly clustered together (Figures 5.4-5.6). In both maximum parsimony trees, clades with support values of even more than 80% were not necessarily from the same site (Figures 5.4-5.5).

5.4 Discussion

Our population genetic studies on *Pollicipes polymerus* from high and low shores of moderate and highly wave-exposed sites did not indicate any genetic structure among populations in Barkley Sound. Neither SNPs nor mitochondrial DNA sequences differed significantly among *P. polymerus* populations (Figure 5.2 and Figures 5.4-5.6). Therefore, the striking morphological differences observed among populations (Marchinko and Palmer, 2003) were not associated with any genetic differences that could be detected with the current data.

Our results are consistent with those from two other studies that did not detect any genetic diversity between two physiological races of *P. polymerus* along the coasts of north and south of the Californian Transition Zone (Miner, 2002; Van Syoc, 1994). However, in those studies, shore height and wave exposure conditions were not considered as factors that might affect population genetic structure.

We found that differences in the wave-exposure regime among sites did not act as barriers to gene flow. Resistance to shear force differs among cyprids of some barnacle species from habitats with quite different topographical features (open coast versus estuary) (Anderson, 1994). These differences are probably genetically-based resulting in morphological or behavioral characteristics of the larvae that make some more tolerant of strong wave action than others. Water-velocity differences among habitats of *P. polymerus* populations are less dramatic, which may allow *P. polymerus* larvae to settle under conditions that are physically different from their parent's environment. After settlement, developmentally plastic responses to differences in wave-exposure regime most likely yield the differences observed in adult body form.

Dispersal ability varies considerably among barnacles, which likely has an affect on the degree of genetic differentiation among populations. Species with lecithotrophic larval stages retain nauplii in their mantle cavity and only release the non-feeding cypris larvae into the water. These cypris larvae usually settle within a few days before consuming all of their stored energy reserves (Anderson, 1994). Therefore, they disperse less, which yields higher genetic diversity among

distant populations, as seen in the acorn barnacle *Tesseropora atlantica* (Pannacciulli, et al., 2009). Species with planktotrophic naupliar stages, like *P. polymerus*, usually spend several weeks swimming and feeding before transforming to a cypris and thus experience greater dispersal. Despite extensive gene flow and low pairwise F_{st} values, we observed some evidence of isolation by distance among *P. polymerus* populations separated by 12 kilometers (Figure 5.3). To confirm that this is a real pattern of isolation over small distances, further work needs to be done using more molecular markers and higher number of samples from more sites.

Finally, the genetic composition of barnacles did not seem to differ between shore-height or years. Older (larger) barnacles from the high shore grouped with younger (smaller) barnacles from low shore in the same clades in many cases (Figures 5.4-5.6). Therefore, neither differential survival nor temporal variation in genetic makeup of the larval pool appeared to yield significant genetic differentiation with shore height. Alternatively, age-associated molecular differences might not have been detected because the offspring of older barnacles likely don't settle close to them on the high shores due to the high dispersal, which homogenizes the populations.

The variation in the SNP markers that were used in this study was low, which may have reduced our ability to detect differences among populations. The frequency of the dominant allele in 11 of 16 SNPs exceeded 80% (Barazandeh and Davis, 2012), which could yield overestimates of genetic similarities between populations even though other genetic differences might exist. On the other hand,

the mitochondrial sequences were highly polymorphic. When considering COI+D-loop, only two out of 191 barnacles shared the same haplotype (~1% of the population). Such high polymorphism of the mitochondrial loci could greatly underestimate genetic similarities and F_{st} values both within and between populations, which could prevent detection of any potential population genetic structure (Jost, 2008). Nevertheless, separate analysis of the COI gene, which was less polymorphic than COI+D-loop and was used as a control to detect cryptic populations, also did not indicate any population structure among sites (Figure 5.5). Although F_{st} statistics suggest some genetic differentiation between the high- and low-shore populations of Kelp Bay ($F_{st} = 0.06$, $p = 0.04$), no such differences were observed at other sites. Therefore, the evidence that genetic differences exist between barnacles located on different shore heights is weak at best.

Pollicipes polymerus larvae seem to use more general chemical and physical cues to choose where to settle rather than specific signals, which could create genetically homogeneous populations. Although settlement of *P. polymerus* cypris larvae is expected to be affected by wave action to a great extent (Barnes and Reese, 1960), our results do not provide any molecular evidence of that. However, using additional, more variable nuclear markers (SNPs and microsatellites), less polymorphic mitochondrial genes, and higher number of individuals per site, might yield a different conclusion. Alternatively, the affect of wave-exposure on the genetic diversity might be better tested using barnacles

species with lecithotrophic larvae, which disperse over shorter distances (Anderson, 1994).

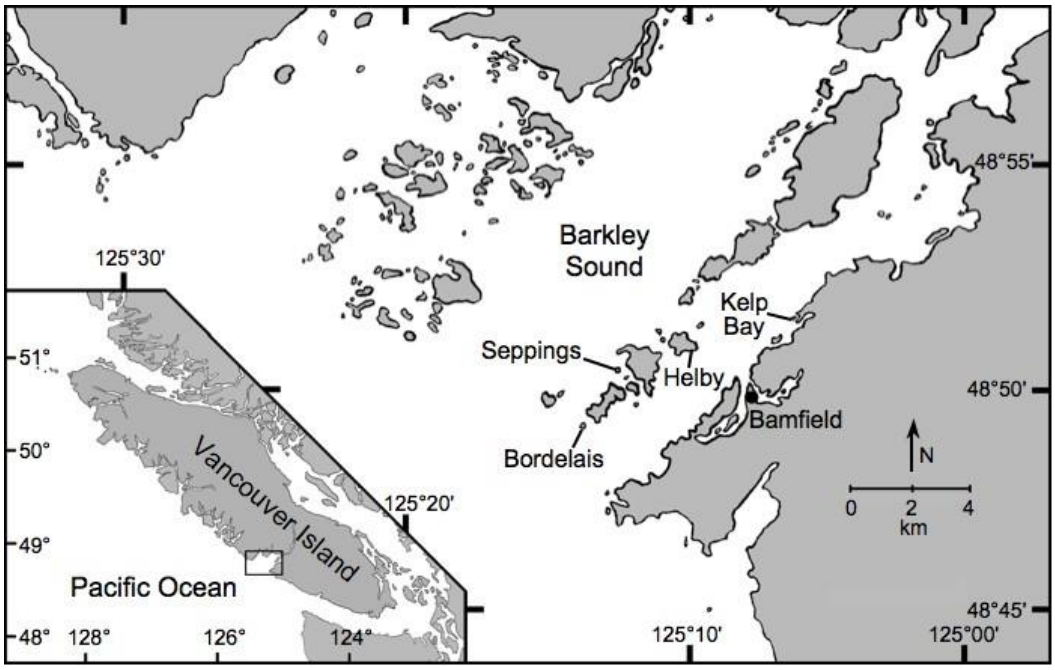

Figure 5.1 Sample localities, Barkley Sound, British Columbia, Canada.

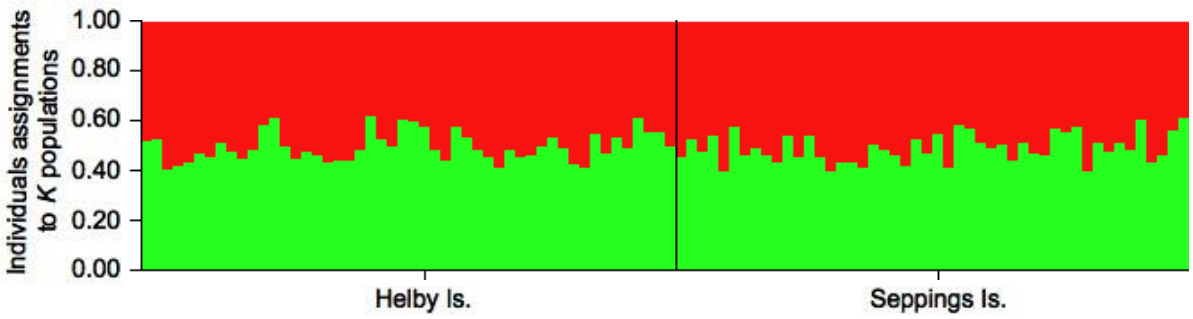

Figure 5.2 STRUCTURE admixture plots for sample set 1: 50 individuals from a wave-protected site (Helby Is.) and 48 individuals from a wave-exposed site (Seppings Is.) genotyped for 16 Single Nucleotide Polymorphism (SNP) markers. Each color indicates a hypothetical genetically distinct population (here *K* = 2; green: Helby Is., red: Seppings Is.). Each vertical bar represents a single individual. The site names on the X-axis indicate the original population (sampling site) of that individual. The height of each color in each vertical bar represents the probability of assignment of that individual to either population.

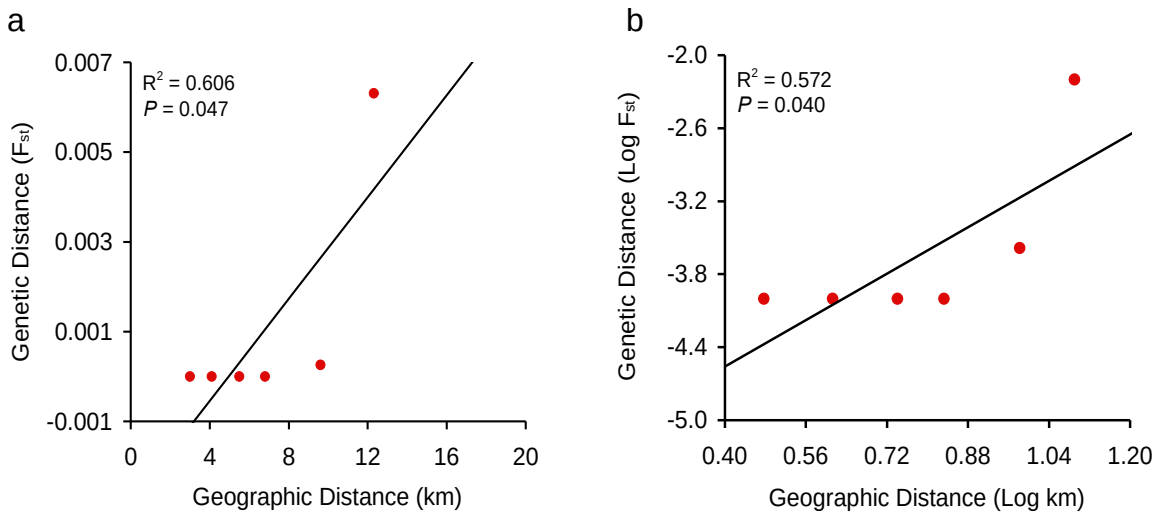

Figure 5.3 Isolation-by-distance of *Pollicipes polymerus* populations from four different sites in Barkley Sound, British Columbia, Canada (sample set 2, 24 individuals per site). Each point represents a pairwise estimate of genetic distance (F_{st} obtained for COI+D-loop) and geographic distance for two populations. Seppings Island and Bordelais Islet had the least and Kelp Bay and Bordelais Islet had the highest geographic distances. (a) Raw estimates, (b) Log-transformed estimates.

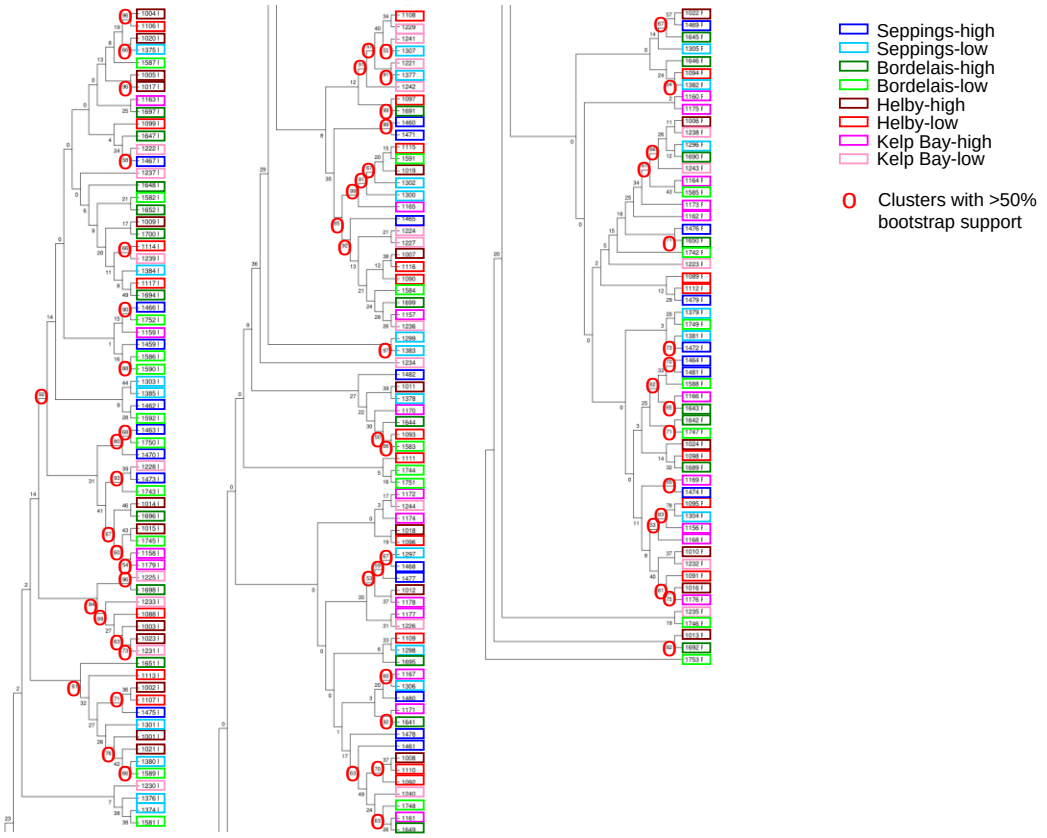

Figure 5.4 Maximum parsimony tree (single shortest tree) of 191 *Pollicipes polymerus* COI+D-loop sequences. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Numerous clusters without any significant geographical connection were obtained.

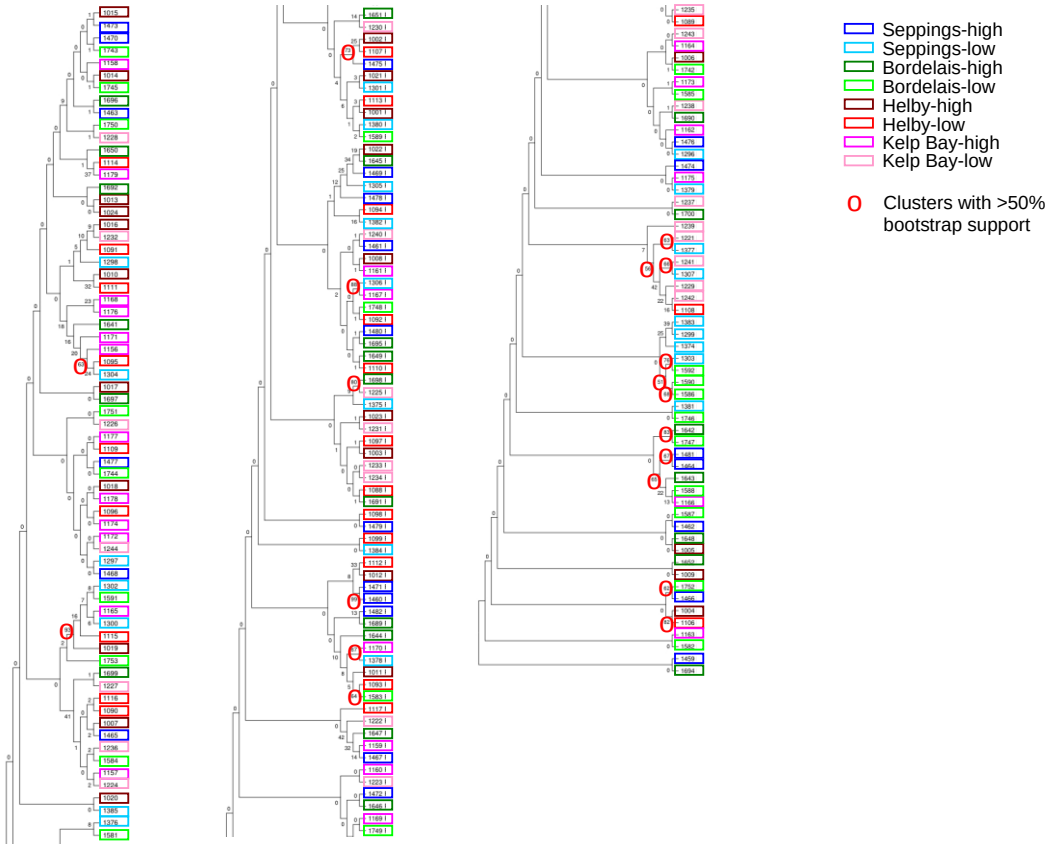

Figure 5.5 Maximum parsimony tree (single shortest tree) of 191 *Pollicipes polymerus* COI sequences. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Numerous clusters without any significant geographical connection were obtained.

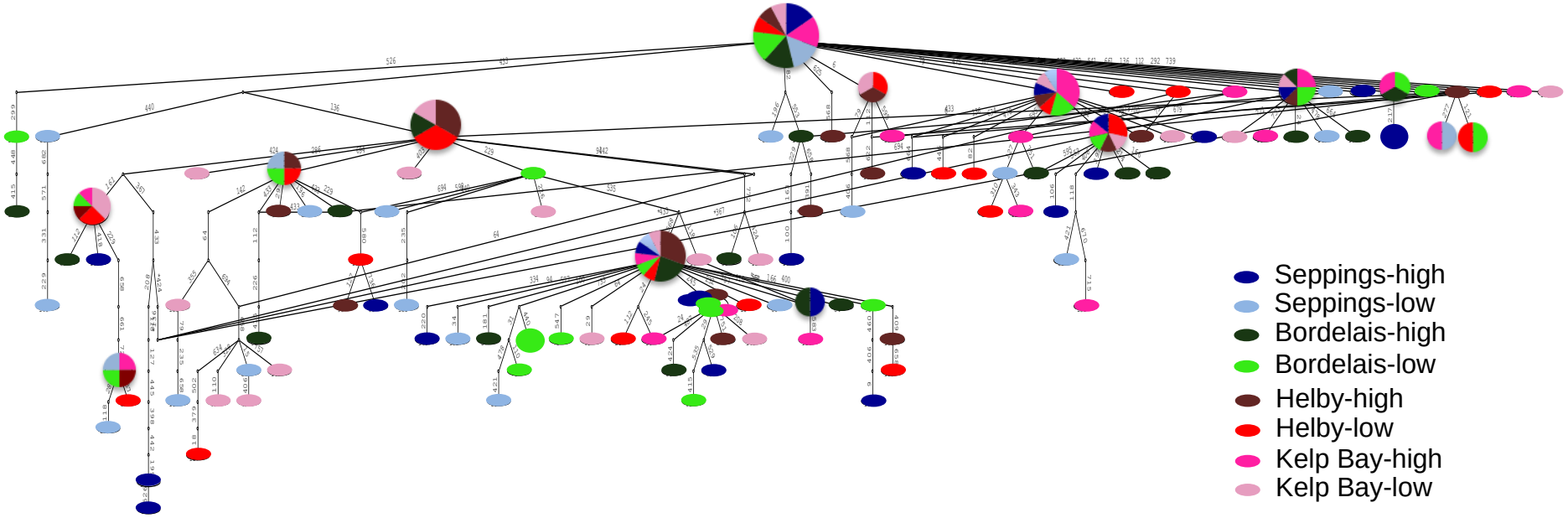

Figure 5.6 Network of haplotypes of 191 *Pollicipes polymerus* COI sequences. Each circle represents one unique haplotype. The size of each circle is proportional to the frequency of its haplotype; the smallest circles indicate haplotypes that were observed in only one individual. The colors of the circles show the original site of the samples. Each branch indicates a single substitution between the haplotypes and the numbers on the branches show the position of the variable nucleotide in the gene.

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Chapter 6. General Conclusions

6.1 Spermcast mating and aerial copulation in barnacles

Barnacle mating systems are more complicated than generally presumed. Despite decades of detailed study (Barnes, et al., 1977; Barnes, 1992; Berger, 2009; Buhl-Mortensen and Høeg, 2006; Burrows, et al., 1992; Darwin, 1851, 1854; Hilgard, 1960; Klepal and Barnes, 1977; Klepal, et al., 1977), novel and unexpected observations continue to emerge.

Spermcast mating is one such novel observation. The results of chapters 2 and 3 of my thesis reveal — for the first time — that spermcast mating can occur in an aquatic arthropod (Barazandeh, et al., In Press; Barazandeh, et al., 2013). Spermcasting occurs in at least three barnacle species (*Pollicipes polymerus*, Sowerby 1833; *Balanus glandula*, Darwin 1854; and *Chthamalus dalli*, Pilsbry 1916) from two orders (Pedunculata, Lamarck 1818 -- stalked barnacles; Sessilia, Lamarck 1818 -- sessile barnacles) and three families (Pollicipedidae, Leach 1817; Balanidae, Leach 1817; Chthamalidae, Darwin 1854) of the subclass Cirripedia, which contains the free-living, non-boring barnacles (Barazandeh, et al., In Press; Barazandeh, et al., 2013; Newman and Abbott, 1980). Barnacles are the only known sessile animals that both a) copulate with neighbors to transfer sperm and b) spermcast at the same time, at least when they do not have access to a mate. This mode of sperm transfer happens more commonly in *P. polymerus*, which has a relatively short penis compared to the other two acorn barnacles studied (Barazandeh, et al., In Press; Barazandeh, et al., 2013).

Aerial copulation in *P. polymerus* — a totally unique behavior among barnacles — is another such novel observation (Chapter 4). This unusual behavior may facilitate mating with short penises when exposed to strong wave action (Barazandeh and Palmer, Submitted). In this chapter, I also discovered that the frequency of sperm leakage in *P. polymerus* is higher at lower population densities, where there may be less opportunity for copulation. In addition, sperm-leakers were more common on the low shore than the high shore of the same sites, probably because higher wave action and immersion time on the low shore facilitate sperm dispersal (Barazandeh and Palmer, Submitted). However, genetic studies (Chapter 5) did not provide any evidence that differences in mating behavior among shores with different flow regimes were associated with any genetic differences (Barazandeh and Palmer, In Preparation). Many questions arise from these observations that could provide answers to persistent enigmas of barnacles reproduction. Although spermcast mating is likely a supplemental mating method to copulation in barnacles, it should be considered in further studies on barnacles' reproductive biology, sex allocation models and population genetics.

6.2 *Evolution* of mating patterns in barnacles: Is spermcast mating restricted to hermaphrodites?

Although most barnacles are hermaphroditic, dimorphism also occurs in some species in the forms of dioecy (dwarf males and large females) and androdioecy (small complemental males and large hermaphrodites) (Yamaguchi, et al., 2007) and it seems to have evolved multiple times in the superorder

Thoracica (Anderson, 1994; Kelly and Sanford, 2010). Some phylogenetic analyses suggest that dwarf and complemental males evolve from hermaphrodites (Anderson, 1994; Perez-Losada, et al., 2008), however, the ancestral mating pattern of many families has not been identified yet. In many cases sexual expression is environmentally controlled (Yusa, et al., 2012), which would complicate evolutionary inferences. For example, theoretically, resource allocation to sperm production increases in hermaphrodites as mating group size (MGS) rises and vise versa, to the point that no sperm is produced in isolated individuals and they become females (Kelly and Sanford, 2010; Yamaguchi, et al., 2012; Yusa, et al., 2012). When MGS is really small, dwarf males can invade the system due to low sperm competition, and hence dioecy arises. Thus, many more isolated individuals are observed in species with separate sexes than in androdioecious and hermaphroditic species (Yusa, et al., 2013). Moreover, hermaphroditism is more common in shallow water barnacles, where more food is available, than in deep-sea species, where food sources are limited. In the latter case, barnacles devote most of their resources to reproduction rather than to growth and thus create dwarf or complemental males attached to large females or hermaphrodites (Yamaguchi, et al., 2008).

Curiously, spermcast mating was never considered to be a possible mode of fertilization in barnacles. Spermcasting increases effective mating-group size, therefore functional males will be competing with more individuals than the nearby ones. All three species that I studied are simultaneous hermaphrodites, so no information is available about the incidence of spermcast mating in dioecious

or androdioecious species. Sperm competition is more intense among the androdioecious than the dioecious species because dwarf males need to compete with the large hermaphrodites in the former group (Yusa, et al., 2013). Therefore, it would be worth studying the possibility of spermcast mating in dioecious species as well to assess the role of sperm competition in this process. Since self-fertilization is automatically excluded in this group, the study of spermcasting would be less challenging.

6.3 Spermcast mating versus self-fertilization

One third of animals (excluding insects) are hermaphrodites, which could lead to self-fertilization in simultaneous hermaphroditic organisms (Jarne and Auld, 2006). Self-fertilization could be beneficial when available mates are scarce, but it could also entail long-term negative consequences including inbreeding depression. Therefore, it evolves only when the benefits outweigh the negative impacts (Slotte, et al., 2013; Wright, et al., 2013). Overall, self-fertilization is advantageous in the complete absence of out-crossing opportunities (Lloyd, 1979).

In barnacles, selfing is assumed to occur when gametogenesis is finished but copulation does not happen (Barnes and Crisp, 1956; Desai, et al., 2006). Self-fertilization has been reported in a few barnacle species such as *Balanus improvisus*, *B. amphitrite*, *B. eburneus*, *B. trigonus*, *B. perforatus*, *Chthamalus stellatus*, *C. fissus*, *C. dalli*, *C. fragilis*, *Verruca stroemia* (Barnes and Crisp, 1956; Barnes and Barnes, 1958; Desai, et al., 2006; Elkomi and Kajihara, 1991). However, most of these reports were based on the observation of fertilized

embryo-masses in the mantle cavity of isolated barnacles and they did not consider spermcasting as a possible source of the sperm. In chapter 3 of my thesis, I found some molecular evidence that both spermcast mating and self-fertilization happen in *C. dalli*. On the other hand, self-fertilization does not appear to happen in *B. glandula* since no fertilized individuals were found beyond a distance of 10 body lengths (Barazandeh, et al., In Press). However, more detailed molecular studies of single embryo-masses or laboratory experiments (such as retaining single individuals in separate aquaria and blocking all potential sources of waterborne sperm) should be done to confirm these results. Other species of *Balanus* and *Chthamalus* could be also studied to determine whether my observations are representative of both genera. *B. glandula* and *C. dalli* usually co-occur on rocky intertidal shores and both bear long penises with potential extension of seven times their body lengths (Barazandeh, et al., In Press). It would be interesting to unearth the factors that allow self-fertilization in one species and not in the other one. In species with short penises, like *P. polymerus*, selfing could be beneficial because the number of potential mates within penis range is less than species with longer penises (Barazandeh, et al., In Press; Barazandeh, et al., 2013). However, *P. polymerus* commonly use spermcasting to overcome this problem. In *P. polymerus*, efficient sperm dispersal appears to be limited to certain distances, because barnacles beyond eight body lengths from other individuals are not fertilized (Chapter 2). This also implies that selfing probably does not occur at all in *P. polymerus* (Barazandeh, et al., 2013). As mentioned before, *C. dalli* commonly copulate with neighbors and they also occasionally

self-fertilize and spermcast, at least when isolated (Barazandeh, et al., In Press). However, we do not know if and under what circumstances *C. dalli* within aggregations self-fertilize. The overall spermcasting rate was also lower in *C. dalli* than in *P. polymerus* and *B. glandula* (Barazandeh, et al., In Press). Why such differences occur among species remains a puzzle.

6.4 Other potential factors affecting barnacle mating behavior

Sperm longevity in the water after release as well as its dilution by the wave action, are among the most important factors affecting the success of spermcast mating (Bishop, 1998). Under similar flow regimes, sperm longevity may have a more significant role. In marine spermcasters or broadcasters, sperm lifespan varies from a few seconds to hours (Bishop, 1998). To increase longevity and mating efficiency, sperm probably stay inactive in the water and gain more motility when entering the body of mates (Bishop and Pemberton, 2006). Spermcasters utilize different methods to gather and store sperm to a significant level that is enough for fertilizing eggs (Bishop, 1998; Bishop and Pemberton, 2006; Bishop, et al., 2000). Although, spermcast mating is not the primary mating pattern in barnacles, they must have developed some adaptations in sperm form that facilitate spermcasting. Extensive studies have been done on barnacle sperm anatomy (Healy and Anderson, 1990), however, nothing is known about how sperm behave out of the body. Are they active in the water? How long can they survive in water before being captured? What is the efficient dilution of sperm to fertilize eggs? Does the fertilization efficiency of sperm decline with increasing time out of the body? The answers to these questions would be crucial in

understanding the observed differences between the rates of spermcast mating among different species.

Penis size also seems to have a significant role in barnacle reproduction. Other than spermcasting, *P. polymerus* exhibits another bizarre mating behavior: aerial copulation. In both *B. glandula* and *C. dalli*, functional mates (both male and female) feed as the tide rises and usually stop feeding and start copulating when they are completely immersed (Anderson, 1994; Personal Observations, 2009-2013). *P. polymerus* behave differently as copulation occurs when the first waves hit their opercular plates and copulation frequency declines when they are completely underwater (Barazandeh and Palmer, Submitted). Both aerial copulation and unusually short penises are likely adaptations to life on wave-swept shores in *P. polymerus* (Barazandeh and Palmer, Submitted). To confirm the impact of penis size on the evolution of new mating patterns, other barnacles with relatively short penises (e.g., shorter than the feeding legs), such as stalked barnacles like androdioecious *Scalpellum* (Darwin, 1851), which live subtidally in high-current areas, and acorn barnacles like the deep-sea *Pachylasma* and the intertidal *Octomeris* (Darwin, 1854) should also be examined for spermcasting or aerial copulation.

6.5 Other future directions

Many other questions remain to be answered regarding the great variation in mating behavior within and among barnacle species in response to different situations: How widespread are spermcasting and aerial copulation among other barnacle species? In spermcasters, do isolated individuals produce any sperm at

all or do they completely lose male function as sex allocation models suggest? Does the viability differ between self-fertilized and cross-fertilized eggs? Does body size influence the incidence of self/cross fertilization? Is early post-settlement mortality higher among cyprids resulting from self-fertilization as a result of inbreeding depression?

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Appendix 1. Identification and characterization of 16 Single Nucleotide Polymorphisms (SNPs) in the northeast Pacific intertidal gooseneck barnacle, *Pollicipes polymerus*[5]

Pollicipes polymerus is a common free-living, hermaphroditic gooseneck barnacle endemic to Pacific Northeast intertidal shores, which is presumed to reproduce exclusively by pseudo-copulation (Barnes and Crisp, 1956; Hilgard, 1960; Strathmann, 1987). However, the presence of egg masses in the mantle cavity of some isolated barnacles (well outside the range a penis could reach) is puzzling (personal observations). Molecular markers, most commonly microsatellites, are commonly used to study mating systems. While microsatellites have been developed for a few species of acorn barnacles (e.g. (Robson, et al., 2009), they often suffer from high rates of HW disequilibrium and may be unreliable. Here, we report on the isolation and characterization of 16 single nucleotide polymorphisms that will be useful for the study of the mating system of *P. polymerus.*

Although more straightforward methods of SNP discovery have been used (Olsen, et al., 2011), we cloned random genomic fragments for SNP discovery using a protocol commonly used to isolate microsatellite markers (Glenn and

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[5] A version of this appendix has been published. Barazandeh, M., Davis, C.S., 2012. Identification and characterization of 16 single nucleotide polymorphisms (SNPs) in the northeast intertidal gooseneck barnacle, *Pollicipes polymerus*. Conserv. Genet. Res. 4 (2), 217-219.

Schable, 2005) but without enrichment. This method was used as we were attempting to isolate microsatellite markers for *P. polymerus* (which was largely unsuccessful) concurrently with the isolation of SNP markers. Briefly, total genomic DNA was extracted from the stalk tissue of 5 *P. polymerus* individuals using a DNeasy® Blood and Tissue Kit (QIAGEN). Approximately 5 µg of pooled DNA was digested with XbaI, ligated to SuperSNX linkers, amplified by PCR and cloned using a TOPO TA cloning kit (Invitrogen). Inserts from 168 clones were amplified and sequenced using BigDye® Terminator v3.1 chemistry (ABI) and resolved on a 3730 ABI DNA analyzer. The resulting sequences were aligned using SeqMan® (DNASTAR) resulting in 112 unique contigs. Primers were designed for thirty fragments with largest product sizes using Primer3 (Version 0.4.0) (Rozen and Skaletsky, 2000).

To identify fragments for SNP discovery by re-sequencing, we attempted amplification of these 30 primer sets in 7 *P. polymerus* individuals. PCR reactions consisted of 1X PCR buffer [500mM Tris-Cl (pH 9.2), 18mM $MgCl_2$, 100mM $(NH_4)_2SO_4$, 1 mg/ml BSA, 0.025% (v/v) B-mercaptoethanol], 0.2mM dNTPs, 0.2µM of each primer and 0.2 units of *Taq* DNA polymerase. PCR was performed using the following thermal cycling program: 4 min at 94°C, 30 cycles of 15 s at 94°C, 30 s at 56°C and 30 s at 72°C, and a final extension at 72°C for 5 min. Seventeen primer sets yielded products of the expected size in all 7 individuals tested and were re-sequenced. The sequences from 11 of 17 fragments contained putative SNPs (each having two alleles with the rare allele observed at least twice and with at least one heterozygote individual – as identified from "double peaks"

in the sequence data). Primers to produce small products (75-200 bp) encompassing SNPs for SNaPshot detection (Filippini, et al., 2007) were designed for 22 putative SNPs in 11 fragments using Primer3 (Version 0.4.0) (Rozen and Skaletsky, 2000) and tested for amplification. Seventeen amplicons with the highest amplification success (single bands in all individuals amplified) were selected and SNaPshot interrogation primers (Filippini, et al., 2007) were designed.

Total genomic DNA was isolated from the stalk tissues of 117 *P. polymerus* adult individuals. Amplification primers were divided into two pools (Table A1) and were co-amplified in two 7.5 µl PCRs with 1x QIAGEN® Multiplex PCR master mix, 0.2 µM amplification primers and 20 ng template DNA. Thermal cycling consisted of 15 min at 95°C, 33 cycles of 30 s at 94°C, 90 s at 50°C and 90 s at 72°C, and a final extension at 72°C (10 min). The ABI PRISM® SNaPshot™ Multiplex Kit was used to genotype SNPs. 1.5 µl of ExoSAP-purified PCR product was added directly to reactions including 2.5 µl of SNaPshot Multiplex Ready Reaction Mix and 0.2 µM (each) pooled SNaPshot interrogation primers. The cycling program was conducted as follows: 25 cycles of 10s at 96°C, 5s at 50°C, 30s at 60°C. Products were purified using 0.5 units of Shrimp Alkaline Phosphatase (USB), diluted 1 in 4 and resolved on an ABI 3730 DNA Analyzer using GeneScan™ 120 LIZ™ Size Standard (ABI) as an internal size standard. Peaks were scored using GeneMapper, v4.0 (ABI).

Sixteen polymorphic loci were successfully amplified all individuals and all had 2 alleles (Table A1), though the frequency of the rare allele in two loci (105-

2, 151-3) was <5% (2% and 4% respectively). These loci were in nine unique fragments. Three loci deviated from Hardy-Weinberg equilibrium after applying Bonferroni correction for multiple comparisons ($P \leq 0.003$; Table A1). Many factors could be the source of heterozygote deficiency in marine sessile organisms such as natural selection, the Wahlund effect and inbreeding (Furman and Yule, 1990). Deviations from linkage equilibrium were calculated using GENEPOP (v4.1) (Raymond and Rousset, 1995; Rousset, 2008). After Bonferroni correction for multiple comparisons, two of 120 pairs showed significant linkage disequilibrium (Pp190-1 and Pp190-2, Pp117-2 and Pp117-5; $P \leq 0.0004$). The results are not surprising as these pairs of loci are located close to each other in the same cloned genomic region.

The SNPs presented in this study are the first nuclear markers to be developed for *Pollicipes polymerus* and will be used for further studies on aspects barnacle biology including reproduction.

Table A1.1 Characteristics of 16 SNPs isolated from 117 *Pollicipes polymerus*.

Locus	Allele Freq	Amplification primers (5'-3')	Tm	Interrogation primer (5'-3')	Tm	H_O	H_E	*P-value*
12a-3*	G/A 0.83/0.17	F: GGTTTTATCTTCGGTCACAT R: CCGGTTTTTCACAGGTAG	54.13 54.03	**(CAGT)$_{12}$**CCCCGTACCGCA	50.85	0.252	0.282	0.262
11-1†	G/A 0.95/0.05	F: GCGAGAACATAACAGATTCA R: ATGCCGTGGTCTAATTGT	53.85 53.75	**(CAGT)$_{9}$**TGTCCGAATTCCATTT	49.69	0.071	0.101	**0.002**
105-1*	T/G 0.75/0.25	F: GCAATATAGACAGAACACATCC R: GTAACTGTGCACCTAGAAGTGA	53.94 54.73	**GACTG**CTGTTTGGTTTTCATCA	47.54	0.302	0.379	0.028
105-2†	C/A 0.98.0.02	F: GGATGTGTTCTGTCTATATTGC R: AATAACCATCAAGCAGTTTTC	53.94 53.70	**GAC**TAGATATTTAAGTCCTTGTGTTT	49.86	0.026	0.042	**2.852E-05**
107-2*	C/T 0.83/0.17	F: GCCTTATGTTAAGATTAGCTAGGA R: GGCAGTAGGTTGATCCATT	55.53 54.96	(**CAGT)$_{4}$GA**TCTGGATCTGATCAAGTTAG	50.07	0.267	0.280	0.632
107-3†	C/T 0.90/0.10	F: CTTTTAGTTCACATGGCTTG R: CGGGAATAAAGTGTTTAATGT	53.59 53.65	**(CAGT)$_{3}$G**GAAATTGGGGATAGCTT	49.74	0.172	0.185	0.448
117-1*	G/T 0.82/0.18	F: ATTTTCTGAACGAGGAGGTA R: GGTGAGCTTAACTTTGGATT	54.02 53.63	**(CAGT)$_{6}$G**AAAGCCTCACAATTTCC	50.65	0.265	0.300	0.207
117-2†	C/T 0.78/0.22	F: GGTGAGCTTAACTTTGGATT R: ATTTTCTGAACGAGGAGGTA	53.63 54.02	**(CAGT)$_{6}$GAC**AGTTTCATGAGTTGAAAACTA	49.28	0.319	0.343	0.450
117-5*	G/A 0.61/0.39	F: TTATTCCATGTAAAATGTGATTTG R: GCAGTTTGCTTGTAGTGCTT	55.92 55.88	**(CAGT)$_{8}$GA**TGGTTTGTAACTCCATGT	48.95	0.419	0.475	0.198
119-1†	T/C 0.86/0.14	F: GGCTGATGTTTCTGGATTA R: TGAAACTTCATAGCTTTCCA	53.56 53.62	**(CAGT)$_{10}$GAC**CCCAGGTGCGGAT	51.68	0.181	0.244	0.005
151-2*	A/C 0.90/0.10	F: AAAGCATACACAAGAGCCTTA R: ATGCCACTGTTTCCTCAT	54.52 53.64	**(CAGT)$_{10}$**ATCTTTTTTGGAAGGG	47.66	0.122	0.187	**0.0002**
151-3†	G/C 0.96/0.04	F: ATGCCACTGTTTCCTCAT R: GCACAGTAAGATGGGTTTC	53.64 53.00	**(CAGT)$_{3}$GAC**GCTCTTGTGTATGCTTTTT	50.56	0.085	0.082	0.629
172-5†	T/C 0.65/0.35	F: TCACACTCGTAGTCCAAACA R: AACGCCTGGTCTGAAAAT	55.10 55.64	**(CAGT)$_{5}$**CAGTGAAAGCACATACTGTC	51.50	0.402	0.453	0.223
172-6*	T/C 0.75/0.25	F: TATGTGCTTTCACTGTGACC R: CTGTGTCACACTCGTAGTCC	54.55 53.94	**(CAGT)$_{4}$**AAGGCAGTGAACGC	48.06	0.325	0.375	0.151
190-1*	A/G 0.93/0.07	F: TTCAAGGTCAGGTGCTGT R: CAGGACTTCGTGAAATATCC	54.94 54.71	**(CAGT)$_{5}$GA**CCGATTGCACGG	47.81	0.112	0.136	0.060
190-2†	G/T 0.91/0.09	F: ACAGTGATTCCTGTCAAACA R: CCCCACTTCATATCTTGC	53.85 53.81	**(CAGT)$_{6}$GA**GATTTAAGTGGTTTTGCC	50.51	0.164	0.165	0.955

* pool 1; † pool 2; P-values in bold indicate significant deviation from Hardy-Weinberg equilibrium

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