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The Roles of Allorecognition and Larval Interactions in the Fusion of Swimming Sponge Larvae

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THE ROLES OF ALLORECOGNITION AND LARVAL INTERACTIONS IN THE FUSION OF SWIMMING SPONGE LARVAE

By

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The members of the Committee approve the thesis of Katie E. McGhee defended on July 9th, 2003.

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The office of Graduate Studies has verified and approved the above named committee members.

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ABSTRACT

For sedentary marine invertebrates, allorecognition systems allow individuals to distinguish between genetically similar and distinct tissue they may encounter and are thought to reduce costly tissue fusion with individuals other than self or kin. In this study, I examined the effects of relatedness on the fusion frequencies of the purple sponge, *Haliclona sp.*, as sedentary adults and as free-swimming larvae. While adult sponges fuse preferentially with related tissue; larvae fuse equally with sibling and non-sibling larvae at an average rate of 13.4% resulting in swimming larval chimeras capable of successful metamorphosis. In contrast to the adult fusion pattern, these results suggest that larvae are unable to distinguish between individuals of varying relatedness. Although the effect of relatedness on larval fusion rate was non-significant, adult sponges differed significantly in the propensity of their larvae to fuse, with some adult sponges producing larvae that are more fusible than those produced by others. Analysis of larval swimming behavior indicates that larvae aggregate and are capable of increasing the probability of encountering other larvae. The pursuit of fusion at this motile stage along with the evidence of a functioning adult allorecognition system, suggests that larvae may not express a recognition system, or that factors other than relatedness, such as potential benefits to larval or adult chimeras, may be involved in larval fusion and a stage-activated allorecognition system.

INTRODUCTION

Allorecognition systems are widespread across taxa and allow individuals to distinguish between genetically similar and genetically distinct tissue. In sedentary marine invertebrates that may encounter other individuals frequently as they grow along the substrate, allorecognition is critical to reacting to competitive encounters with individuals they come in contact with (Buss 1981, 1990; Buss and Grosberg 1990). In addition to competition at the individual level for space, clonal invertebrates that do not sequester a germ line may also be competing at the cellular level for access to the germ cell lineage and gamete production (Buss 1982, 1990). Recognition and the expression of compatibility when an individual encounters self-tissue, or that of closely related individuals, prevents tissue damage from an accidental rejection reaction and allows rapid expansion by tissue fusion (reviewed in Grosberg 1988). By recognizing self or kin, a genetically based allorecognition system minimizes tissue fusion events between unrelated neighbors and the substantial costs thought to be associated with a chimeric existence, such as somatic cell parasitism, resource competition, or reduced competitive ability. Most studies investigating allorecognition systems have focused on the adult stage although in clonal marine invertebrates with complex life cycles involving a larval stage, many traits that may play a role in allorecognition and fusion, such as motility and size, differ between the larval and adult stages.

Haliclona sp. is a common, encrusting sponge found in the intertidal environment in temperate Pacific waters. It broods free-swimming larvae that aggregate at the water surface after release from the parent sponge and often fuse to form chimeric larvae without loss of swimming or metamorphic ability. In this thesis, I investigated the roles of allorecognition and larval interactions in the fusion of these swimming *Haliclona sp*. larvae. In Chapter 1, I describe the stage-specific differences in the allorecognition response and the effect of relatedness on the fusion frequencies of both sessile *Haliclona sp.* adults and their swimming larvae. In addition, I present hypotheses to explain why

larvae that can easily avoid larval interactions by swimming away may undergo larval fusion. In Chapter 2, I describe how larvae modify their swimming behavior in the presence of related and unrelated conspecific larvae. In addition, I describe how larval behavior is likely to increase the larval encounter rate and the likelihood of larval fusion in nature.

CHAPTER 1

ALLORECOGNITION AT THE ADULT AND LARVAL STAGES OF THE PURPLE SPONGE, *HALICLONA SP*.

Introduction

Often consisting of highly polymorphic histocompatibility loci (Scofield et al. 1982; Rinkevich et al. 1995; Grosberg et al. 1996), allorecognition systems allow individuals to distinguish between genetically similar and distinct tissue and mount the appropriate immune response against foreign invaders or parasites (Burnet 1971). These recognition systems are widespread across taxa and have been demonstrated in numerous marine invertebrates, including sponges, bryozoans, ascidians, and cnidarians (reviewed in Grosberg 1988). In sedentary marine invertebrates that may encounter other individuals frequently as they grow along the substrate, allorecognition is critical to reacting to competitive encounters with both heterospecific and conspecific individuals with which they come in contact (Buss 1981, 1990; Buss and Grosberg 1990). In addition to competition at the individual level for space, clonal invertebrates that do not sequester a germ line may also be competing with conspecifics at the cellular level for access to the germ cell lineage and gamete production (Buss 1982, 1990). Thus allorecognition may also be critical in maintaining genetic integrity (Buss 1983, 1990).

For clonal sessile marine invertebrates, recognition and the expression of compatibility when an individual encounters self tissue, or that of closely related

individuals, prevents tissue damage from an accidental rejection reaction and allows rapid expansion by tissue fusion (reviewed in Grosberg 1988). The potential benefits of tissue fusion between closely related individuals and the formation of a single chimeric individual are primarily associated with an increase in colony size and the resulting increases in competitive ability, fecundity, and/or survival (reviewed in Grosberg 1988; Foster et al. 2002; but see Rinkevich and Weissman 1992; Maldonado 1998). However, unless fusion occurs between self or kin, there may be substantial costs of fusion due to resource competition between genotypes (Rinkevich and Loya 1983), resorption of one member of the chimera by the other (Rinkevich and Weissman 1987a, b, 1989; Rinkevich et al. 1993; Barki et al. 2002; but see Fuchs et al. 2002), decreased competitive ability (Foster et al. 2002), or somatic cell parasitism where one genotype dominates the germ cell lineage and hence gamete production of a chimera, while the other genotype is limited primarily to the somatic cell lineage (Buss 1982, 1990; Stoner and Weissman 1996; Stoner et al. 1999; but see Pancer et al. 1995). By recognizing self or kin, genetically based allorecognition systems are thought to allow benefits through tissue fusion with related individuals while minimizing the costs associated with fusion events between unrelated neighbors (Feldgarden and Yund 1992; Grosberg 1992).

In clonal marine invertebrates with complex life cycles involving a larval stage, many traits that are likely to play a role in fusion undergo an ontogenetic shift from the larval stage to the adult stage. For example, adults are sessile and thus, an adult that encounters tissue of another individual is essentially forced to either accept or reject the tissue because it is unable to escape contact by moving away. In contrast, larvae are motile, and thus potentially capable of escaping or pursuing encounters with other larvae. In addition, although adult size may be variable due to the amount of substrate available, established adults have nonetheless survived the extremely high mortality that occurs at the small larval and juvenile stages (Connell 1973; Highsmith 1982; Davis 1988). Due to this high size-dependent mortality, a doubling in size at these small early stages may potentially result in greater settlement and survival benefits than a doubling in size at a larger adult stage (Connell 1973; Highsmith 1982). Thus, the costs and benefits of fusion are likely to change as individuals progress from the motile larval stage to the sessile adult stage.

Experimental evidence for a functioning allorecognition system in adults and post-metamorphic individuals involves forced tissue contact via grafting or by manipulating individuals in space and allowing them to grow into each other. In contrast to that of adults, evidence for a functioning allorecognition system in earlier stages is mixed. Some studies have demonstrated non-kin fusion and suggest the presence of an ontogenetically changing recognition system (Hidaka 1985; Ilan and Loya 1990; Shenk and Buss 1991; Lange et al. 1992; Frank et al. 1997; Barki et al. 2002; Fuchs et al. 2002; but see Bishop and Sommerfeldt 1999) while other studies have shown evidence for sibling recognition associated with gregarious settlement (Keough 1984; Grosberg and Quinn 1986; but see Maldonado 1998; Barki et al. 2002). Many of these studies however, do not draw a clear distinction between larvae and post-metamorphic individuals, which are likely to have different costs and benefits to fusion. For example, studies using post-metamorphic individuals, or juveniles, that are attached to the substratum are likely to affect larval settlement decisions and potential fusion rates. In addition, if the presence of conspecifics is a settlement cue for swimming larvae, larvae may be more likely to settle on, or near, individuals that have already settled, with fusion being a byproduct of proximity.

The observation that some sponges produce swimming larvae that fuse and form larval chimeras (Lévi 1956; Warburton 1958; S. Leys, pers. comm. 2001) suggests an alternate approach for investigating allorecognition at earlier stages that may avoid potential biases. Contact between pre-metamorphic swimming larvae is not forced by the investigator and can easily be avoided by the larvae swimming away. In addition, chimeric larvae continue to swim and thus, fusion is unlikely to be affected by future settlement opportunities. Thus, using both pre- and post-metamorphic individuals allows an investigator to examine the allorecognition system at different stages of an organism's life cycle. To explore the stage-specific differences in the allorecognition response, here I investigate the effect of relatedness on the fusion frequencies of both sessile adults and swimming larvae in the purple sponge, *Haliclona sp.*.

Methods

(a) Study Species

The purple sponge, *Haliclona sp.* (Class Demospongiae) is a viviparous, encrusting sponge found on partially exposed rocks in the intertidal along the Pacific coast of North America. Reproductively mature sponges brood free-swimming parenchymella larvae that are assumed to be sexually produced (Berquist 1978; Simpson 1984; Fell 1993; but see Bergquist et al. 1970). These parenchymella larvae are uniformly ciliated with a ring of longer cilia at one end, possess directional swimming with constant rotation along their longitudinal axis, and are expelled through the oscula of the parent sponge by the outgoing water current (pers. obs.; Lévi 1956; Bergquist et al. 1970; Bergquist 1978; Woollacott 1990, 1993). Larvae are released in the late spring to early summer and range from approximately 200 to 250 μm in size. A small piece (~5 cm^2) of ripe sponge can release an average of 30 larvae to a maximum of >100 larvae over the course of several days. When transferred forcefully through a pipette, larvae undergo obvious shape changes, such as flattening or elongating, but recover their original spherical shape after several minutes (pers. obs.; Maldonado et al. 1997). Under laboratory conditions, larvae swim at the surface of the water for several hours and then, as has been found in other larvae, begin an exploratory, or creeping, phase where they swim near the bottom of the container until settlement (pers. obs.; reviewed in Bergquist et al. 1970; Bergquist 1978). At this stage, the larvae periodically touch the substrate with these episodes of contact increasing as the larvae age, resulting in times when the larvae spin in one place on the substrate for several minutes. After release from the parent sponge, larvae aggregate at the water surface (see Fig. 1.1A) and often fuse to form chimeric larvae without loss of swimming or metamorphic ability (see Figs.1.1B, C). Under laboratory conditions, larvae settle and metamorphose into juvenile sponges after approximately 2-4 days.

All of the following experiments were conducted under the assumption that genetic relatedness decreases with increasing distance between adult individuals. Thus I am assuming that an individual is more closely related to neighboring individuals at the

same site than individuals at a distant site and that individuals separated by more than a kilometer are unrelated. It should be noted that individuals at the same-site may potentially be members of the same genetic clone. Although the dispersal capabilities of marine invertebrate larvae in nature remains largely unknown due to the inherent difficulties of tracking and observing miniscule larvae in a dynamic environment, support for the presence of a negative relationship between relatedness and distance has been demonstrated with a variety of marine invertebrate taxa with propagules of limited dispersal ability (Jackson 1985, 1986; reviewed in Grosberg 1988; Hellberg 1995; Mariani et al. 2000).

(b) Adult Collection

All adult work was conducted during November 2002 at the Bamfield Marine Station, Vancouver Island, B.C., Canada. Sponges were collected from 2 sites in the Barkley Sound area (see Fig. 1.2) with different sites separated by approximately 5 km and by the deep, high-flow channels between the islands. Using a metal spatula, a ~5 cm^2 piece of tissue was removed from the rock and immediately submerged in seawater. To avoid collecting tissue from members of the same genet, sponges collected from the same site were at least 1 m apart.

(c) Adult Fusion Experiment

The tissue of twelve focal sponges from two sites was paired with: 1) self tissue, 2) tissue from a same-site sponge separated by at least 1 meter from the focal individual, and 3) tissue from a distant site sponge from an island located over 1 km away (see Fig. 1.3A). Sponges were used only once. Pieces of sponge (~1 cm^2) were cut with a razor blade and using monofilament line, tissue pairs were attached side by side with their external surfaces facing outwards to glass microscope slides that were maintained in a flowing seawater table (see Fig. 1.3B). After 10 days, sponge pairs were scored as either fusion or rejection. I a priori defined fusion as healthy tissues joined by a continuous superficial epithelium that cannot be separated by gentle pulling, and rejection as the tissues being easily separated by touch or having an obvious gap between them (e.g. Hildemann et al. 1980a, b; Kaye and Oritz 1981; Curtis et al. 1982; Jokiel et al. 1982;

Neigel and Schmahl 1984; Neigel and Avise 1985). Assuming that neighbors within a few meters of one another are more likely to be related than individuals on distant islands separated by over a kilometer, I used a Cochran-Armitage trend test (Agresti 1996) to test for a linear trend between the adult fusion rates and the treatment.

(d) Larval Collection

All larval work was conducted during May and June 2001 at the Bamfield Marine Station, Vancouver Island, B.C., Canada. Reproductively mature sponges were collected from four sites in the Barkley Sound area (see Fig. 1.2) with different sites separated by distances ranging from 1 to 5 km and by the deep, high-flow channels between the islands. At each site, I took compass measurements and distances between collected sponges. Sponges were collected as described above (see *Adult Collection*) and maintained in a flowing seawater table on a 14:10 hr light:dark regime with exposure to an approximate 2 hr "low tide" once a day.

(e) Larval Fusion Experiment

Eight focal sponges from four sites were paired with a same-site sponge separated by at least 1 meter from the focal individual, and a distant site sponge from an island located over 1 km away. I chose sponges for this experiment based on their initial larval production and potential future production. Larvae were collected following natural release from the parent sponge and larval pairs of approximately the same age were put in Falcon tray wells (diameter= 1.6 cm, height= 1.7 cm) of unfiltered seawater. These larval pairs consisted of a focal sponge larva and a second larva representing one of three treatments: 1) a sibling larva from the same parent sponge, 2) a larva from a same-site sponge, and 3) a larva from a distant site sponge (see Fig. 1.4). Larvae were used only once. I performed a total of 24 sponge crosses (8 focal sponges X 3 treatments) and to obtain fusion frequencies, each sponge cross consisted of 15.2 larval crosses on average (range = 10-22 larval crosses). Thus, for each focal sponge, I performed an average of 45.6 total larval crosses (15.2 larval crosses X 3 treatments).

The Falcon trays containing the larval pairs were maintained at ambient seawater temperature and ¾ of the water in each well was changed every two days. I checked

larvae several times daily with a dissecting scope for evidence of fusion. Although some larvae settled on top of already metamorphosing individuals, I excluded these from the analysis and recorded only larvae that fused prior to settlement and resulted in a swimming chimera as fusions. I monitored all larvae until metamorphosis regardless of fusion status.

I square-root arcsine transformed the data and used a two-way ANOVA without replication to test for the effects of treatment and focal sponge on larval fusion rates. In addition, I used a linear regression to test for the effect of distance between the same-site sponges on larval fusion rates.

Results

(a) Adult Fusion Experiment

There was a significant nonzero correlation between the adult fusion rates for the three treatments with self tissue fusing in 100% of trials, same-site sponge tissue fusing in 25% of trials, and distant site sponge tissue never fusing (M^2= 24.45, P<0.0001, see Fig. 1.5). All fused sponges were joined by continuous, healthy tissue and could not be separated (see Fig. 1.6) while the tissues of the nonfused sponges remained unjoined.

(b) Larval Fusion Experiment

The mean larval fusion rate over all treatments was 13.4% (S.E.=0.018). Fusion rates were not significantly different among the three larval treatments ($F_{2,21}$=1.24, P=0.310, see Fig. 1.7). In addition, the distance between same-site sponges and the focal sponge did not affect the fusion rate of their larvae (R^2=0.285, P=0.173, see Fig. 1.8). The proportion of larval fusions differed significantly among the focal sponges ($F_{7,16}$=4.52, P=0.008, see Tables 1.1, 1.2). This was examined further using a linear regression to see if variation in fusion frequencies across focal sponges could be explained by differences in the fusion frequencies between siblings. The pattern of larval fusion indicates that larvae from focal sponges that exhibit high fusion rates with siblings also exhibit high fusion rates with larvae from distant sites and this trend is significantly different from zero (R^2=0.680, P=0.012, see Fig. 1.9). Fusion rates ranged from 0% to

37.5% (see Table 1.2) with the highest fusion rate occurring between a focal sponge with average sized larvae (200-250 μm) and a same-site sponge with larvae smaller than those of all other sponges (approximately 100 μm). When the focal sponge with this high fusion rate was removed from the analyses, results were unchanged: treatments did not differ significantly ($F_{2,18}$=0.43, *P*=0.663), distances between same-site sponges did not significantly affect fusion rates (R^2=0.008, *P*=0.851), and focal sponges remained significantly different ($F_{6,14}$=3.13, *P*=0.044). All metamorphosed individuals and chimeras survived for the duration of the experiment (approximately 27 days).

Chimeric larvae were approximately double the size of single larvae, and upon metamorphosis, chimeric tissue was noticeably denser (opaque rather than transparent) and covered a larger area than non-chimeric tissue. Since accurate settlement and metamorphosis times were not measured, these potential differences between chimeras and non-chimeras were not quantified.

Discussion

The results of the adult fusion experiment, where self-tissue always fuses, tissue from individuals at the same site fuse occasionally, and tissue from individuals at distant sites never fuse, are consistent with other studies demonstrating a functioning allorecognition system based on relatedness at the adult stage (see Fig. 1.5) (reviewed in Grosberg 1988). Although precautions were taken to avoid collecting tissue from two ramets of the same genet, it is possible that tissue pairs from the same-site may have been self-tissue pairs of a fragmented colony. Previous studies forcibly maintaining tissue contact in the lab have found fusion frequencies between adult individuals separated by at least 1m are often under 7% (Karakashian and Milkman 1967, 4.5%; Hildeman et al. 1980a, 1980b, 0%; Jokiel et al. 1982, 0%; 4.7%; Neigel and Schmahl 1984, 0%; Neigel and Avise 1985, 0%; Grosberg and Quinn 1986, 4.2%; Amano 1990, 4.8%; Jokiel and Bigger 1994, 0%; Rinkevich et al. 1995, 0%, 1.2%, 2%, 6.6%; but see Curtis et al. 1982, 24%, 44%; Heyward and Stoddart 1985, 12.8%, 100%; Rinkevich and Saito 1992, 12.1%) thus the increased same-site tissue fusion rate of 25% found here may be due to these self-tissue pairings rather than the presence of many related individuals. If the

fusions in the same-site treatment are simply self-tissue fusions, these results suggest that adults may exhibit a self-, rather than a kin-recognition, system and that fragments of a colony are more likely to be near each other than on different islands as would be predicted. If instead, tissue pairs from the same-site are between two genetically different individuals, these results suggest that a negative relationship between relatedness and distance likely exists in *Haliclona sp.*. Although it is impossible to distinguish between these scenarios without genetic data, these results show evidence for either a functioning self- or kin-recognition system at the adult stage.

In the larval fusion experiment, fusion frequencies did not differ significantly among sibling larvae, larvae from sponges at the same site, and larvae from sponges at distant sites suggesting that the degree of relatedness did not influence fusion frequencies among larvae (see Fig. 1.7). Since no formal breeding design was employed, it is likely that the siblings in the present study represent an unknown combination of full- and half-sibs that may vary between parent sponges based on the number of sires. This ratio of full- to half-sibs can potentially affect fusion rates and in the hydroid *Hydractinia symbiolongicarpus*, full-sibs have a fusion rate of ~30% while half-sibs have a fusion rate of 2% and unrelated individuals have a fusion rate of 0.5-1% (Grosberg et al. 1996). It is possible that *Haliclona sp.* also exhibits this pattern and that the sibling fusion rate in this study represents a mix of the full- and half-sib fusion rates. However, siblings did not have a greater average fusion rate, or greater variation around the mean, than larvae from the same-site or distant sites (see Table 1.2) suggesting that if both full- and half-sibs are being produced by this sponge species, larval fusion rates do not differ between them.

The results of the adult and larval fusion experiments are consistent with previous allorecognition studies suggesting the presence of ontogenetic changes in compatibility (Hidaka 1985; Ilan and Loya 1990; Shenk and Buss 1991; Lange et al. 1992; Frank et al. 1997; Barki et al. 2002; Fuchs et al. 2002). Unlike previous studies however, the swimming larvae were able to interact freely with each other and fusion between individuals was not driven by unavoidable encounters or settlement decisions. If substantial costs are associated with indiscriminate fusion, then larvae should avoid all encounters and possible resultant fusions. However, larvae fuse at a rate of 13.4% regardless of relatedness, which is relatively high when compared to the fusion

frequencies of adult tissues (Karakashian and Milkman 1967, 4.5%; Hildeman et al. 1980a, 1980b, 0%; Jokiel et al. 1982, 0%; 4.7%; Neigel and Schmahl 1984, 0%; Neigel and Avise 1985, 0%; Grosberg and Quinn 1986, 4.2%; Amano 1990, 4.8%; Jokiel and Bigger 1994, 0%; Rinkevich et al. 1995, 0%, 1.2%, 2%, 6.6%; but see Curtis et al. 1982, 24%, 44%; Heyward and Stoddart 1985, 12.8%, 100%; Rinkevich and Saito 1992, 12.1%).

It is possible that larvae do not yet possess a functional allorecognition system, or are physiological unable to mount an allorecognition response until after metamorphosis and increased cellular differentiation, and larval fusion is simply a nonadaptive result of close proximity. It is also possible however, that larvae are pursuing fusion for adaptive benefits such as increased survival and rapid age at first reproduction (Connell 1973; Highsmith 1982; Davis 1988; reviewed in Grosberg 1988; but see Harvell and Grosberg 1988), or accelerated organizational processes and spicule formation (Lévi 1956).

In addition, it is possible that there are benefits to larval fusion that occur at the adult chimera stage. For example, work with the colonial ascidian *Botryllus* suggests that fusion may increase the specificity of the chimeric allorecognition system. With cells of different genotypes dispersed throughout their tissue (Stoner et al. 1999; Stoner and Weissman 1996; Pancer et al. 1995; Fuchs et al. 2002), chimeras will reject any colony that does not share at least one allele at the histocompatibility locus with each of the genotypes present in the chimera (Oak and Watanabe 1957; Tanaka 1973; Mukai and Watanabe 1975a, b) and as a result, decrease the probability of fusing with individuals other than self or kin that may be disadvantageous to both members of the chimera.

Another possibility, suggested by the result that fusion frequencies differ significantly among focal sponges indicating that parent sponges produce larvae that differ in their propensity to fuse (see Fig. 1.9, Tables 1.1, 1.2), is that larval fusion may benefit some individuals and not others. These fusibility differences in larvae may be a result of differences in particular larval traits, such as larval swimming behavior or average larval size that may be controlled by the parent sponge. If outcomes of germ cell competition within a chimera are heritable (e.g. Stoner et al. 1999), perhaps the production of offspring that pursue or avoid fusion with other larvae is based on the parent's competitive ability as a member of a chimera. Producing larvae with high fusion

rates may be advantageous if, on average, they fuse with competitively inferior individuals, and if, as adults, they will be capable of dominating the germ cell lineage by competitively displacing a partner's genotype within a chimera.

Because of the difficulties in tracking and observing the behavior of miniscule propagules in the field, the extent to which marine invertebrate larvae interact in nature, as well as their dispersal capabilities, remain largely unknown. Although adult *Haliclona sp.* occur in the intertidal environment and many individuals are located in tide pools where synchronously released larvae may be more likely to interact for prolonged periods, it is unknown whether this actually occurs. Despite the short dispersal time and weak swimming ability of *Haliclona sp.* larvae, the large distances between sites, and the strong currents within the separating channels, without genetic data, the key assumption, that genetic relatedness decreases with increasing distance, remains untested.

Determining whether larvae express any kind of recognition system, whether allorecognition undergoes an ontogenetic shift, or whether larval fusion results in benefits to either the larval or adult chimeras are readily testable and may explain why larvae fuse with one another. Testing these hypotheses, as well as determining how common genetically chimeric adults are in nature (e.g. Stoner and Weissman 1996), could lead to an explanation for the presence of a stage-activated allorecognition system.

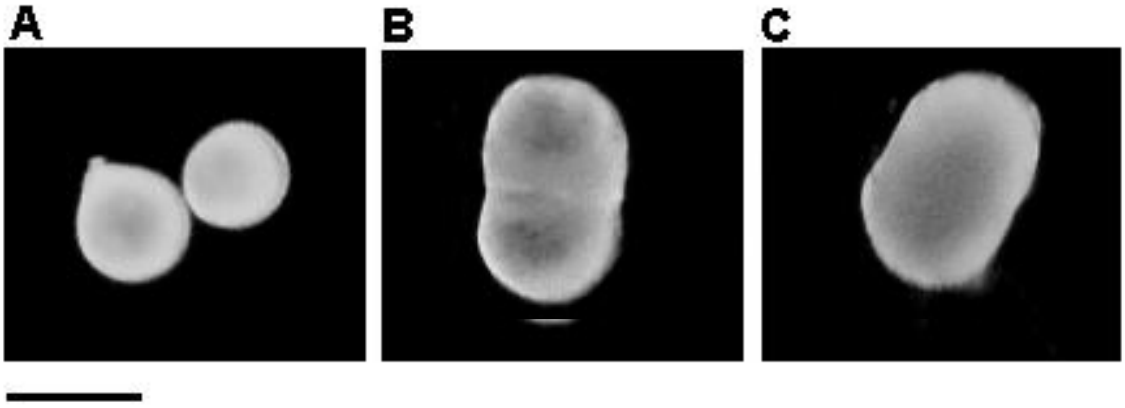

Figure 1.1 Sponge larvae and fusion. (A) Two *Haliclona sp.* larvae swimming in close proximity of each other. A swimming chimeric larva approximately (B) 20 minutes, and (C) 60 minutes after initial fusion. Scale bar ≅ 250 μm.

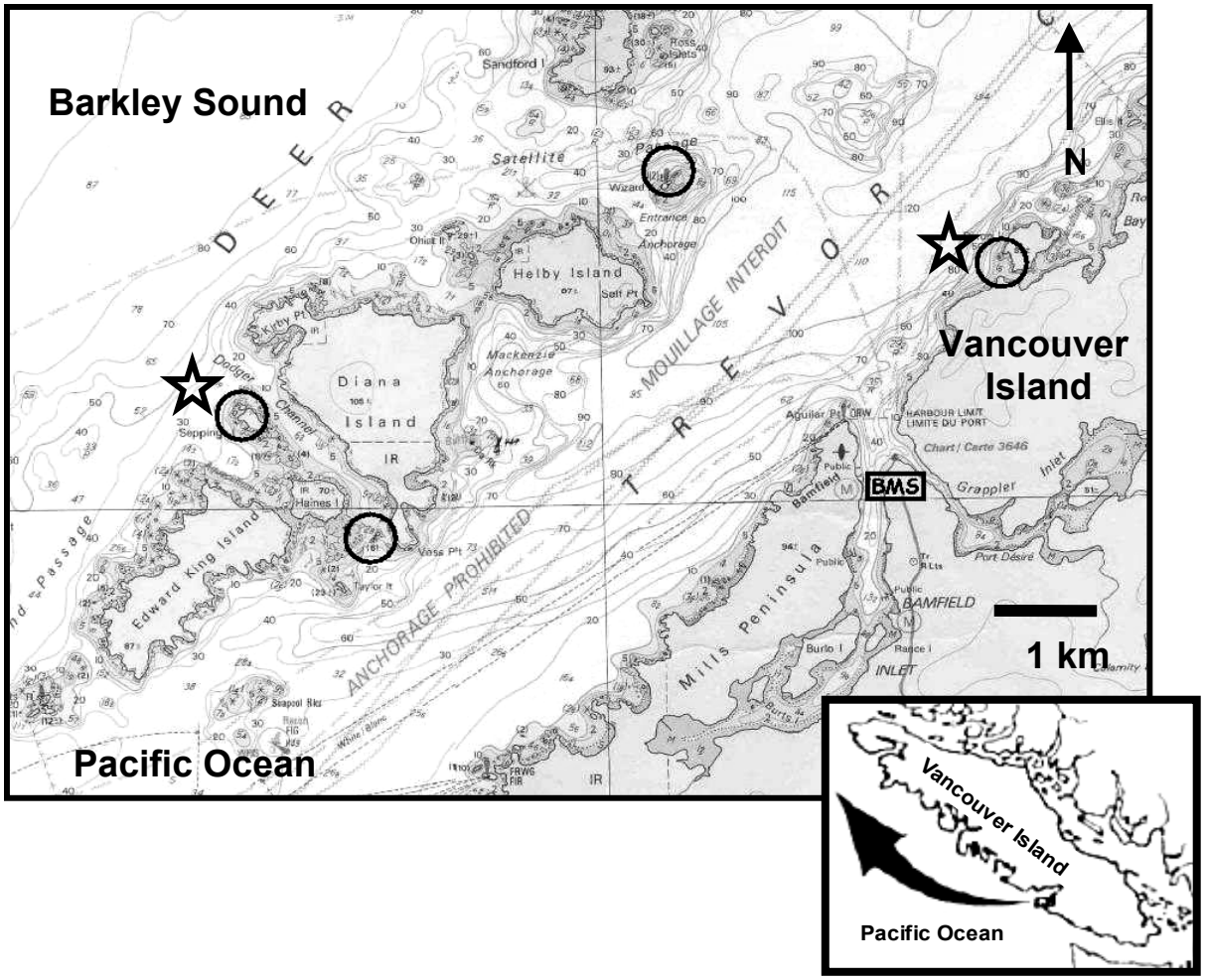

Figure 1.2. A map of Barkley Sound, Vancouver Island, B.C., Canada, with the four collection locations of sponges for the larval experiment circled and the two collection locations of the sponges for the adult experiment indicated with a star. Scale bar = 1 km.

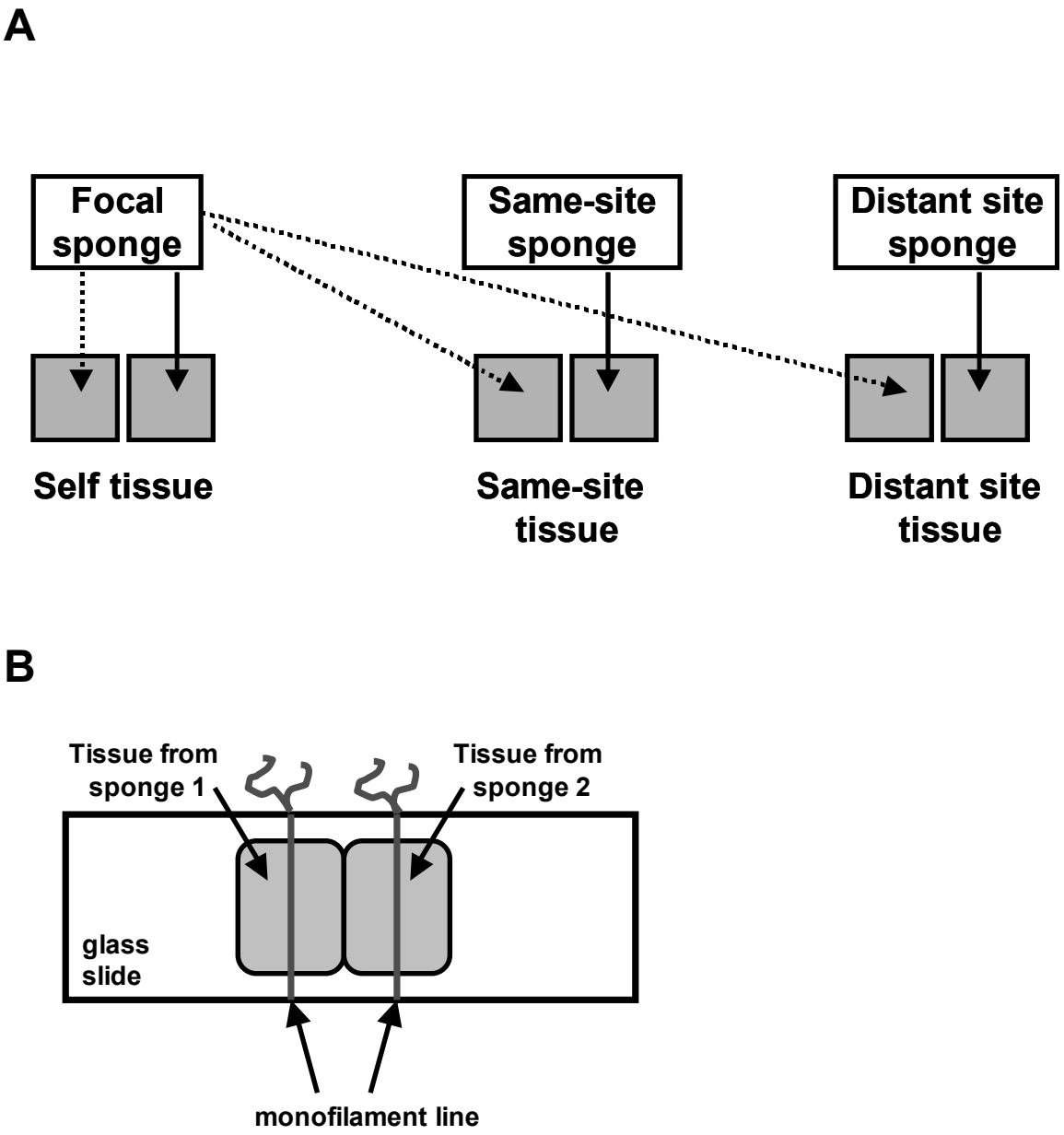

Figure 1.3. The adult fusion experimental design: (A) a schematic of the three treatments and (B) a schematic of one tissue pair. The shaded squares represent pieces of sponge tissue.

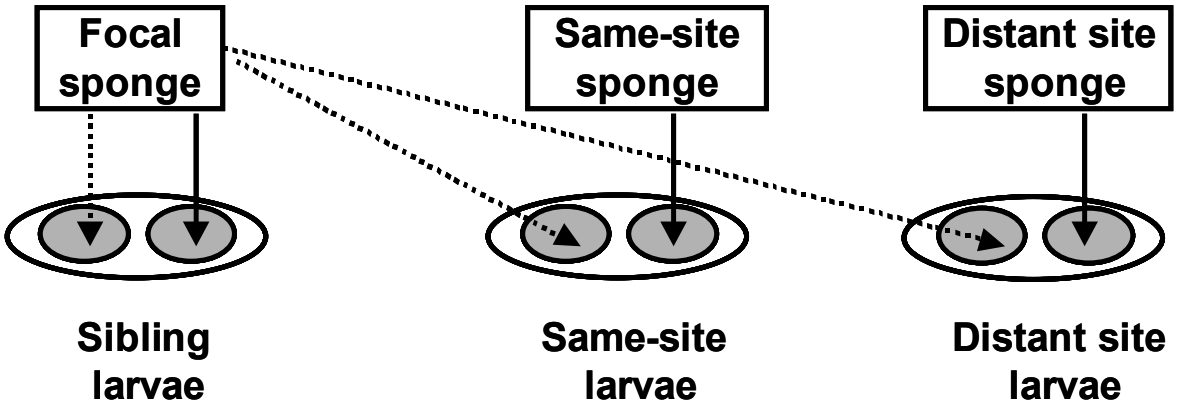

Fig 1.4. The larval fusion experimental design. The small, shaded ovals represent larvae and the larger ovals represent the Falcon tray wells in which the larval pairs were placed.

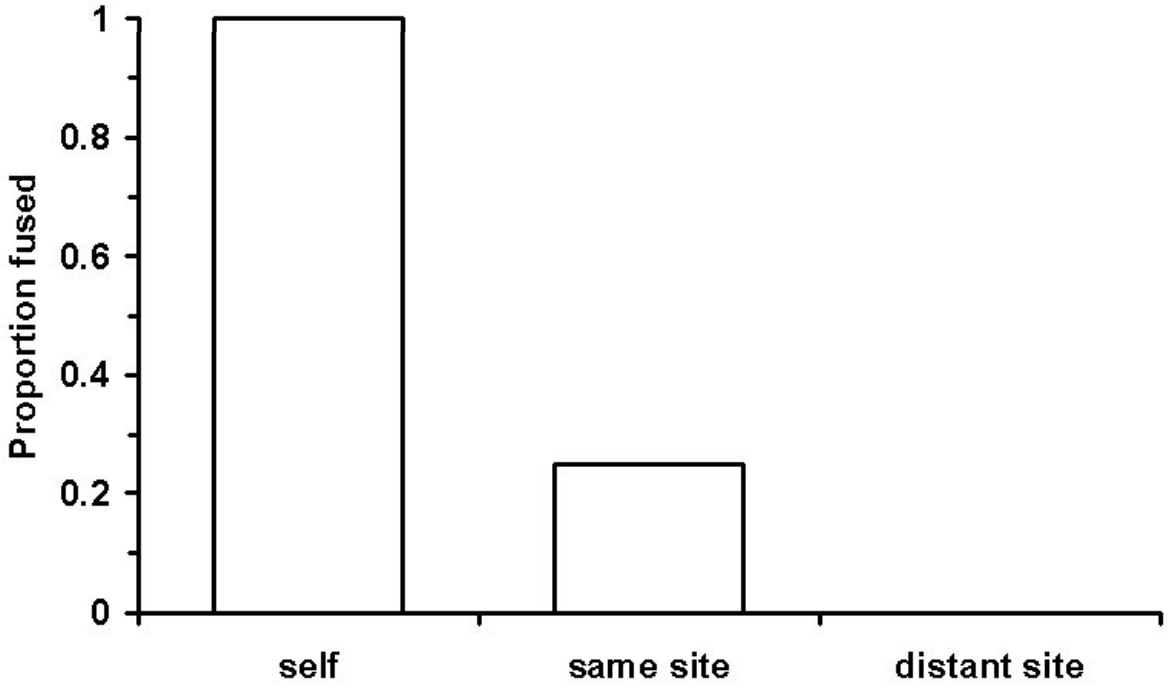

Figure 1.5. The proportion of adults whose tissue fused when paired with self tissue, tissue from a sponge at the same site, and tissue from a sponge at a distant site (n=12).

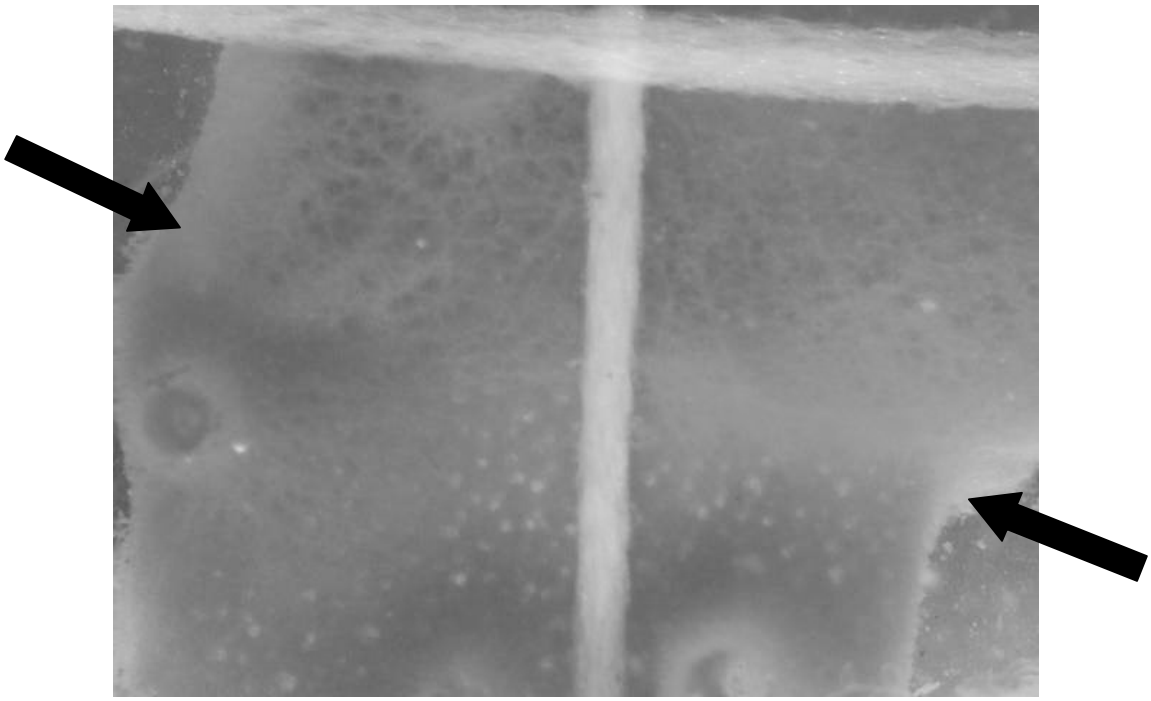

Figure 1.6. An example of fusion of an adult tissue pair from the adult fusion experiment. The arrows indicate the site of fusion and the white lines are monofilament thread.

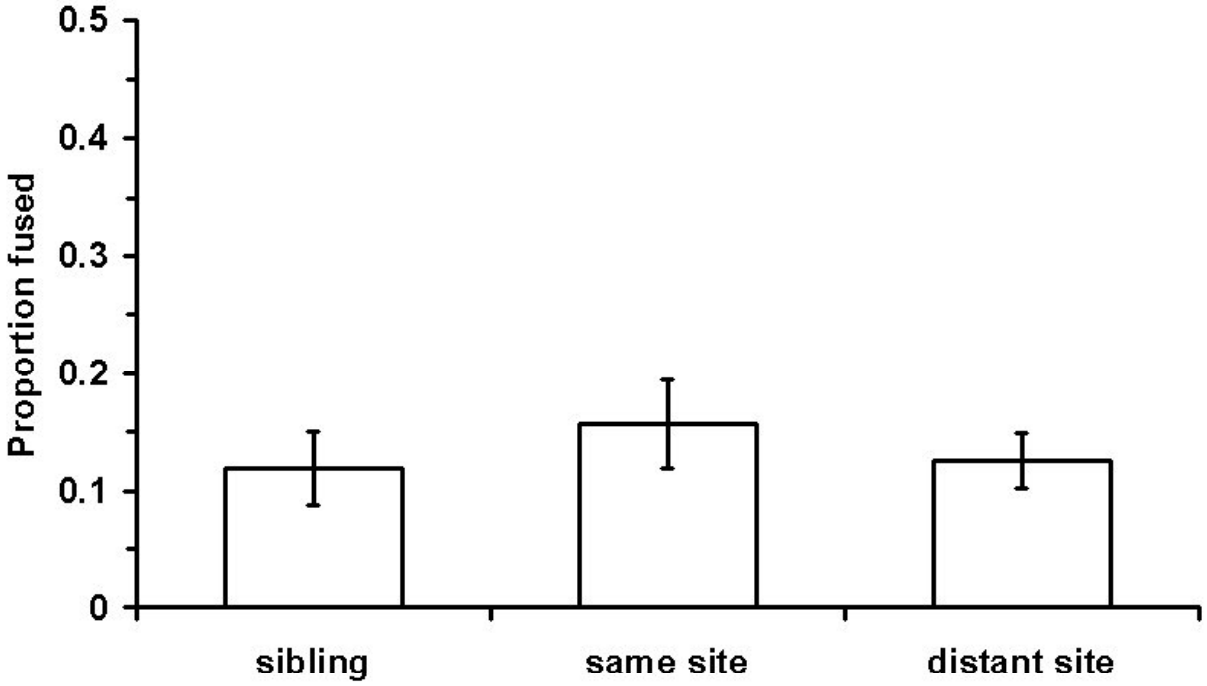

Figure 1.7. The average proportion of larvae that fused when paired with a sibling larva, a larva from a sponge at the same site, and a larva from a sponge at a distant site (n=8). The proportion of larvae that fused for each focal sponge per treatment was calculated using an average of 15.2 larval crosses. Bars indicate the standard error.

Table 1.1. The effects of treatment and focal sponge on the proportion of larval fusions based on the two-way ANOVA.

Source	d.f.	Type III Sums of Squares	Mean Sum of Squares	F value	P value
Treatment	2	0.029	0.015	1.27	0.312
Focal sponge	7	0.367	0.052	4.50	0.008
Error	14	0.163	0.012		

Table 1.2. The average proportion of larval fusions for all focal sponges across the three treatments. Unless indicated as standard errors, the number of larval crosses that generated the proportion of fused larvae is indicated in parentheses.

Focal Sponge	**Treatment**			**Average (S.E.)**
	Sibling	**Same-site**	**Distant Site**	
1	0.235 (17)	0.182 (22)	0.200 (15)	0.206 (0.016)
2	0 (15)	0.063 (16)	0.067 (15)	0.043 (0.022)
3	0.176 (17)	0.077 (13)	0.133 (15)	0.129 (0.029)
4	0.200 (10)	0.231 (13)	0.133 (15)	0.191 (0.031)
5	0.133 (15)	0.133 (15)	0.133 (15)	0.133 (0)
6	0 (15)	0.133 (15)	0 (15)	0.044 (0.044)
7	0.143 (14)	0.375 (16)	0.200 (15)	0.239 (0.070)
8	0.067 (15)	0.059 (17)	0.133 (15)	0.086 (0.024)
Average (S.E.)	0.119 (0.031)	0.158 (0.038)	0.125 (0.023)	0.134 (0.018)

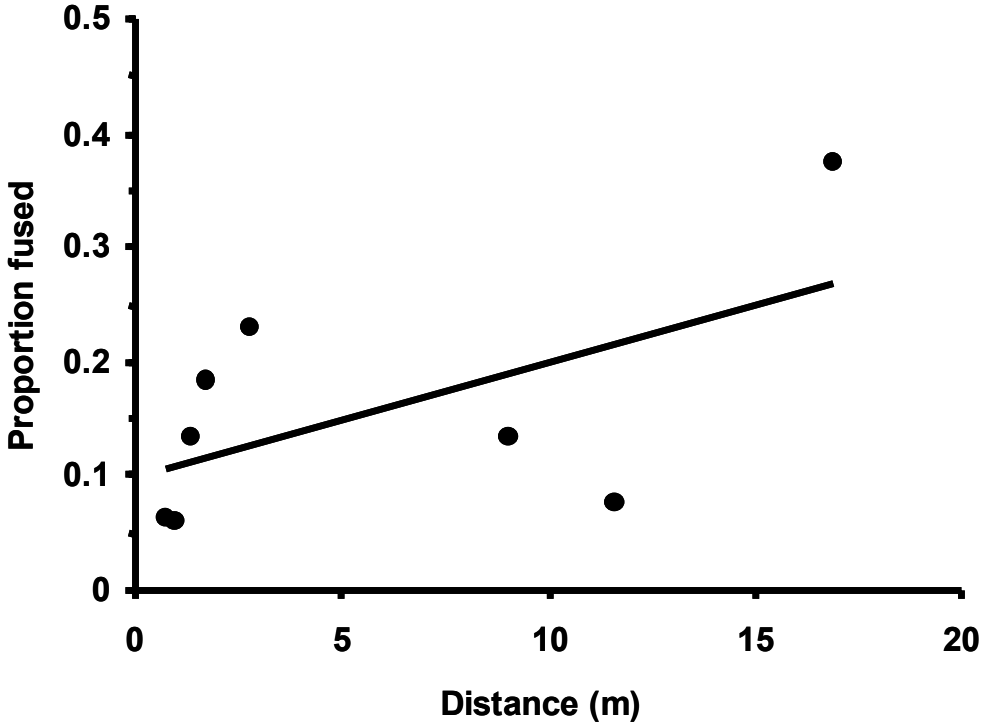

Figure 1.8. The average proportion of each focal sponge's larvae that fused with larvae from sponges at the same-site and the distance separating the focal sponge and the other parent sponge from the same-site (n=8). The regression line shown is based on the untransformed data, however all statistics were performed on the arcsine square root transformed data ($y = 0.0111x + 0.3164$).

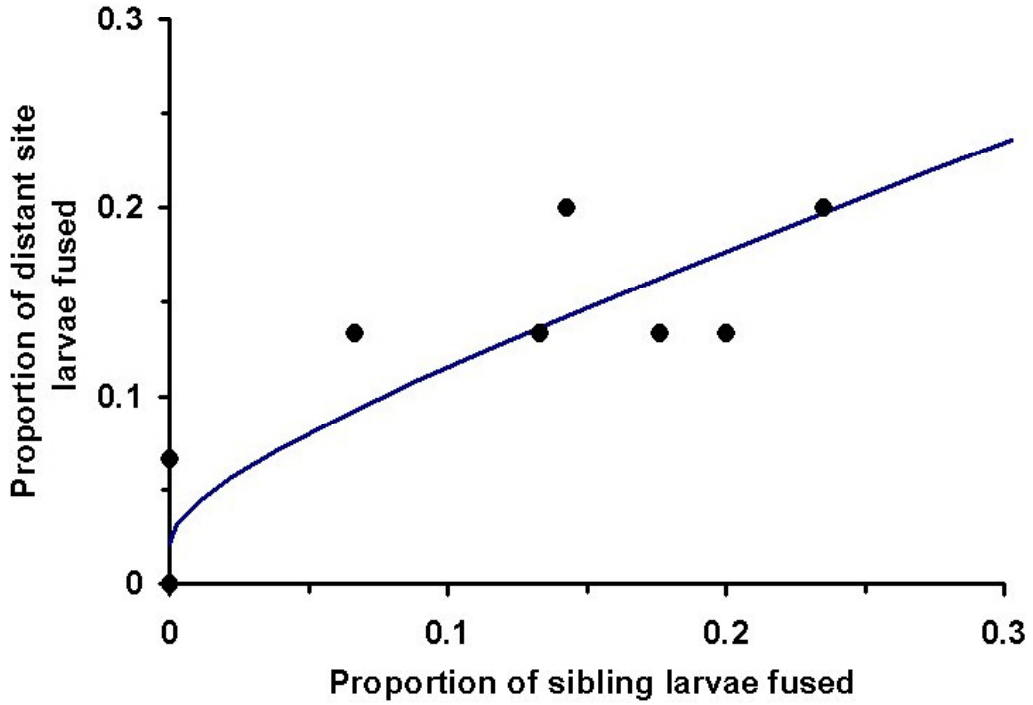

Figure 1.9. The average proportion of each focal sponge's larvae that fused with sibling larvae and larvae from distant sites (n=8). Each point represents a focal sponge and the line represents the best fit line of the back transformed arcsine square root transformed data ($y = 0.6152x + 0.1489$).

CHAPTER 2

LARVAL INTERACTIONS OF THE PURPLE SPONGE, *HALICLONA SP.*

Introduction

For almost 100 years, researchers have studied the behavioral responses of marine invertebrate larvae to abiotic and biotic attributes of their environment (reviewed in Young 1990, 1995). Tactic responses of larvae to abiotic factors, such as light (phototaxis), gravity (geotaxis), or waterflow (rheotaxis), are widespread across marine invertebrate taxa and are used in larval orientation in a three-dimensional dynamic environment (reviewed in Thorson 1964, Sulkin 1990, Young 1990, 1995; e.g. sponges: reviewed in Bergquist 1978: Bergquist et al. 1970; Woollacott 1990, 1993; Kaye and Reiswig 1991; Maldonado and Young 1996; Maldonado et al. 1997; Leys and Degnan 2001; bryozoa: Pires and Woollacott 1983; ascidians: Kajiwara and Yoshida 1985; polychaetes: Young and Chia 1982; molluscs: Bayne 1964; crustaceans: reviewed in Forward 1988). It is hypothesized that these larval behaviors allow larvae to avoid predators and move into waters where they are most likely to encounter appropriate settlement sites (Thorson 1964; Young and Chia 1987; Forward 1988).

In addition to abiotic factors, many larvae also modify their behavior in response to chemical cues from a variety of biotic sources (reviewed in Pawlik 1992). For example, the presence of competitors (Grosberg 1981; Young and Chia 1981), certain species of algae (Heyward and Negri 1999), and settled conspecific adults, or at least

fragments of previously settled conspecifics (e.g. crustaceans: Knight-Jones 1953; Crisp 1961; polychaetes: Toonen and Pawlik 1994, 1996; sponges: Uriz et al. 1998; corals: Heyward and Negri 1999; but see Walters et al. 1997), have been shown to act as important settlement cues in a variety of taxa (reviewed in Thorson 1964, Svane and Young 1989, Pawlik 1992). In many species, larvae will delay metamorphosis, often indefinitely, until they encounter the appropriate chemical cue(s) (reviewed in Pechenik 1990). Behaviors that can be modified by biotic factors in the immediate environment are thought to aid larvae in selecting a favorable environment and as a result, obtain the best conditions for settlement and their future survival (Thorson 1964).

Although several studies have examined larval responses to the above-mentioned abiotic and biotic factors, few studies have investigated the behavioral interactions that may occur between swimming conspecific larvae. In Chapter 1, I found that swimming *Haliclona sp.* larvae often swim around each other in a tight circle separated by less than 50 μm and fuse to form chimeras, regardless of the degree of relatedness between the larvae. It is possible that *Haliclona sp.* larvae alter their swimming behavior in response to the presence of related and unrelated conspecific larvae to either pursue or avoid fusion. Based on the results from Chapter 1, if the costs of fusion, such as somatic cell parasitism, outweigh the potential benefits of fusion, larvae should avoid encountering other larvae and the risk of fusion, and distribute themselves in an overdispersed fashion in the environment. If fusion benefits exceed the costs however, larvae should aggregate, thereby increasing their encounter rate and potential contact time. Here I investigate whether larvae of the purple sponge, *Haliclona sp.*, modify their swimming behavior in the presence of other conspecific larvae and if so, whether it is affected by the degree of relatedness between the larvae.

Methods

(a) Study Species

The purple sponge, *Haliclona sp.* (Class Demospongiae) is a viviparous, encrusting sponge found on partially exposed rocks in the intertidal along the Pacific coast of North America. Reproductively mature sponges brood free-swimming

parenchymella larvae that are assumed to be sexually produced (Berquist 1978; Simpson 1984; Fell 1993; but see Bergquist et al. 1970). These parenchymella larvae are uniformly ciliated with a ring of longer cilia at one end, possess directional swimming with constant rotation along their longitudinal axis, and are expelled through the oscula of the parent sponge by the outgoing water current (pers. obs.; Lévi 1956; Bergquist et al. 1970; Bergquist 1978; Woollacott 1990, 1993). Larvae are released in the late spring to early summer and range from approximately 200 to 250 μm in size. A small piece (~5 cm^2) of ripe sponge can release an average of 30 larvae to a maximum of >100 larvae over the course of several days. Under laboratory conditions, larvae swim at the surface of the water for several hours and then, as has been found in other larvae, begin an exploratory, or creeping, phase where they swim near the bottom of the container until settlement (pers. obs.; reviewed in Bergquist et al. 1970; Bergquist 1978). At this stage, the larvae periodically touch the substrate with these episodes of contact increasing as the larvae age, resulting in times when the larvae spin in one place on the substrate for several minutes. As described in Chapter 1, larvae aggregate at the water surface after release and often fuse to form chimeric larvae without loss of swimming or metamorphic ability. After approximately 2-4 days, larvae will settle and rapidly metamorphose into juvenile sponges.

As described in Chapter 1, all of the following experiments were conducted under the assumption that genetic relatedness decreases with increasing distance between individuals. Thus I am assuming that an individual is more closely related to neighboring individuals at the same site than individuals at a distant site and that individuals separated by more than a kilometer are unrelated. Although the dispersal capabilities of marine invertebrate larvae in nature remains largely unknown due to the inherent difficulties of tracking and observing miniscule larvae in a dynamic environment, support for the presence of a negative relationship between relatedness and distance has been demonstrated in a variety of marine invertebrate taxa with propagules of limited dispersal ability (Jackson 1985, 1986; reviewed in Grosberg 1988; Hellberg 1995; Mariani et al. 2000).

(b) Larval Collection

All larval work was conducted during May and June 2001 at the Bamfield Marine Station, Vancouver Island, B.C., Canada. Reproductively mature sponges were collected from four sites in the Barkley Sound area (see Fig. 2.1) with different sites separated by distances ranging from 1 to 5 km and by the deep, high-flow channels between the islands. Using a metal spatula, a ~5 cm^2 piece of tissue was removed from the rock and immediately submerged in seawater. To avoid collecting tissue from members of the same genet, sponges collected from the same site were at least 1 m apart. Sponges were maintained in a flowing seawater table on a 14:10 hr light:dark regime with exposure to an approximate 2 hr "low tide" once a day.

(c) Larval Behavior

To determine whether the behavior of a larva is altered by the presence of another larva, I compared the swimming pattern of larval pairs and single larvae. Larvae were collected following natural release from parent sponges and placed either singly or in pairs chosen at random in Falcon tray wells of unfiltered seawater. Larvae were used only once. Larvae were videotaped continuously using a microscope-mounted camera for 10 minutes on a background grid. To determine the location of the larva(e), each videotape was stopped every 10 seconds during playback and the occupied grid square number(s) were recorded, resulting in approximately 60 larval location points per video.

To quantify the behavior of larval pairs (n=20), I randomly chose 10 larval location points and for each one, I calculated the distance between the two larvae by measuring the distance between the middle of each of the occupied grid squares (see Fig. 2.2A). I then averaged these 10 distances for each pair. If larvae occupied the same grid square simultaneously, I recorded the distance between them as 0 mm. To quantify the behavior of single larvae swimming without the influence of a second larva, the videotapes of single larvae were randomly matched up to form "virtual pairs" (n=5). I then superimposed the 10 minute video of each member of a "virtual pair" on the other so that at 10 randomly chosen larval location points, I could determine the location of each member of the "virtual pair" and measure the distance between them (see Fig. 2.2B). I

then averaged these 10 distances for each "virtual pair". In this way, I could compare the average distance between the larvae of these "virtual pairs" with the average distance between the larvae of the true larval pairs. I used a t-test to compare the average distance between the larvae of larval pairs and "virtual pairs".

To determine whether the average distance between larvae (both larval pairs and "virtual pairs") differs from that predicted by a random larval distribution, I calculated the distance between two points distributed at random within the videotaped area using the equation (Vandermeer 1981):

$$\bar{r}_{\text{random}} = \frac{1}{2(N/A)^{1/2}}$$

where A is the total area (A= 99 mm), N is the total number of individuals (N=2), and thus, $\bar{r}_{\text{random}}$ = 3.518 mm. Using a one-sample t-test, I compared this predicted distance with the average distances between larval pairs and "virtual pairs". To look at the pattern of larval distribution, I calculated a measure of aggregation, R, by dividing the average measured distance between two larvae by the $\bar{r}_{\text{random}}$, where the distribution of two larvae is random if R=1, overdispersed if R>1, and clumped if R<1 (Vandermeer 1981).

Since the larval pairs videotaped (n=20) were paired at random, they consisted of a mix of sibling pairs (n=8) and non-sibling pairs (n=12). To determine whether relatedness affects larval swimming behavior, I used a t-test to compare the average distance between sibling larval pairs and non-sibling larval pairs.

Results

Larval Behavior

The average distances between larvae of the larval pairs and larvae of the "virtual pairs" differed significantly (t_{23}=5.60, P=0.027). The larvae of the larval pairs were separated by an average of 2.058 mm (S.E.=0.259) while the larvae of the "virtual pairs" were separated by an average of 3.466 mm (S.E.=0.597). The distance predicted between

two larvae distributed at random within the videotaped area ($\bar{r}_{random}$ = 3.518 mm) did not differ significantly from the average distance between larvae of "virtual pairs" (t_4=0.087, P=0.935) but did differ significantly from the average distance between larvae of larval pairs (t_{19}=5.642, P<0.0001, see Fig. 2.3). The measure of aggregation, R, for the "virtual pairs" was 0.985, indicating a random distribution, and the R for the larval pairs was 0.585, indicating a clumped distribution. The larvae of the sibling larval pairs were separated by an average of 1.954 mm (S.E.=0.589) while the larvae of the non-sibling pairs were separated by an average of 2.123 mm (S.E.=0.211). The average distances between larvae of the sibling pairs and the non-sibling pairs did not differ significantly (t_{18}=0.10, P=0.753, see Fig. 2.4). However, this test had very low statistical power (1-β=0.061) due to the small effect and low sample size, and would have required a sample size of over 700 pairs to reject the null hypothesis if it were false.

Discussion

The results from this study show that sponge larvae actively modify their swimming behavior when in the presence of a second larva. Larvae swimming alone tend to distribute themselves randomly while two larvae swimming together tend to distribute themselves in a clumped, or aggregated, fashion (see Fig. 2.3). In addition, this tendency to aggregate occurs despite the degree of relatedness between the larvae (see Fig. 2.4).

Larval stages of some species show aggregation in the lab, although this swarming behavior tends to disappear as the larvae age (polychaetes: Young and Chia 1982; ascidians: Kajiwara and Yoshida 1985; but see Young 1995). There are accounts of swarming in the field for some crustaceans that may alter swimming behavior to stay within visual contact (reviewed in Young 1995; krill (euphausiids) and shrimp-like animals (mysids): Komaki 1967, reviewed in Mauchline 1980, Jillet and Zeldis 1985), however many of these animals remain pelagic throughout their life and swarms in these cases may consist of mixed age classes. Although it is hypothesized that by forming larval clouds, larvae may decrease their vulnerability to predators and increase their chances of finding suitable substrate (reviewed in Mauchline 1980; Young 1995), it remains unknown whether this occurs. Surprisingly, larval interactions have not been

investigated in species that are capable of fusion, where the distance between larvae may directly affect the probability of fusion.

In Chapter 1, I found that swimming larvae occasionally fuse despite the potential cost of somatic cell parasitism. Based on the results from this Chapter, *Haliclona sp.* larvae modify their behavior to decrease the distance between them. By exhibiting such aggregative behavior, larvae may increase their likelihood of encountering other larva in the field, thereby increasing their chances of undergoing fusion. Alternatively, it is possible that larvae aggregate for another reason, such as predator avoidance, and fusion itself is a byproduct of this close contact. Sponges possess no integrated nervous system and the tactile or chemical receptors to allow detection of cues are poorly understood (Lévi 1956; Bergquist 1978), therefore how these larvae are capable of detecting other larvae nearby remains unknown. Although *Haliclona sp.* larvae may be able to somehow detect other larvae, similar to the results in Chapter 1, larvae seem unable to distinguish between siblings and unrelated larvae.

Although my results indicate that *Haliclona sp*. larvae swim in a clumped distribution in the lab, it is unclear what effect this behavioral modification may have on larval dispersal in the field at a larger scale. Faced with coastal currents on the order of cm to 10's of cm per second (Shanks 1995), it is doubtful that ciliated larvae can actively control their dispersal by swimming at speeds on the order of mm per second (Chia et al. 1984; Woollacott 1990, 1993; Shanks 1995; Maldonado and Young 1996; but see Stoner 1990). A study by Koehl and Powell (1994) investigating the transport of larval mimics in the wave-exposed, rocky intertidal environment, where turbulent mixing overwhelms larval motion, suggests that passive dispersal by water currents may be more important in concentrating larvae than their active swimming. They found that larvae remain concentrated near their release point over time due to water oscillating back and forth, rather than flowing unidirectionally, as waves move across the habitat. In addition to low advective velocities near shore and the formation of eddies, topographically induced fronts (Kingsford et al. 1991), tidally forced internal waves (Shanks 1983), and interactions between the wind and the water (Langmuir 1938) have also been shown to be involved in larval transport and the concentration of larvae in certain regions. For example, Langmuir circulation, the alternating clockwise and counterclockwise vortices

that form due to the action of the wind on the water, concentrates passively buoyant particles and plankton in the convergence zones of the vortices, or Langmuir cells (Orton 1937; Langmuir 1938; Jillett and Zeldis 1985; Hamner and Schneider 1986; Kingsford et al. 1991). Larval swarming may then be more likely where oceanographic features, such as waves, eddies, and Langmuir cells, tend to concentrate rather than disperse particles (Orton 1937; Shanks 1983, 1995). Thus, the encounter rate of larvae might be increased if a behavior, such as larval aggregation, keeps larvae in the same, rather than different, eddies.

With larvae often being released synchronously once gravid adults detect their own cues (Simpson 1984; Mariani et al. 2000), it seems likely that larvae will interact with one another as they disperse away from the parent or neighboring individuals. In addition, larvae using similar abiotic and biotic cues to modify their behavior may interact with one another as they progress towards metamorphosis. Without knowing the temporal and spatial pattern of larval release however, the likelihood of unrelated larvae getting trapped in the same eddy remains unclear. If siblings are much more likely to get transported together and unrelated larvae rarely interact, then the costs of pursuing fusion might be lower and larvae may not benefit from discriminating against these rarely encountered unrelated larvae. Adult *Haliclona sp.* occur in the intertidal environment and many individuals are located in tide pools where synchronously released larvae may be more likely to interact for prolonged periods, especially if larval release coincides with the falling tide (Bergquist et al. 1970). Although it is unknown whether this actually occurs, it is hypothesized that traits such as viviparity and larvae with short swimming periods ensure that many larvae are maintained in the nearby region (Bergquist et al. 1970; Schmidt 1982; Young and Chia 1987). Because of the difficulties in tracking and observing the behavior of miniscule propagules in the field, the extent to which marine invertebrate larvae interact in nature, as well as their dispersal capabilities, remain largely unknown and despite the limitations of experimental studies conducted in small containers of still water, these lab studies are still essential in observing and quantifying subtle larval behaviors that may influence larval interactions and dispersal in the field (Butman 1987; Forward 1988; Sulkin 1990).

To my knowledge, this study is the first attempt at quantifying the larval interactions that may play a role in the likelihood of larval fusion and chimera formation. Although the mechanism by which larvae detect one another is still unknown, the results from this study suggest that *Haliclona sp.* larvae actively modify their swimming behavior when in the presence of other larvae to decrease the distance separating them. This behavior is likely to increase the encounter rate between larvae trapped in small eddies or Langmuir cells, and based on the results from Chapter 1, increase the probability of larval fusion. Whether the aggregative behavior shown in this study occurs frequently in the field, how it affects the probability of larval fusion, and whether it plays a role in the distribution of the adults and the genetic structure of the population remain open questions with important consequences to larval ecology.

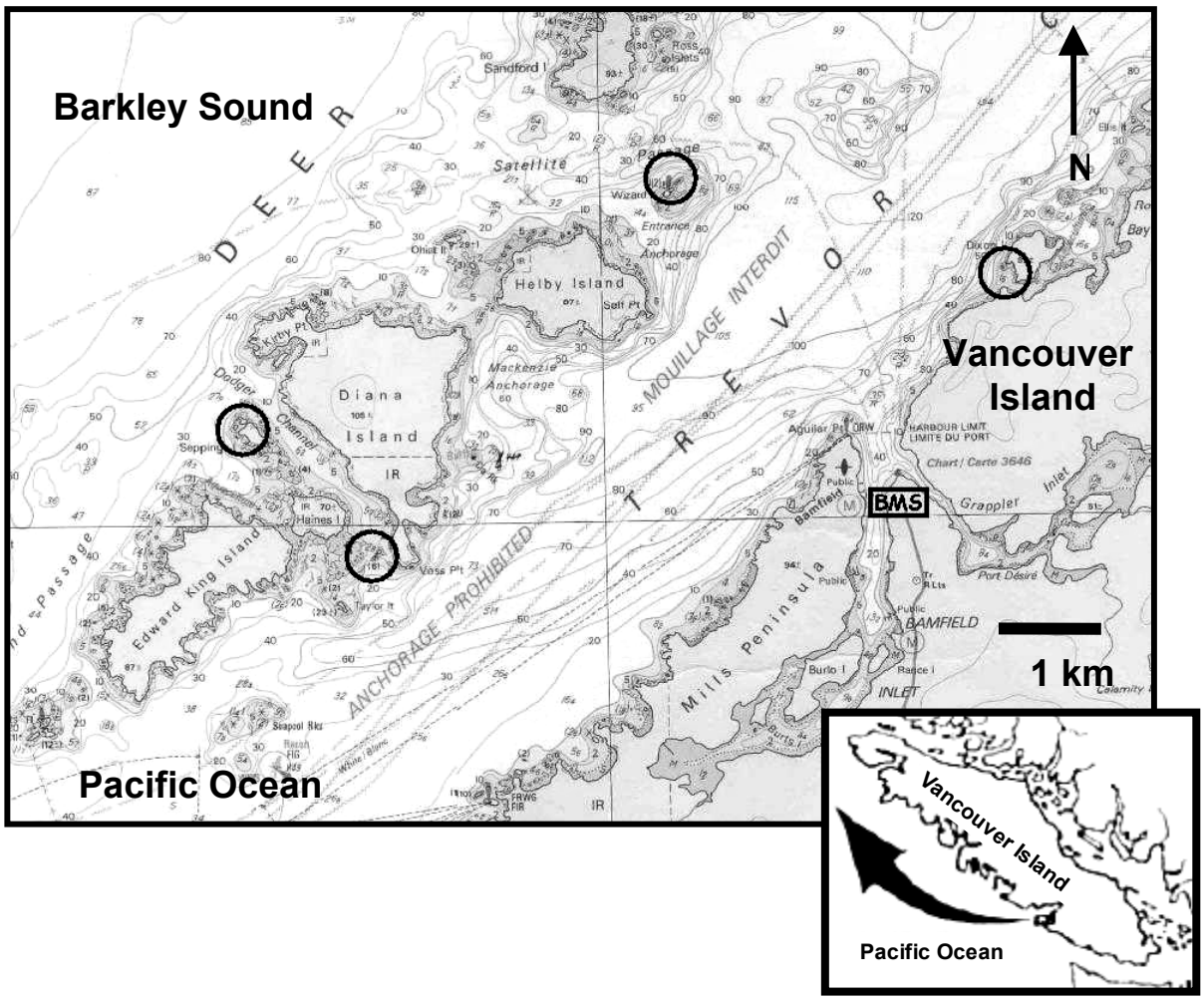

Figure 2.1. A map of Barkley Sound, Vancouver Island, B.C., Canada, with the four collection locations of sponges for the larval experiment circled. Scale bar = 1 km.

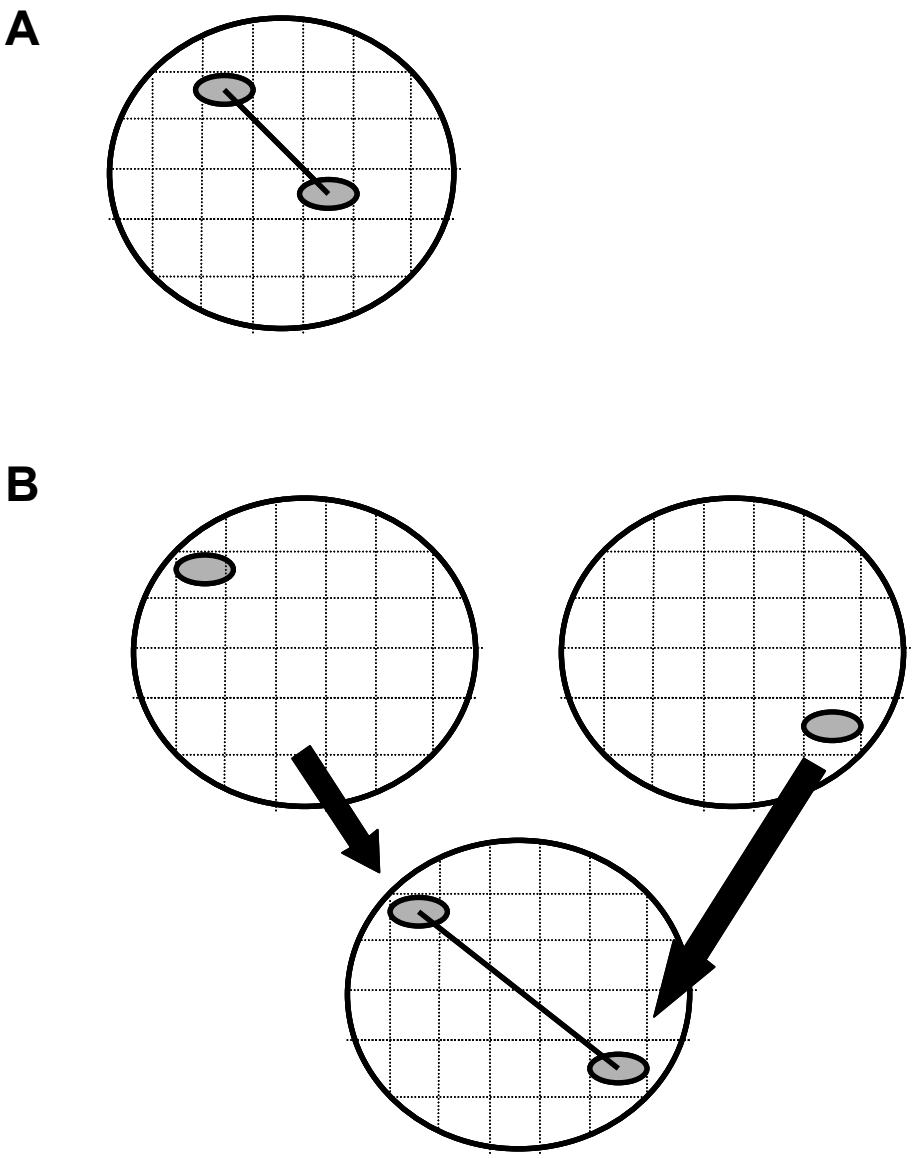

Figure 2.2. The larval behavior experimental design: (A) measuring the distance between a larval pair and (B) measuring the distance between single larvae put into "virtual pairs" by superimposing the videos. The small, shaded ovals represent larvae and the large circles represent the Falcon tray wells with background grids of 1mm².

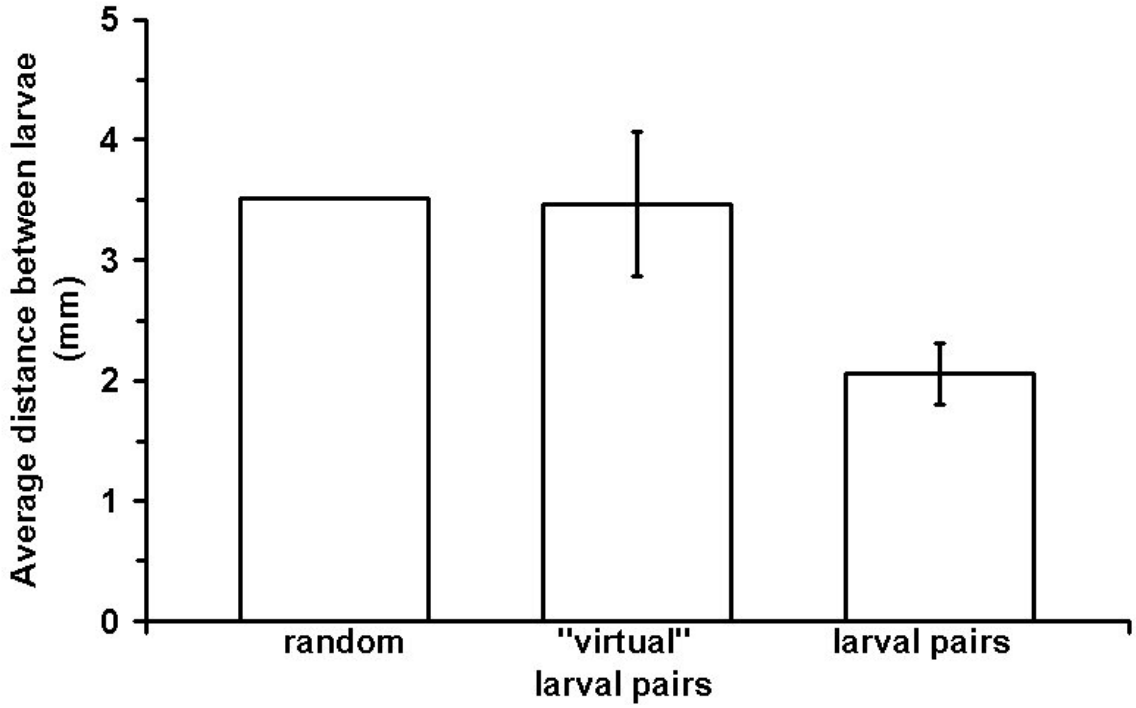

Figure 2.3. The distance between larvae as predicted by random movement within the videotaped area, the average distance between "virtual pairs" of single larvae (n=5), and the average distance between larval pairs (n=20). The average distance between larvae was calculated using the position of the larvae at 10 random times taken from 10 minute videos. Bars indicate the standard error.

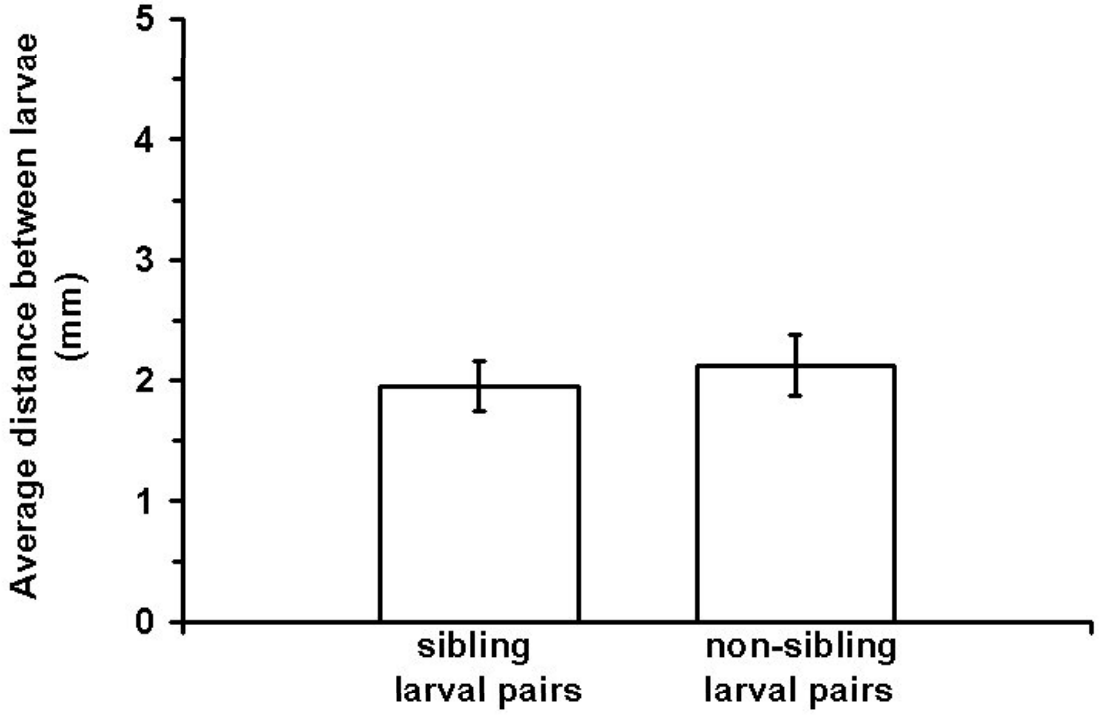

Figure 2.4. The average distances between sibling larval pairs (n=8) and between non-sibling larval pairs (n=12). The average distance between larvae was calculated using the position of the larvae at 10 random times taken from 10 minute videos. Bars indicate the standard error.

CONCLUSION

In Chapter 1, I found that the allorecognition system of the temperate encrusting sponge, *Haliclona sp*. differs between the adult and larval stages. While adults fuse preferentially with related tissue, larvae fuse equally with sibling and non-sibling larvae at an average rate of 13.4%, with these larval fusions resulting in swimming larval chimeras capable of successful metamorphosis. In addition, I found that adult sponges differ significantly in the propensity of their larvae to fuse, with some adults sponges producing larvae that are overall more fusible than those produced by other adult sponges. In Chapter 2, I found that larvae are capable of modifying their swimming behavior to increase their encounter rate. In the presence of a second larva, larvae aggregate and tend to decrease the distance separating them. In addition, the degree of relatedness between the larvae did not affect this clumping behavior.

Together, these results suggest that either larvae are unable to express the allorecognition system and are aggregating for an unknown reason, or larvae are indeed pursuing fusion despite the potential costs of indiscriminate fusion. The pursuit of fusion with unrelated individuals at this motile stage along with the evidence of a functioning allorecognition system at the adult stage, suggest that factors other than relatedness may be involved in determining the likelihood of larval fusion. Factors such as unique fusion costs and benefits present at the larval stage where size may be critical, strategies of the parental genotype based on heritable competitive ability, and the future specificity of the adult chimeric allorecognition system, may all play a role in the benefits of larval fusion and a stage-activated allorecognition system. Testing these hypotheses, determining how common genetically chimeric adults are in nature, and investigating whether larval aggregative behavior affects larval fusion frequencies in the field, remain open questions with important consequences to our understanding of allorecognition systems, larval ecology, and the genetic structure of marine invertebrate populations.

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Publications:

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Manuscripts in Preparation:

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Research Experience:

2000, 2001 Caribbean Marine Research Center, Lee Stocking Island, Bahamas
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Honours and Awards:

2000	Bamfield Marine Station Student Scholarship
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2002-2003	Assistant Lab Coordinator	Animal Diversity
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2001-2002	Instructor, Saturday-at-the-Sea, Florida State University's outreach program for local middle school children
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