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Effects of Sperm Environment on the Evolution of Gamete Traits in Ciona Robusta

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EFFECTS OF SPERM ENVIRONMENT ON THE EVOLUTION OF GAMETE TRAITS IN *CIONA ROBUSTA*

By

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For Carlos, whose love, selflessness, and support made this possible.

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ABSTRACT

Fertilization is a complex process, and gamete traits can affect the rate at which sperm and egg collide and fuse, making them prime targets for selection. This is particularly true for broadcast spawners, whose fertilization success mainly depends on interactions between gametes. Gamete traits can modify collision and fusion rates affecting fertilization success, but sperm availability can affect the rate at which gametes can interact. Because of this the strength and direction of selection on gamete traits to optimize fertilization success is dependent upon sperm availability.

While many studies have examined differences in selection pressures due to sperm availability on individual traits, rarely has the effect of interactions between traits been examined. Yet, interactions between traits may have an important impact on selection by modifying the focal trait's effect on fertilization success, and that modification can be contingent on the sperm environment. For instance, eggs that increase collisions and are quicker to fuse with sperm may be more prone to polyspermy (reproductive failure due to multiple sperm fusions) at lower sperm availabilities than eggs that increase collisions but are slower to fuse with sperm. Therefore, determining how interactions between traits can affect fertilization success, and how that effect can change across different sperm environments, is important for understanding the selective pressures a particular trait may face for a given sperm environment. Additionally, while many studies have postulated that interactions between different male and female gamete recognition protein (GRP) variants can affect fertilization success by altering fusion rates, it has yet to be examined.

In this dissertation, I present the results of manipulative and observational experiments designed to determine how interactions between a suite of gamete traits may affect fertilization success. I conducted a series of no-choice fertilization assays over a range of sperm availabilities, in order to determine whether collision rates, genetic variability in GRPs (which mediate compatibility), or interactions between the two were the most important in determining fertilization success. I also attempted to determine if there was a difference in compatibility between different male and female GRP variants by examining whether individuals garnered a greater share of paternity based on their respective genotypes. This was accomplished by conducting fertilization assays, in which the eggs were offered a mix of two males' sperm. I also

examined whether there might be a functional link between genetic variation in GRPs and chemoattractant-mediated differences in sperm behavior. This was accomplished by examining whether there was a difference in sperm chemotaxis or chemokinesis based on the respective GRP genotypes of the eggs and sperm using video analysis and dichotomous chambers. Finally, I looked at whether the trends seen in the laboratory were found in a natural population. I correlated changes in settler density (as a proxy for sperm availability) with changes in collision trait values, as well as examining for increases in assortative mating based on GRP identity with increasing settler density.

I found that interactions between traits tended to explain most of the variance in fertilization success for most sperm environments. While I was unable to determine differences in compatibility, my results suggest that sperm that have the same female GRP genotype as the eggs they were exposed to tended to garner a higher share of the paternity. Additionally, my results also suggest that sperm will aggregate around eggs when they both share the same GRP genotype at the receptor locus, offering a mechanism by which assortative mating between gametes based on GRP genotype could occur. I then found that assortative mating based on the female receptor genotype does occur in a natural population, as more homozygous settlers were produced than expected under random mating as settler density increased. Additionally, while I found that collision rate traits changed in the directions predicted from previous studies based on increasing settler density, that relationship could be modified by GRP genotype. This suggests that assortative mating based on multiple trait values can occur, most likely due to the fact that different combinations of traits can maximize fertilization success.

Overall, interactions of gamete traits with compatibility played a large role in fertilization success, particularly as sperm density increased. These results highlight the importance of examining the interplay of multiple traits under differing spawning conditions, in order to truly understand how they can affect fitness, and shape trait evolution.

CHAPTER 1

INTRODUCTION

Sexual selection is a major force in shaping many traits associated with mating, and may also have profound effects on speciation and the evolution of mating systems (Dobzhansky 1940, Gavrilets and Waxman 2002, Bode and Marshall 2007, Henshaw et al. 2014, Cotto and Servedio 2017). Studies of sexual selection have focused on mate choice and male-male competition in highly motile, terrestrial organisms, but understanding how sexual selection operates in broadcast spawners can also be crucial to gain information on the different mechanisms of sexual selection. For example, examining how sperm competition and cryptic female choice can shape reproductive traits can be studied in broadcast spawners more easily that in internally-fertilizing species (Andersson and Iwasa 1996, Evans and Sherman 2013), as direct manipulations of their gametes are feasible. Fertilization in broadcast spawners ultimately depends on interactions between the gametes themselves, making them a prime target for sexual selection (Levitan 1998, Evans and Sherman 2013).

Fertilization is a complex process that consists of several steps, each of which depends on the interactions of multiple gamete traits. First, sperm and egg must collide. Sperm-egg collision rate can depend on numerous physical and biological factors, such as water turbulence, density of gametes in the water, as well as the target size of the egg and swimming behaviors of sperm (Levitan 1996, 1998, Crimaldi and Zimmer 2014, Hussain et al. 2016). Next primary binding, where ligands from the sperm bind to receptors on the egg's vitelline coat, occurs after collision, and self-nonself recognition may also occur at this step (Sawada et al. 2004, Saito et al. 2012). Genetic variation in gamete recognition proteins (GRPs), which are involved in primary binding, can mediate compatibility between egg and sperm and affect sperm-egg binding rate (Palumbi 1999, Levitan and Ferrell 2006). They are also known to be some of the fastest evolving proteins, and have been estimated to evolve at 2-4 times the rate of other proteins involved in fertilization (Lessios and Zigler 2012, Vicens et al. 2014). After primary binding, the acrosome reaction occurs and sperm move through the previtelline space to fuse with the egg plasma membrane (Lambert 1982, Evans and Sherman 2013).

The rate at which these steps are completed can depend on the gamete traits involved with each step, as well as the overall availability of sperm, which can affect the rate of sperm-egg interactions. Sperm availability has long been recognized as an important factor shaping the evolution of gamete traits (Levitan 2002, Levitan 2012, Evans and Sherman 2013, Crean and Marshall 2015). If eggs are released but there is not enough sperm to fertilize them, sperm limitation can occur (Levitan and Peterson 1995, Yund 2000). Conversely, if there are too many sperm available, an egg may experience cell death due to polyspermy; that is, multiple sperm fusing with the egg and causing reproductive failure (Styan 1998, Yund 2000, Franke et al. 2002). Selection on gamete traits affect collision and fusion rates can modify how many sperm are needed for successful fertilization to optimize fertilization success for a particular sperm environment (Levitan 1996, Levitan et al. 2007).

Sperm limitation can select for traits that can increase the probability of a sperm colliding with an egg. These can include increasing effective target size for eggs (Farley and Levitan 2001, Podolsky 2001, Crean and Marshall 2008), and/or increasing sperm longevity and the probability of encountering an egg (Levitan 2000, Fitzpatrick et al. 2012). It can also select for traits that increase sperm-egg fusion rates, which can result in the reduction of genetic variation in gamete recognition proteins (GRPs), as mutations can decrease compatibility between egg and sperm (Metz et al. 1998, Levitan and Stapper 2010, Tomaiuolo and Levitan 2010). On the other hand, polyspermy can select for traits that decrease the probability of polyspermy by decreasing collision rate or separating sperm arrival times. This can be accomplished either through decreasing egg target size (and thus collision rate), or by increasing the distance a sperm has to travel to get to the ovum by increasing the jelly coat or follicle cell thicknesses (Frank 2000, Podolsky 2001, Crean and Marshall 2015). Additionally, selection for decreasing sperm-egg compatibility can decrease fusion rate, reduce polyspermy, and result in an increase in genetic variation in GRPs as mutations are maintained within a population (Levitan and Ferrell 2006, Levitan and Stapper 2010, Tomaiuolo and Levitan 2010).

In addition to polyspermy, high sperm environments can also increase the probability of sperm competition and/or cryptic female choice occurring, as eggs are exposed to more sperm from different individuals (Levitan 2018). Sperm traits that are responsible for increasing collision rates, such as sperm velocity, motility, and reactivity to chemoattractants, are expected to be favored when there is competition among sperm from different individuals for an egg, as

being the male able to locate and fuse with an egg first would increase fitness, even if some eggs become polyspermic as a result (Levitan et al. 2007, Johnson et al. 2013). Similarly, males would be selected to outcompete other males by increasing compatibility with female GRPs, even as females are selected to decrease compatibility to reduce polyspermy (Gavrilets 2000, Haygood 2004, Tomaiuolo and Levitan 2010). This can result in a co-evolutionary arms race, with one or more pairs of high-affinity sperm and egg proteins being maintained within a population (Gavrilets and Waxman 2002, Haygood 2004, Tomaiuolo and Levitan 2010). However, there is an absence of empirical data on how genetic variation in egg GRPs can affect fitness, nor are there measurements of the relative affinities for different receptor-ligand pairings. Most studies have focused on how variation in the sperm protein affect fitness in both males and females and inferred the effects of male-female protein interactions from those results (Palumbi 1999, Levitan and Ferrell 2006).

Although sperm limitation, competition, and polyspermy's effects on the evolution of gamete traits have been studied in the laboratory for many years, studies of interactions between traits based on the sperm environment are rare, and almost non-existent between traits that affect different fertilization processes, like sperm-egg collision rates and fusion rates (Evans and Sherman 2013). However, it is possible that in different sperm environments the importance of traits that govern different fertilization processes in determining fertilization success changes. For instance, in low sperm environments traits that increase collision rates may play a greater role in determining fertilization success than compatibility, as decreased access to sperm means fewer interaction between different male protein variants and the egg receptor has less of an ability to exert choice (Sherman et al. 2015, Levitan 2018). Conversely at higher sperm availabilities, interactions between collision rate traits and compatibility may determine whether or not an egg is more likely to become polyspermic based on the combination of trait values involved.

Traits that affect collision rates may become temporarily or permanently associated with gametes of differing compatibilities (e.g. smaller target size, faster swimming speeds), as sperm competition and the probability of polyspermy increases. There is some evidence that collision rate traits may have become associated with traits that affect compatibility, as sperm have been found to be more reactive towards the chemoattractants of eggs that they are more compatible with (Evans et al. 2012, Hussain et al. 2016, Lymbery et al. 2017), although direct tests of how

genetic variation in compatibility may be used to predict sperm behavior as mediated by chemoattractants are lacking.

By examining a suite of gamete traits across multiple sperm environments in an external fertilizer (the ascidian, *Ciona robusta*), I examined how the sperm environment and trait interactions can influence fertilization success. In Chapter 2, I explored how interactions between compatibility and traits that affect collision interact to affect fertilization success through a series of no-choice fertilization assays. This was done to determine how the importance of such interactions might change across different sperm environments. I also attempted to assess how interactions between male and female GRP variants might affect fertilization success; the first time that interactions between male and female proteins have been directly examined.

In Chapter 3, I further explored interactions between male and female GRPs by attempting to determine the relative compatibility of different receptor-ligand variants. This was accomplished by allowing eggs a choice between two males' sperm and measuring relative paternity based on GRP identity. In chapter 4, I determined if variation in GRPs could be used to predict changes in sperm behavior as mediated through egg chemoattractants. Differences in sperm behavior based on the relative compatibility between individuals can provide a mechanism by which assortative mating can occur and may help mediate sperm competition (Evans et al. 2012, Lymbery et al. 2017).

These laboratory experiments allowed me to make hypotheses about which combinations of traits should be favored in different sperm environments, which I then examined in chapter 5. In this chapter, I examined whether fluctuations in population density affected trait values in the directions predicted by previous laboratory studies. I also determined whether temporary associations between traits were more likely to occur during high population densities, when eggs can exert more choice (Levitan 2018). Overall, these experiments allowed for a greater understanding for how gamete trait evolution may be shaped by selection, by offering insights into the importance of trait interactions on fertilization under different selection regimes and examining how selective pressures may be magnified or mitigated by such interactions.

1.1 Study Organism

Ciona robusta is an important model organism in developmental biology and genetics (Dehal et al. 2002, Hendrickson et al. 2004). Like many ascidians, it is hermaphroditic and reproduces by broadcast spawning. Life-span and growth rate is typically dependent on water temperature, with individuals from warmer waters (20°C) sexually maturing in 1 month, and living for 3 to 6 months, while colder water (<8°C) individuals can live for 2 to 3 years (Yamaguchi 1975, Carver et al. 2006). After sexually maturing, adults can spawn every two to three days, and larvae can settle within 18 hours of fertilization (Yamaguchi 1975).

Ascidian eggs possess follicle cells embedded in the vitelline coat that provide a primary binding site for sperm, and where species recognition and self/non-self discrimination occurs (Marino et al. 1999, Lambert 2009, Yamada et al. 2009, Yamaguchi et al. 2011). In addition, *C. robusta* eggs possess chemoattractant that can alter sperm swimming behavior (Miller 1982, Bolton and Havenhand 1996, Yoshida et al. 2002). This change in sperm swimming behavior is somewhat species-specific, as sperm from *C. robusta* increase their swimming behavior in the presence of chemoattractants from some heterospecific tunicates, but not in others (Bolton and Havenhand 1996, Yoshida et al. 1993, Yoshida et al. 2013). In the absence of chemical cues from eggs, sperm remain relatively motionless and viable for over 13 hours (Bolton and Havenhand 1996).

While the male and female gamete recognition proteins have been identified in this species (Yamaguchi et al. 2011), no formal work has been done quantifying how much intraspecific genetic variation occurs and how it may affect fitness. The male GRP gene in this species is approximately 1,800 bp long and consists of a single exon, while the female protein is approximately 5,700 bp long and has seven exons (Yamada et al. 2009, Yamaguchi et al. 2011). *C. robusta* is an ideal model organism for testing hypotheses about the evolution of gamete compatibility developed in longer-lived species, because of their relatively short generation time, brief larval period, and the relative ease with which both male and female GRP genes can be sequenced.

CHAPTER 2

THE ROLE OF INTERACTIONS BETWEEN GAMETE TRAITS IN FERTILIZATION SUCCESS IN BROADCAST SPAWNERS

2.1 Introduction

Broadcast spawning, or the release of eggs and sperm into the sea for external fertilization, can result in highly variable encounter rates between gametes both within and between spawning events (Yund 2000, Franke et al. 2002, Levitan 2002, Marshall 2002). The density and abundance of individuals participating in a spawning event (Levitan and Young 1995, Levitan 2002, Marshall 2002) and turbulent water motion (Petersen et al. 1992, Crimaldi and Zimmer 2014) can affect the sperm concentration in a parcel of water. This variation in sperm availability can result in eggs being surrounded by too few sperm, leading to reduced fertilization success due to sperm limitation, or too many sperm, leading to polyspermy, reproductive failure due to multiple sperm fusing with the egg (Figure 2.1A; Franke et al. 2002, Marshall 2002, Levitan 2004, 2005). Because of this, selection on adult spawning behaviors and gamete traits that affect fertilization processes can occur to maximize reproductive success for a given sperm environment (Levitan et al. 2004, Levitan et al. 2007, Levitan 2008, Evans and Sherman 2013).

The question of how sperm availability may shape the evolution of gamete traits has been a topic of intense study (Levitan 1996, Farley and Levitan 2001, Podolsky 2001, Fitzpatrick et al. 2012, Evans and Sherman 2013, Johnson et al. 2013, Crean and Marshall 2015, Lymbery et al. 2018). However, in many cases gamete traits affecting fertilization have been examined in isolation, and little is known about how traits from the different steps of fertilization can interact to affect fertilization success; much less how the importance of those interactions might change across different sperm environments (Evans and Sherman 2013). My goal is to examine how traits that affect two different fertilization processes, namely, sperm-egg collision and fusion rates, can interact to affect fertilization success. I also aim to determine how the relative importance of collisions rates versus fusion rates can change across different sperm environments. As eggs and sperm must collide before fusion can occur, I hypothesized that

collision rates may be more important at low sperm availabilities, but become less important as sperm numbers increase, whereas fusion rates would become increasingly important in determining fertilization success as eggs are exposed to more sperm (Figure 2.1, Sherman et al. 2015, Levitan 2018).

Gamete traits that can affect collision rates included the effective target size of an egg (Levitan 1993), which itself can consist of several traits such as ovum size, accessory structure size (e.g. jelly coats and follicle cells), and chemoattractant production (Jantzen et al. 2001, Podolsky 2001, Levitan 2006). Increases in effective target size of the egg can cause an increase in collision rates (Styan 1998, Podolsky 2001, Levitan 2006; Table 2.1). In male gametes, collision rates can be affected by sperm velocity and swimming behaviors like chemotaxis, with faster sperm increasing collision rates (Bolton and Havenhand 1996, Levitan 2000, Evans et al. 2012, Crimaldi and Zimmer 2014). Fusion rates can be affected by compatibility between gametes, which is mediated by the relative binding ability of ligand and receptor gamete recognition proteins (GRPs) expressed on the surface of sperm and eggs (Palumbi 1999, Levitan 2012).

Studies that have examined gamete traits that affect collision and fusion rates in isolation have found that at low sperm availability, traits that increase either collision or fusion rates are favored (Table 2.1). For collision rates, larger target sizes (either by increasing the ovum or accessory structure sizes) are favored in female gametes (Podolsky 2001, Levitan 2006). In male gametes, longer-lived sperm increase the probability of a sperm-egg collision while the sperm is still viable. As there is a trade-off between sperm velocity and longevity, it results in slower, longer-lived sperm being favored at low sperm availability (Levitan 2000). For fusion rates, selection for increased compatibility is favored at low sperm availability. This can result in purifying selection within the population and decreased variation in both male and female GRPs, as any novel mutations would more likely reduce fusion rates (Swanson et al. 2001a, Tomaiuolo and Levitan 2010).

At high sperm availability traits that decrease the probability of polyspermy, by decreasing collision or fusion rates, are generally favored in the absence of direct sperm competition for an egg (Table 2.1). For collision rates, this generally means selection for slower sperm and smaller ova (Styan 1998, Podolsky 2004, Fitzpatrick et al. 2012). However, there is some evidence that accessory structures may actually help prevent polyspermy by slowing sperm

movement, and so increase in accessory structure size may be favored at high sperm availability despite the fact that they increase overall target size (Farley and Levitan 2001, Podolsky 2004, Crean and Marshall 2015). Selection for a reduction in fusion rates can result in increased genetic variation, as mutations that reduce compatibility are favored in either the male or female GRP the absence of sperm competition (Levitan and Stapper 2010, Tomaiuolo and Levitan 2010). However, in the presence of sperm competition for an egg, sperm traits that increase collision or fusion rates are favored, despite the fact that they may increase the probability of polyspermy for that egg. This can result in selection for faster, more motile sperm (Crean and Marshall 2015, Lymbery et al. 2018), and for male GRP proteins that increase fusion rate with female receptor proteins (Levitan and Ferrell 2006, Tomaiuolo and Levitan 2010, Levitan 2012).

Despite our knowledge on how sperm availability can influence the evolution of an individual gamete trait, there are still some questions remaining. For instance, no study to date has been able to characterize differences in fertilization success based on interactions between variant sperm ligand and egg receptor GRPs. Additionally, because many studies have only examined one gamete trait at a time, or traits within the same process (only collision rate or fusion rate traits separately), a general understanding of how different combinations of these traits may interact to affect fertilization success is lacking (Evans and Sherman 2013). If interactions between traits change a trait's effect on fertilization success, it may have important ramifications for the direction of selection on that trait.

At the extremes of either end of the sperm availability continuum, selection on all gamete traits may be in the same direction; either working to increase collision and fusion rates at sperm limiting conditions or reduce them at high sperm availability. However, at intermediate sperm availabilities, multiple combinations of trait values might optimize fertilization success. Thus, selection may work in different directions on a trait, depending upon the combination of other traits the focal trait is interacting with (Figure 2.1B). For instance, increasing ovum size might be selected against if that egg is compatible with the sperm it is interacting with. Alternately, eggs with low compatibility may select for faster swimming sperm, or larger target sizes (Figure 2.1B, Levitan et al. 2007, Levitan and Stapper 2010). Thus, at intermediate sperm availabilities several different trait combinations can be favored, resulting in a complex fitness landscape.

I performed a series of no-choice crosses using the broadcast spawning hermaphroditic tunicate, *Ciona robusta*, to explore how interactions between multiple gamete traits can affect

fertilization success. These crosses manipulated both sperm concentration and sperm-egg contact time, both of which can affect the number of collisions that can occur (Levitan et al. 1991, Hodgson et al. 2007), in order to create a gradient of sperm availabilities. My main goal was to use these crosses to determine how interactions between collision and fusion rate traits affected fertilization success, and to examine how the relative importance of collision and fusion rates changed over the range of sperm availabilities. A second goal was to examine how the selective landscape changed across the sperm availability gradient for collision rate traits. Finally, I aimed to document how variation in the female GRP can affect fertilization success.

2.2 Methods

Ciona robusta is a broadcast-spawning hermaphroditic tunicate, with a disjointed global distribution (Brunetti et al. 2015). *C. robusta* eggs lack a jelly coat but possess follicle cells embedded in the vitelline coat, which provides a binding site for sperm where species recognition and self/non-self discrimination occurs (Marino et al. 1999, Lambert 2009, Yamada et al. 2009, Yamaguchi et al. 2011). The male ligand protein (CiUrabin) involved in primary sperm-egg binding and its female receptor protein (CiVC57) have been identified in this species (Yamada et al. 2009, Yamaguchi et al. 2011). CiUrabin (ligand) is approximately 1,800 bp long and consists of a single exon that and had approximately 18 variable sites, 6 of which were non-synonymous substitutions. CiVC57 (receptor) is approximately 5,700 bp long and has seven exons. Exon 1 and 7 had the most variable sites; exon 1 having approximatly12 polymorphic sites, 5 of which were non-synonymous polymorphisms, and exon 7 having over 20 polymorphic sites, 15 of which were non-synonymous. Preliminary screening identified a non-synonymous mutation (SNP) in both CiUrabin and CiVC57, where both alleles from each gene were present at roughly equal frequencies (indicative of possible frequency-dependent selection on compatibility; Levitan and Stapper 2010). These SNPs (one for the ligand and one for the receptor) were used as the genotype for each locus for subsequent analyses.

2.2.1 Gamete Collection and Collision Traits Measurements

Gametes were obtained from adult *C. robusta* collected from San Diego, CA from fall of 2012 through summer of 2015. For each individual, eggs were removed from the gonoduct and

rinsed in artificial seawater. Egg concentration per mL was estimated from the stock solution by counting and averaging the number of eggs in three 25 μL subsamples. Sperm was pipetted directly from the gonoduct and kept undiluted in a microcentrifuge tube. Sperm concentration was estimated using a hemocytometer prior to experimentation. For the duration of the experiment, both eggs and sperm were kept either on ice, or in a temperature-controlled room at 19°C.

For each individual used as a female, the average ovum diameter and follicle cell length was calculated from the photographs of 15 eggs, using the computer-imaging program, ImageJ (ver. 1.43; Schneider et al., 2012). Sperm were recorded in seawater where *C. robusta* eggs from at least four different individuals had been soaked for at least 1 hour to allow for chemoattractants to diffuse into the water. This was done because active sperm swimming occurs only in the presence of egg chemoattractants (Bolton and Havenhand 1996, Yoshida et al. 2002). Sperm were recorded at a concentration of 10^7 cells per mL with a Fujifilm Finepix HS30exr camera mounted on a Leitz microscope. For each recording, 15 seconds were analyzed using a computer assisted sperm analysis program (CASA) in ImageJ. Average path curvilinear velocity (VCL), as an estimate of sperm swimming velocity, was recorded for each individual based on the number of sperm paths tracked by the CASA program (generally over 100 paths per individual for 15 seconds).

2.2.2 No-Choice Fertilization Assays

To determine how the importance of gamete traits from different fertilization processes (collision and fusion) may change over different sperm concentrations, a series of no-choice fertilization assays were performed. Three different sperm concentrations and two different sperm contact times were used, in order to create a gradient of five different sperm availabilities.

In the first assay, eggs from an individual were fertilized at two sperm concentrations, $1*10^4$ sperm mL^{-1} and $1*10^6$ sperm mL^{-1} and exposed to sperm for 2 minutes, before rinsing them on a 60 μm mesh in artificial seawater to remove the sperm. This assay represented the two lowest sperm availabilities. For this design, four individual tunicates were crossed in an incomplete diallel design with reciprocal crosses, such that each individual was used as both a male and a female and crossed with every other individual in that block, but not with themselves. Approximately 17 blocks were performed, but only 194 crosses from these blocks were utilized

in the analysis due to missing data on either fertilization success or gamete trait values. A second set of no-choice fertilization assays were performed at a longer contact time and comprised the 3rd and 4rth sperm availability. For these crosses, four individuals per block were fertilized at the same two sperm concentrations ($1*10^4$ sperm mL^{-1} and $1*10^6$ sperm mL^{-1}) in the manner described above (an incomplete diallel design), but the eggs were exposed to sperm for ten minutes before rinsing with artificial seawater.

A final set of individual crosses was conducted at three different sperm concentrations: $1*10^4$ sperm mL^{-1} (3rd), $1*10^6$ sperm mL^{-1} (4rth), and $1*10^8$ sperm mL^{-1} (5th). In this design, individual tunicates were used only twice, once as male and once as female rather than in an incomplete diallel design due to the limited amount of sperm that could be gathered from a single individual. The sperm contact time for these crosses was also 10 minutes. There were a total of 173 crosses conducted at the sperm concentration of $1*10^4$ sperm mL^{-1} (the 3rd sperm availability, intermediate), 245 crosses for the sperm concentration of $1*10^6$ sperm mL^{-1} (the 4rth sperm availability, 2nd highest), and 142 crosses at the sperm concentration of $1*10^8$ sperm mL^{-1} (the 5th sperm availability, highest).

For all crosses, an egg density of 800 eggs mL^{-1} was used. Fertilization success was estimated by counting the number of eggs undergoing cleavage from a subsample of 100 eggs. Eggs were scored approximately 75 minutes after fertilization, when the majority of the fertilized eggs had reached the 4- and 8- cell stage.

2.2.3 Sequencing Gamete Recognition Proteins

Tissue collected from the siphon for each adult was digested in CTAB and proteinase K in a 64°C hot water bath for 12-14 hours. DNA was purified using magnetic beads, and stored at -20°C. For CiVC57, a 372 bp region was amplified using the primers developed from the initial sequencing scan to target the non-synonymous mutation of interest located in exon 7 (CiVC57Exon7F: 5' –TTCTAGGCATGCCCTGGTGATTCT-3' and CiVC57Exon7R: 5'-CCATAGTGTGAACCCGCCTTTACT-3'). For CiUrabin, a 594 bp region was amplified using primers developed to target the non-synonymous mutation of interest (CiUrabinCF: 5'-GTAGTTCCATCTGCGAGTAACA-3' and CiUrabinFR: 5'-ACATAAGTGCGGAGAGTGTAAT-3').

Both genes were amplified using an initial denaturation for 2 min at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 64°C, 45 sec at 72°C, and a 2 min 72°C final extension. The reaction mixture consisted of 2.4 µL of 5x buffer, 1.3 µL of magnesium chloride at 25 mM concentration, 1.2 µL of DNTPs at 2 mM concentration, 0.5 µL of the forward and reverse primer for the loci targeted at 10 mM concentration, 0.2 µL of BSA at 10 µg/µL, and 0.15 µL of GoTaq at 5 µg/µL, plus autoclaved, double-distilled water for a total reaction volume of 10 µL. All individuals used in no-choice crosses were sequenced for both the male and female locus and the targeted SNP was recorded as the individual's genotype for that locus.

2.2.4 Interactions Between Collision and Fusion Traits Across Differing Sperm Availabilities

The data from the no-choice fertilization assays were analyzed in two ways, to address the two main questions: does the relative importance of collision and fusion rate traits change across sperm environments and how does the selective landscape change across sperm environments for collision rate traits? For the question, the effect of collision number (estimated from sperm swimming speed and egg size, see below) and fusion rate (mediated by sperm and egg GRP genotypes) and their interactions on fertilization success was tested for each of the five different sperm availabilities. This allowed me to examine how the importance of collision and fusion processes (and interactions between them) changed across different sperm environments.

The total number of collisions was estimated using the equation (Vogel et al. 1982):

$$S_e = S_o (1-e^{-BEt})$$

Where:

S_e = total number of collisions

S_o = sperm concentration

B = total cross-sectional area of the egg (in this case calculated by adding 2 times the follicle cell length to the ovum diameter) multiplied by sperm velocity

E = egg concentration

t = sperm contact time

A binomial generalized linear mixed effects model was fitted to each of the five different sperm availabilities to determine how collision rate, fusion rate, or interactions between the two affected fertilization success at each of the five different sperm concentrations and contact times

combinations. By fitting an equation to each sperm availability, I controlled for sperm concentration and contact time allowing me to focus on how egg target size and sperm swimming velocity together (parameter "B") influenced fertilization. For each sperm availability, collision number, male GRP genotype for individuals whose sperm were used, and female GRP genotype for individuals whose eggs were used, were included as fixed effects in the model. Male and female identity were included as random effects. A binomial link function was used, with fertilization scored as the number of success and failures for the 100 eggs assessed for fertilization. To help with model convergence, total number of collisions was standardized for each sperm concentration and contact time to have a mean of zero and a standard deviation of 1.

2.2.5 Changes in Selection on Collision Traits Across Differing Sperm Availabilities

For the second analysis, I generated three different hypotheses for how multiple gamete traits may affect fertilization success based on sperm environment using the conclusions from previous studies (Table 2.1). To determine whether selection on collision traits occurred in the directions predicted based on sperm availability, I defined a linear combination of traits corresponding to each hypothesis and then evaluated the strength of selection in each of those three directions for each of the five sperm availabilities. Axis 1 consisted of fast sperm swimming speeds combined with small eggs and small follicle cells (small total target size), as sperm velocity was positively loaded on to this axis, while egg diameter and follicle cell length were negatively loaded. This axis represented a combination of traits that may be selected for at high sperm densities if follicle cells do not act to slow sperm but did act to increase egg target size (Podolsky 2001, Lymbery et al. 2018). Axis 2 consisted of fast sperm swimming speeds combined with small eggs and large follicle cells, as sperm velocity and follicle cell length were positively loaded on to this axis, while egg diameter was negatively loaded. This axis represented a combination of traits that may be selected for at high sperm densities if follicle cells act to reduce the probability of polyspermy by slowing sperm movement despite increasing egg target size (Crean and Marshall 2015). The final axis, axis 3, represented slow sperm swimming speeds combined with small eggs and large follicle cells, as follicle cell length was positively loaded on to this axis, while egg diameter and sperm velocity was negatively loaded. This combination of traits may be favored either at high sperm availabilities when there is no

direct sperm competition for an egg (as slower sperm will be less likely to cause polyspermy, but would lose in a direct competition), or at low gamete availabilities if eggs are rare (egg-limiting) and sperm must survive longer to find them (as sperm swimming speed can trade off with longevity; Bolton and Havenhand 1996, Levitan 2000).

Prior to creating the axes, each collision rate trait (ovum diameter, follicle cell length, and sperm velocity) was transformed into a z-score, and the linear and non-linear selection gradients on the axes were estimated using the R package GSG (Lymbery et al. 2018). To test whether there was significant selection in the directions indicated, a permutation test where fitnesses (as measured by fertilization success) were shuffled randomly and the linear and non-linear selection gradients were calculated 1,000 times for the randomized fitnesses to generate a null distribution, to which the selection gradients estimated from the three axes were then compared against. Tests for selection in the direction indicated by the three axes were performed for each of the five sperm availabilities.

2.3 Results

2.3.1 Interactions Between Collision and Fusion Traits Across Differing Sperm Availabilities

The three-way interaction between collision number and male and female GRPs was highly significant for four out of five sperm availabilities (Table 2.2). It was not significant at the highest sperm availability; 10^8 sperm per mL^{-1} with a 10 min contact time (Table 2.2: $p = 0.078$). In every case where the three-way interaction was significant, it explained the majority of the variation in fertilization success. Interactions between male and female proteins were significant at the three intermediate sperm availabilities (Table 2.2: $p = 0.006$, $p = 0.002$, $p < 0.001$, respectively), while collision number was significant at the same three sperm availabilities (Table 2.2: $p < 0.001$, $p < 0.001$, $p = 0.017$, respectively) and marginally significant at the highest sperm availability (Table 2.2: $p = 0.044$).

Male GRP genotype significantly affected fertilization success at high sperm availabilities, both alone (Table 2.2: $p = 0.002$) and in conjunction with collision number (Table 2.2: $p = 0.001$). In general, sperm that were homozygous for Methionine (MM) tended to have the highest fertilization success, while heterozygous individuals (TM) had intermediate success,

and sperm that were homozygous for Threonine (TT) had the lowest success at the highest sperm availability. At the highest sperm availability (10^8 sperm per mL^{-1} with a 10 min contact time), the least-squared mean fertilization success for sperm that were homozygous for Methionine (MM) was 60.8 $\pm$ 5.4% across all females. Sperm that were homozygous for Threonine (TT) had a lower least-squared mean fertilization at 42.2 $\pm$ 3.0% at that sperm availability, while heterozygous individuals had a least-squared mean fertilization equivalent to TT individual at 42.7 $\pm$ 3.4% across all females. Within a sperm availability, variation in GRPs caused up to a 34% difference in fertilization success between the highest performing male and female GRP combination (Figure 2.2).

The significant interaction between male and female GRPs at the three intermediate sperm availabilities (10^6 sperm per mL^{-1} with a 2 min contact time and 10^4 and 10^6 sperm per mL^{-1} with a 10 min contact time), suggested that the general order of fertilization success between male and female protein pairs changed at lower sperm availabilities. This shift was apparent in females who were homozygous for glutamic acid (EE), where heterozygote sperm had the lowest fertilization success at 10^6 sperm per mL^{-1} with a 2 min contact time, but higher or equal to MM sperm at higher sperm availabilities (Figure 2.2). For heterozygote eggs, heterozygote sperm had the lowest fertilization success at 10^6 sperm per mL^{-1} with a 10 min contact time, with a least-squared mean fertilization rate of 31.8 $\pm$ 5.0 %, compared to a fertilization rate of 54.4 $\pm$ 8.3 % with MM sperm and 43.0 $\pm$ 7.7 % with TT sperm, and continued to be lower or equivalent to TT sperm rather than intermediate at higher sperm availabilities (Figure 2.2).

2.3.2 Changes in Selection on Collision Traits Across Differing Sperm Availabilities

Significant selection occurred in the direction delineated by the three hypothetical axes in four of the five sperm availabilities. Axis 3, indicating that the combination of slower (longer-lived) sperm, smaller ovum diameters, and larger follicle cells was significant at the lowest and second lowest sperm availabilities (10^4 and 10^6 sperm per mL^{-1} with a 2 min contact time), suggesting that these traits are favored (Table 2.3: $p = 0.040$ and $p = 0.004$, respectively). Axis 1, the combination of fast sperm, small ovum diameters, and smaller follicle cells was only significant at the second lowest sperm availability, along with Axis 3 (Table 2.3: $p > 0.001$). Axis 2, the trait combination of fast sperm, small ovum diameters, and large follicle cells was

favored at the intermediate and the second highest sperm availabilities, 10^4 and 10^6 sperm per mL^{-1} with a 10 min contact time (Table 2.3: $p = 0.002$ and $p > 0.001$, respectively). No significant selection in the direction defined by any axis tested was detected at the highest sperm availability (Table 2.3).

There was also evidence for disruptive selection at the intermediate sperm availability (10^4 sperm per mL^{-1} with a 10 min contact time) for axis 3, which had the trait combination of slow sperm, small ovum diameter, and large follicle cells (Table 2.3: $p = 0.002$). Thus, out of the directions examined by the axes, significant selection in a direction defined by a single axis was found at the lowest sperm availability, favoring the combination of slower (longer-lived) sperm, smaller ovum diameters, and larger follicle cells, and in a direction defined by a single axis at the second highest sperm availability, favoring fast sperm, small ovum diameters, and large follicle cells. However, for the second lowest and intermediate sperm availabilities, directions defined by multiple axes were significant, suggesting that multiple different combinations of traits could be favored.

2.4 Discussion

Sperm availability is known to affect the strength and direction of selection on traits that affect collision and compatibility separately (Levitan 1996, 1998, Podolsky 2001, Levitan and Ferrell 2006, Crean and Marshall 2008, Sherman et al. 2015). This is the first time interaction between traits that affect collision and compatibility have been examined across different sperm availabilities. I had hypothesized that collision rates would play a greater role in determining fertilization success at low sperm availabilities, compatibility play a larger role at high sperm availabilities, while at intermediate sperm availabilities interactions between those processes would be most important in determining fertilization success. This hypothesis was formed by the idea that at lower sperm availabilities whether or not a sperm hits an egg would be more important than whether that sperm binds well with it, but as more sperm are available it may allow for greater discrimination among those sperm due to differences in binding affinity (Sherman et al. 2015). My results did not support this hypothesis. The three-way interaction between male and female compatibility and collision number was the most important factor in explaining variation in fertilization success for most sperm availabilities. Thus, it seems that

fertilization success, especially at low sperm densities, is dependent not only upon whether a sperm strikes the egg, but also the ability of the sperm and egg to fuse.

Previous studies on GRPs have found evidence in sea urchins that individuals that have matching sperm ligands tend to experience higher levels of polyspermy but have not directly assessed how interactions between ligand and egg receptor protein variants may affect fertilization success (Levitan and Ferrell, 2006; Levitan 2012). Interactions between the male and female proteins may affect fertilization success, as compatible pairs will reduce fertilization due to polyspermy, while non-compatible pairs will have increased fertilization success in the absence of sperm completion at high sperm availability (Levitan and Ferrell, 2006; Levitan 2012). My results show directly that interactions between different male and female protein pairings can result in a ~34% difference in fertilization success between highest and lowest performing protein pairs at the highest sperm availability.

Rather interestingly, I found that interactions between the male and female proteins were not as important as genotype of the male protein alone at the highest sperm availability, which suggests the presence of a single compatible male ligand. However, the presence of significant male by female interactions at the second lowest, intermediate, and second highest sperm availabilities seems to suggest that there may be a difference in compatibilities between male and female GRP pairings. While there was a significant male by female interaction at moderate sperm availabilities, it was difficult to determine which pairs may be more compatible than others, in part due to the fact that there was no significant male by female interaction at the highest sperm availability.

In no-choice settings, compatible genotype pairings would be expected to have high fertilization at low sperm densities, and low fertilization at high sperm densities due to increasing polyspermy (Levitan and Ferrell, 2006; Levitan 2012). This pattern was not seen in any of the male and female genotype pairings, with the possible exception of when females who were heterozygous at the receptor were crossed with males who were heterozygous at the ligand. When compared to males that where homozygous for Threonine (TT) at the ligand, heterozygous males (TM) had slightly higher fertilization success at the three lowest sperm availabilities but had the lowest fertilization success at the two highest sperm availabilities. This result suggests that sperm from individuals who were heterozygous at the ligand may be more compatible with eggs from individuals who were heterozygous at the receptor. In order to test this hypothesis and

to determine whether the change in ranking for fertilization success observed between heterozygous ligands and heterozygous receptors with increased sperm availability is due to increased compatibility, choice fertilization assays that would directly test compatibility between the different protein pairings must be performed (see Chapter 3).

An alternate explanation is that there is no difference in compatibility between male and female genotype pairings; rather one male variant does well across all females at high sperm concentrations. While this can explain the importance of male GRP identity on fertilization success at the highest sperm availability, it does not explain why there was a difference in the rank order of which male genotype had the highest success based on female genotype at the lower sperm availabilities. In either case, the presence of sperm competition for an egg would help determine whether there was a difference in fertilization success based on compatible male-female GRP variants, or whether there is a single male ligand that is generally more compatible across all female GRP variants, as higher affinity will offer a competitive edge to compatible sperm (Palumbi 1999, Levitan and Ferrell 2006).

Selection on traits that influence collision numbers (ovum diameter, follicle cell length, and sperm velocity) have been studied extensively in many species (Levitan and Irvine 2001, Podolsky 2001, Lymbery et al. 2018), allowing me to make predictions on which combination of traits should be favored at differing sperm availabilities. I found that at the extremes, selection on axes in the directions I had predicted would occur, had occurred. At the lowest sperm availability, significant selection in the direction of slow sperm, large follicle cells, and small eggs was found, matching patterns seen in laboratory studies examining these traits independently. Slower sperm can survive longer, and thus maybe more likely survive long enough to find and fertilize an egg under low gamete density conditions (Levitan 2000, Fitzpatrick et al. 2012, Johnson et al. 2013). Large accessory structures, like follicle cells, can offer a relatively lost-cost way to increase collision rate at low densities by increasing the effective target size of the egg (Levitan and Irvine 2001, Podolsky 2001, Lambert 2009).

At the second highest sperm availability, significant selection in the direction of fast sperm, large follicle cells, and small eggs was found. This result supports the hypothesis that accessory structures, like follicle cells and jelly coats, may increase fertilization success at high sperm concentrations by slowing sperm movement and decreasing the probability of polyspermy (Farley and Levitan 2001, Crean and Marshall 2015). That fast sperm was also favored suggests

that even though sperm availability was high, it was not high enough to lead to slower sperm being favored, as would be expected if a significant amount of polyspermy was occurring in the absence of sperm competition (Fitzpatrick et al. 2012). It may be that in this species the probability of polyspermy causing an appreciable drop in fertilization success is very low save at extreme sperm concentrations (Figure A1), as the result of an evolutionary history favoring polyspermy-reducing traits like increased follicle cell size (Levitan et al. 2007). However, it should also be noted that polyspermy can occur even in spawning situations where eggs are sperm-limited (i.e., no drop in the overall fertilization success may be observed), so the probability of polyspermy occurring and its possible effect on the evolution of gamete traits should not be discounted even if a drop in fertilization success is not observed (Franke et al. 2002). In order to know for certain how much polyspermy occurred, a measurement of the number of eggs fertilized by multiple sperm would have had to have been made.

Interestingly, at moderate sperm availabilities significant selection in the direction of more than one combination of traits was found. This confirms my hypothesis that multiple combinations may yield equivalent fertilization success at sperm saturating conditions. If multiple trait combinations are favored, I hypothesize that increases in temporary associations between traits that increase fertilization success would be more likely to occur at these sperm saturating conditions via assortative mating. This can have important consequences on the evolution of gamete traits, as it can result in the maintenance of variation within traits.

In summary, by incorporating multiple traits and examining them over a range of sperm availabilities, I was able examine the shifting dynamics between the interactions of these traits. My results suggest that interactions between fusion and collision rates are important in mediating fertilization success in over a wide range of sperm availabilities. Additionally, there are many different combinations of traits that can be important in affecting fertilization success, which may result in complex interactions between differing selection pressures on any one trait based on other gamete trait values, particularly as sperm availability increases (Levitan 2006, Tomaiuolo and Levitan 2010, Evans and Sherman 2013). While more work remains to be done to determine the mechanisms behind how exactly variation in male and female proteins can affect compatibility, my results show that these interactions between male and female proteins, as well as collision rate traits, are important in determining fertilization success across a broad range of sperm environments.

Table 2.1: Hypotheses for how gamete traits from differing processes should change based on selection pressures due to sperm availability and the presence of sperm competition. These hypotheses were generated based on studies that examined each of these traits individually (see text for study citations).

Process	Trait	Low Sperm Availability	High Sperm Availability
Collision Rate	Ovum Diameter	Increase size for increased collisions	Decrease size for decreased collisions
	Accessory Structure (jelly coat, follicle cell)	Increase size for increased collisions	Decrease size for decreased collisions Increase, if structure slows sperm and prevents polyspermy
	Sperm Velocity	Decrease speed for increased longevity to increase probability of surviving long enough to fertilize a sperm.	Decrease speed for decreased collisions in absence of sperm competition. Increase speed for increased collisions, if sperm from multiple males are directly competing to fertilize an egg
Fusion Rate	Receptor (female) Protein	Increase compatibility to increase fusion rate	Decrease compatibility to reduce fusion rate
	Ligand (male) Protein	Increase compatibility to increase fusion rate	Decrease compatibility to reduce fusion rate in absence of sperm competition. Increase compatibility to increase fusion rate with female protein in presence of sperm competition

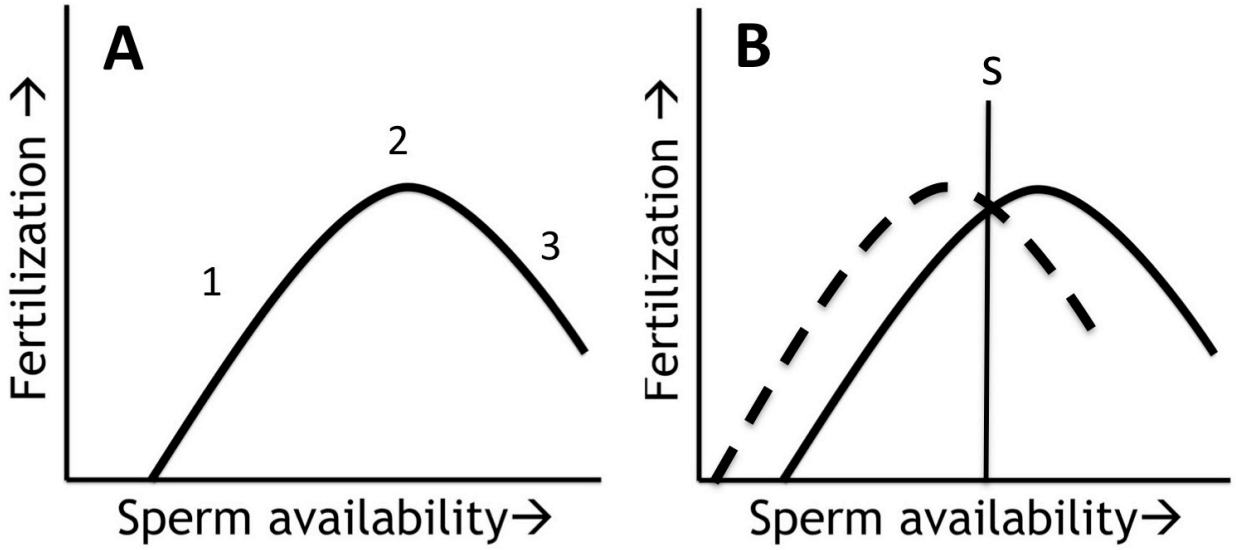

Figure 2.1: Hypothetical fertilization curves based on sperm availabilities. A: At low sperm availabilities (1), fertilization may be reduced due to a lack of sperm-egg encounters, so traits that influence collision rates may have a greater effect increasing fertilization success than those that influence fusion rates. At some sperm availability (2), fertilization is maximized, but beyond that (3) fertilization may decrease due to an over-abundance of sperm-egg encounters leading to egg death through polyspermy. In this case, traits that influence collision rates may not be as important as those that influence fusion rates in increasing fertilization success. B: At intermediate sperm availabilities, which traits are favored may depend upon interactions with other traits. For example, traits that increase collision rates (like egg size) decrease the amount of sperm needed to fertilize the egg shifting the hypothetical fertilization curve to the left (dashed curve). At intermediate sperm availability (s), fast sperm, which increases collision rates, may not be favored in combination with large eggs, but would be with small eggs in the absence of sperm competition.

Table 2.2: Results for the generalized linear effects model used to examine interactions between collision rate traits and fusion rate traits (gamete recognition proteins) at the five different sperm contact times and concentrations. Male and female identities were included in the full model as random factors. Shown here are the fixed effects and interactions presented in a type II ANOVA table using a Wald Chi-square test of significance.

Factor	DF	Chi-Sq	*P*
10^4 mL^{-1} Sperm Concentration 2 min Sperm Contact Time			
Collision Number	1	2.928	0.087
Female GRP genotype	2	2.470	0.291
Male GRP genotype	2	2.185	0.335
Collision: Female GRP	2	0.366	0.833
Collision: Male GRP	**2**	**10.505**	**0.005**
Female GRP: Male GRP	4	5.620	0.229
Collision: Female GRP: Male GRP	**4**	**25.238**	**< 0.001**
10^6 mL^{-1} Sperm Concentration 2 min Sperm Contact Time			
Collision Number	**1**	**26.226**	**< 0.001**
Female GRP genotype	2	1.190	0.552
Male GRP genotype	2	3.220	0.200
Collision: Female GRP	**2**	**15.941**	**< 0.001**
Collision: Male GRP	**2**	**31.105**	**< 0.001**
Female GRP: Male GRP	**4**	**14.403**	**0.006**
Collision: Female GRP: Male GRP	**4**	**60.747**	**< 0.001**

Table 2.2: Continued

Factor	DF	Chi-Sq	*P*
10^4 mL^{-1} Sperm Concentration 10 min Sperm Contact Time			
Collision Number	**1**	**20.499**	**< 0.001**
Female GRP genotype	2	1.156	0.561
Male GRP genotype	2	2.333	0.311
Collision: Female GRP	**2**	**7.268**	**0.026**
Collision: Male GRP	2	1.001	0.606
Female GRP: Male GRP	**4**	**17.470**	**0.002**
Collision: Female GRP: Male GRP	**4**	**23.078**	**< 0.001**
10^6 mL^{-1} Sperm Concentration 10 min Sperm Contact Time			
Collision Number	**1**	**5.684**	**0.017**
Female GRP genotype	2	0.174	0.917
Male GRP genotype	2	1.551	0.461
Collision: Female GRP	2	1.712	0.425
Collision: Male GRP	2	3.321	0.190
Female GRP: Male GRP	**4**	**34.209**	**< 0.001**
Collision: Female GRP: Male GRP	**4**	**37.513**	**< 0.001**
10^8 mL^{-1} Sperm Concentration 10 min Sperm Contact Time			
Collision Number	**1**	**4.050**	**0.044**
Female GRP genotype	2	0.664	0.718
Male GRP genotype	**2**	**12.186**	**0.002**
Collision: Female GRP	2	0.866	0.648
Collision: Male GRP	**2**	**13.798**	**0.001**
Female GRP: Male GRP	4	6.899	0.141
Collision: Female GRP: Male GRP	4	8.411	0.078

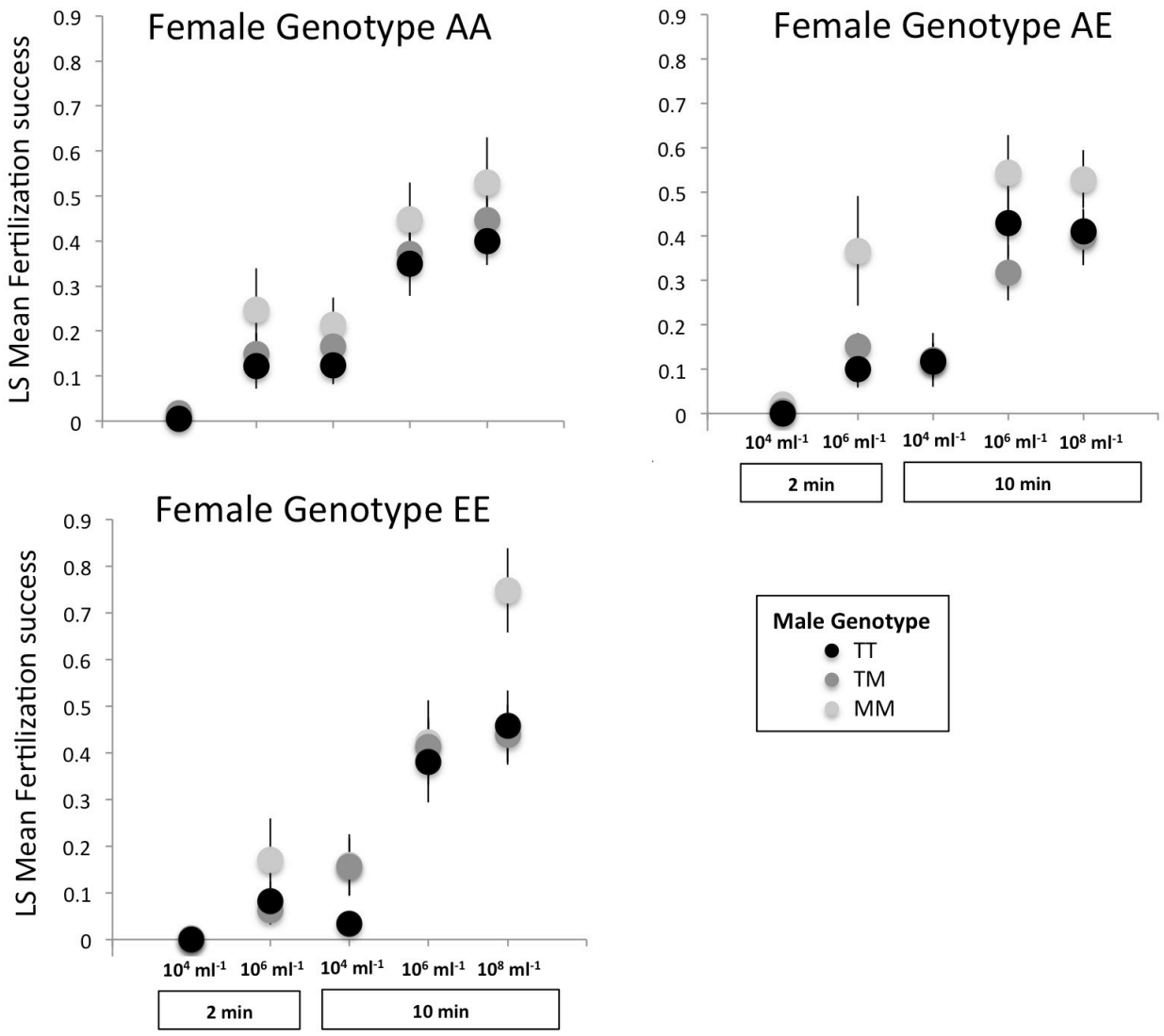

Figure 2.2: Least-squared mean fertilization success for each of the nine male by female genotype combinations across the five different sperm contact time and concentration combinations. The three male genotypes (TT, black; TM, grey; MM, light grey) are shown as a function of the three female genotypes, homozygous for alanine (AA, top left), homozygous for glutamic acid (EE, bottom left), and heterozygous (AE, top right).

Table 2.3: Estimates and standard errors of linear (Θ) and non-linear (λ) selection gradients for the three hypothetical trait axes. Axis 1 represented fast sperm swimming speeds combined with small eggs and small follicle cells (velocity positively loaded, egg diameter and follicle cell length negatively loaded). Axis 2 represented fast sperm swimming speeds combined with small eggs and large follicle cells (velocity and follicle cell length positively loaded, egg diameter negatively loaded). Axis 3 represented slow sperm swimming speeds combined with small eggs and large follicle cells (follicle cell length positively loaded, egg diameter and velocity negatively loaded).

Axis	Θ	*P*	λ	*P*
10^4 mL^{-1} Sperm Concentration	*2 min Sperm Contact Time*			
Axis 1	0.273	0.140	-0.040	0.454
Axis 2	0.366	0.760	-0.482	0.164
Axis 3	**0.597**	**0.040**	0.810	0.806
10^6 mL^{-1} Sperm Concentration	*2 min Sperm Contact Time*			
Axis 1	**0.397**	**< 0.001**	-0.101	0.150
Axis 2	0.128	0.108	-0.188	0.318
Axis 3	**0.203**	**0.004**	0.619	0.132
10^4 mL^{-1} Sperm Concentration	*10 min Sperm Contact Time*			
Axis 1	0.111	0.524	-0.181	0.514
Axis 2	**0.366**	**0.002**	-0.372	0.066
Axis 3	0.034	0.384	**0.016**	**0.002**
10^6 mL^{-1} Sperm Concentration	*10 min Sperm Contact Time*			
Axis 1	0.075	0.402	-0.031	0.498
Axis 2	**0.265**	**< 0.001**	-0.175	0.380
Axis 3	0.024	0.202	0.098	0.054
10^8 mL^{-1} Sperm Concentration	*10 min Sperm Contact Time*			
Axis 1	-0.150	0.148	0.013	0.110
Axis 2	-0.148	0.082	-0.145	0.600
Axis 3	0.016	0.748	0.132	0.176

CHAPTER 3

THE EFFECTS OF VARIATION IN GAMETE RECOGNITION PROTEINS ON COMPATIBILITY

3.1 Introduction

Gamete recognition proteins (GRPs) are involved in the recognition and binding of gametes and are expressed on the outer surfaces of eggs and sperm (Vacquier and Moy 1977, Yamaguchi et al. 2011, Vicens et al. 2014). GRPs are known to be some of the most rapidly evolving proteins in many species (Metz et al. 1998, Palumbi 1999, Levitan and Ferrell 2006, Lessios and Zigler 2012), with some estimates finding a four-fold increase in the evolutionary rate, as measured by dN/dS, when compared to other genes involved in the fertilization process (Swanson and Vacquier 2002, Vicens et al. 2014). Mutations in these proteins can have relatively large impacts on compatibility between gametes. For instance, as few as 10 amino acid substitutions led to complete gametic incompatibility among sea urchin species (Zigler et al. 2005).

Variation in GRPs among species may be selected for via post-zygotic isolation in some species, as levels of protein divergence can predict hybridization success better than neutral genetic markers (Swanson and Vacquier 2002, Zigler et al. 2005). However, selection for post-zygotic isolation does not explain why there is often a high amount of variation maintained within a species, particularly as such variation can negatively affect individual reproductive success (Palumbi 1999, Levitan and Ferrell 2006). Although there are several possible explanations for why high intraspecific variation in GRPs exists (Vacquier and Swanson 2011), the most likely explanation is that decreased compatibility may be favored in environments where polyspermy (egg death by multiple insemination) occurs (Gavrilets 2000, Tomaiuolo and Levitan 2010, Levitan 2012). Evidence from laboratory studies have found that less compatible proteins need more collisions for successful fertilization to occur; but have higher fertilization success in high sperm environments because they are more resistant to polyspermy (Levitan and Ferrell 2006, Levitan 2012). Additionally, interspecific comparisons suggest that species that spawn under conditions that are prone to polyspermy, i.e. that aggregate in high densities, live in

intertidal areas, or lack electrical blocks to polyspermy, tend to maintain high levels of genetic variation in their GRPs (Moy et al. 2008, Hellberg et al. 2012, Sunday and Hart 2013).

Thus, the sperm environment can play an important role in the maintenance of variation in and the evolution of GRPs. Models have shown that a less-compatible egg receptor can invade and be maintained within a population based mainly on sperm availability, with less compatible receptors being favored at high sperm concentrations as a way of reducing polyspermy (Gavrilets 2000, Haygood 2004, Tomaiuolo and Levitan 2010). However, the successful invasion and maintenance of a novel sperm ligand tends to depend on multiple factors. In cases where there is no sperm competition between males for an egg, a novel ligand may invade as easily as a novel protein receptor, because the cost of polyspermy on fertilization success is equal for both males and females (Tomaiuolo and Levitan 2010). This would result in either a ligand or receptor protein that performs well with all individuals at high sperm availabilities, but poorly with all individuals at low sperm availabilities, but would be out-competed by the more compatible protein if sperm competition occurred. With strong sperm competition, a novel ligand may only invade if there is a mutant receptor protein that is more compatible with it already established within the population, resulting in compatible pairs of receptor-ligand proteins being maintained (Haygood 2004, Tomaiuolo and Levitan 2010). This would result in context-specific differences in fertilization success between individuals based on their respective GRP genotype affinities. Thus, patterns in affinity may offer some insight into the sperm environment that may have shaped GRP evolution.

Direct examination of how genetic variation may affect relative affinities between different receptor and ligand protein pairs has been hampered by the difficulty in sequencing the relatively large and complex receptor proteins in the species examined to date. However, evidence of genetic linkage disequilibrium between these ligand and receptor proteins has been found, suggesting that differences in affinities between different ligand and receptor pairs might be expected in those species (Clark et al. 2009, Hellberg et al. 2012, Sunday and Hart 2013, Stapper et al. 2015). Additionally, field and laboratory studies examining how genetic variation may affect fertilization success have found that the ligand genotype which is unexpressed in females can be used to predict fertilization success with males, with male-female pairs that have matching ligand genotypes having higher fertilization success at both low sperm concentrations and in the presence of sperm competition (Palumbi 1999, Levitan and Ferrell 2006, Levitan and

Stapper 2010). That female fertilization success can be predicted based on the degree of similarity between their unexpressed ligand genotype and their partner's expressed genotype, indirectly supports the idea that linkage disequilibrium between different receptor-ligand gene variants may be due to assortative mating between higher affinity receptor-ligand protein pairings (Palumbi 1999, Levitan and Ferrell 2006, Levitan and Stapper 2010).

I aim to directly examine affinities between genetically variable male and female GRPs in the hermaphroditic tunicate *Ciona robusta.* This will be accomplished by determining if there is a difference in the relative number of larvae sired by an individual in the presence of a competitor, based on the genotypes of the egg receptor and the sperm ligands of the individual whose eggs were used, the focal individual whose sperm was used and their competitor. Both CiUrabin, which encodes the sperm ligand, and CIVC57, which encodes the egg receptor protein that is the binding partner for CiUrabin, are known to have high levels of genetic variation (Yamada et al. 2009, Yamaguchi et al. 2011). Previous work has identified a single non-synonymous polymorphism in both the male and female genes that can affect fertilization success in conjunction with collision rate, but the affinities of these GRP variants were not elucidated at that time (Chapter 2). Patterns of siring success may also provide insight into the evolutionary history of the proteins. If one male GRP genotype dominates paternity regardless of female GRP genotype, this would indicate that the alternate GRP ligand is less compatible and may be maintained within the population due to the presence of polyspermy alone. If paternity is dependent upon interactions between male and female GRP genotypes, this would suggest that GRP evolution may be shaped by both polyspermy and sperm competition.

3.2 Methods

3.2.1 Gamete Collection and Egg Choice Assays

Gametes were obtained from adult *C. robusta* collected from Quivera Basin in San Diego, CA from summer of 2015 through winter of 2017. For each individual, eggs were removed from the gonoduct, rinsed, and egg concentration per mL was estimated by averaging the number of eggs from three 25 μL subsamples of the egg stock. Sperm were pipetted directly from the gonoduct and stock concentration was estimated using a hemocytometer prior to

experimentation. For the duration of the experiment, both eggs and sperm were kept cool on ice or in a cold room at 19°C.

Eggs from an individual were diluted to a concentration of 800 eggs per mL and exposed to an equal mixture of sperm from two individuals for ten minutes, prior to rinsing the sperm away with fresh seawater. The sperm mixture was created from equal mixture of sperm from two individuals at a total sperm concentration of 10^8 sperm per mL and was mixed prior to addition to the eggs. The same amount of sperm from each male used in the mixture was also pipetted and retained in separate vial for each male. The sperm concentration was estimated from this vial using a hemocytometer and recorded, to ensure that sperm numbers were equal truly between males and that any differences in sperm number among males due to errors from the initial sperm concentration estimation or subsequent pipetting errors that occurred could be included in the data analysis. The eggs from two different individuals were exposed to each sperm mixture to increase the probability of a relatively rare male genotype pairing being tested across multiple female genotypes, and only the crosses where the males used in the mixture had differing genotypes were retained for data analysis (47 crosses total). Eggs were allowed to develop for ~18 hours into tadpole larvae, before they were collected from each cross and stored in 95% ethanol for parentage analysis.

3.2.2 Sequencing Gamete Recognition Proteins

For each cross, 10-12 larvae were selected and DNA extracted using a Tween and proteinase K cocktail. Larvae were then sequenced for their male GRP genotype using primers designed to amplify a 594 bp region of CiUrabin (CiUrabinCF: 5'-GTAGTTCCATCTGCGAGTAACA-3' and CiUrabinFR: 5'-ACATAAGTGCGGAGAGTGTAAT-3'). This region had 18 single nucleotide polymorphisms, and was used to assign parentage because of its variability.

Tissue collected from the siphon for each adult was digested in CTAB and proteinase K in a 64°C hot water bath for 12-14 hours. DNA was purified using magnetic beads, and stored at -20°C. All individuals used were sequenced for both the male GRP genotype and their female GRP genotype at exon 7 of CiVC57. The primers used were designed to target a 372 bp region of the receptor gene that contained the SNP known to affect fertilization success from previous experiments (Chapter 2; CiVC57Exon7F: 5' –TTCTAGGCATGCCCTGGTGATTCT-3' and

CiVC57Exon7R: 5'-CCATAGTGTGAACCCGCCTTTACT-3'). This SNP was recorded as the GRP genotype for the female locus for use in later data analysis. All adults in the cross as well as the larvae had all variable regions of their male GRP genotypes recorded for paternity assignment, but only the SNP known to affect fertilization success was used as the adult's male GRP genotype for data analysis.

3.2.3 Data Analysis

Paternity assignments were made using the program Cervus (Kalinowski et al. 2007), and the percentage of larvae sired per individual out of the total number of larvae sequenced was recorded for each male. A binomial generalized linear model was fitted for each of the three male genotypes, to determine if the percentage of larvae sired by an individual was affected by either the GRP genotype of the individual whose eggs were used, or the GRP genotype of the competitor whose sperm was used in the mixture, or an interaction between the two. A separate model was fitted for each male GRP genotype to account for the fact that each ligand genotype could occur in only two of the three possible ligand GRP pairings (i.e. TT males only appear in two of the three possible competitive ligand pairs: TT vs TM and TT vs MM, but not TM vs MM). The percent sired by an individual was treated as a binomial function, with the percentage of larvae being sired by that male or not. For each model, male and female identity was included as a random effect, to account for the fact that each sperm mixture was used on eggs from two different individuals, and the ratio of the two males' sperm concentrations used in the mixture was included as a covariate.

As previous studies have only examined whether more larvae were sired by males based on whether the eggs and sperm share the same ligand genotype, I also examined whether having the same GRP genotype influenced paternity, but for both the ligand and receptor genotypes. Since this examined whether individuals had matching genotypes at the receptor or ligand gene, rather than actual genotype of those proteins, a single binomial generalized linear model could be fitted to the entire dataset. However, to account for the fact that both males from a sperm mixture were included in the same analysis, male identity was nested inside cross identity as a random effect. Female identity was also included as a random effect.

3.3 Results

There was no significant effect of female receptor GRP genotype on paternity for any of the three male GRP genotypes (Table 3.1), nor was there an effect of the challenger individual's ligand genotype on the focal male's percent paternity (Table 3.1). Although non-significant, males who were homozygous for Threonine (TT) tended to sire a higher percentage of larvae (72.2 $\pm$ 18.6%) when the challenger male genotype was homozygous for Methionine (MM) across all females. When the challenger male's genotype was heterozygous, only 42.0 $\pm$ 20.3% of the larvae were sired by TT males across all females. When exposed to females that were heterozygous at the receptor locus, heterozygous males tended to sire more of the larvae (68.6 $\pm$ 17.1%) when the challenger male was homozygous for Threonine (TT), than when the challenger was homozygous for Methionine (MM: 44.9 $\pm$ 22.0%, Figure 3.1), suggesting that heterozygous males may out-compete TT males when eggs came from an individual who was heterozygous at the receptor locus.

There was no significant effect of genotype matching at either the ligand or receptor GRP genotype on patterns of paternity (Table 3.1: $p = 0.745$ and $p = 0.311$, respectively), nor was there an interaction between the two (Table 3.1: $p = 0.489$). Sperm from males that had the same receptor genotype as the female whose eggs they were exposed to but had a different ligand genotype than the female sired the most larvae at 64.8 $\pm$ 13.2% (Figure 3.2). Sperm exposed to eggs from an individual that had a non-matching genotype at the receptor, but a matching one at the ligand sired 47.4 $\pm$ 17.0% of the larvae in their crosses (Figure 3.2). Finally, sperm from males that matched the GRP genotypes of the female whose eggs they were exposed to at both the ligand and receptor locus sired 46.3 $\pm$ 20.4% of the larvae in their crosses, while sperm that did not match the female whose eggs they were exposed to at either the ligand or receptor locus sired 44.3 $\pm$ 9.0% of the larvae in their crosses (Figure 3.2).

3.4 Discussion

Under conditions of direct sperm competition, I found no clear evidence for higher or lower compatibility pairings between different male and female gamete recognition proteins. Models suggest the evolution of high compatibility receptor and ligand pairings can occur when

polyspermy affects fertilization success and direct sperm competition for an egg occurs (Gavrilets and Waxman 2002, Tomaiuolo and Levitan 2010). In the absence of sperm competition, a mutant ligand can invade when polyspermy is prevalent (Tomaiuolo and Levitan 2010), but this would result in one 'higher' affinity ligand and one 'lower' affinity ligand, and the higher affinity ligand would have a higher paternity share in the presence of sperm competition (regardless of genetic variation in the receptor) a pattern that was also not detected in this study. This suggests that either the difference in binding affinities between variants was too slight to be detected in this study, or that the variation in these proteins is maintained by some other mechanism than the polyspermy-driven, frequency-dependent selection found in other species (Moy et al. 2008, Levitan and Stapper 2010).

Given that theory predicts a novel receptor protein will be more likely to invade when differences in binding affinities between the novel and resident receptor proteins are small (Gavrilets and Waxman 2002,Tomaiuolo and Levitan 2010), it is possible that this study did not have the sample size necessary to detect the differences in affinities between receptor variants, particularly as some genotype combinations were very rare (n=3 for the rarest combination). Although not significant, there were some trends that suggested that differences in binding affinities between protein variants might be revealed with larger sample sizes. For instance, individuals homozygous at the sperm ligand locus for Threonine (TT) tended to sire almost ~2.5 more larvae in their crosses when they were in competition with individuals homozygous at the sperm ligand locus for Methionine (MM: 72.2 $\pm$ 16.6%), across all female receptor genotypes. This suggests that individuals homozygous at the sperm ligand locus for Threonine (TT) are better at siring larvae than individuals homozygous at the sperm ligand locus for Methionine (MM). Yet, this was opposite to the pattern seen in the no-choice assays, where individuals homozygous at the sperm ligand locus for Methionine (MM) had significantly higher fertilization success (~60%) than either of the other male ligand genotypes (~42% for each) across all female receptor genotypes at high sperm availabilities (Chapter 2). Compatible proteins are more prone to polyspermy at high sperm availabilities, but out-compete less compatible proteins when in direct competition for an egg, leading to a pattern of low fertilization success in conditions of high sperm concentrations and no sperm competition and high fertilization success in high sperm concentrations and when sperm competition occurs (Levitan 2012). Thus reversal in the order of fertilization/parentage success between choice and no-choice assays is predicted to occur if

differences in compatibility between proteins exist and polyspermy is occurring (Levitan 2012), suggesting that TT individuals may possess a more 'compatible' ligand than MM individuals.

In general, individuals heterozygous at the ligand did not appear to have such a clear advantage with any receptors, although they tended to sire more offspring (68.6 $\pm$ 17.1%) with individuals heterozygous at the egg receptor locus when in competition with TT males. Again, this pattern was reversed in the no-choice assays, where sperm from males that were heterozygous at the sperm ligand locus had the lowest fertilization success out of the three male genotypes when exposed to eggs from females that were heterozygous at the egg receptor locus at high sperm availabilities (Chapter 2: Figure 2.2). If a greater sample size confirms these trends, it would suggest that individuals that are homozygous at the sperm ligand locus for Threonine (TT) produce sperm that are more compatible with most egg receptors, with the exception of eggs produced by individuals heterozygous at the egg receptor locus. It may also suggest that individuals that are heterozygote at the ligand may have equivalent binding affinities as sperm produced by either TT or MM individuals with most receptor types, but higher binding affinities with individuals that are heterozygous at the receptor locus.

In sea urchins, the unexpressed male ligand can predict female fertilization success, and that individuals whose ligand genotypes match generally have higher compatibility and are more prone to polyspermy (Palumbi 1999, Levitan and Stapper 2010). I did not find any evidence for a competitive advantage based on genotype matching in this species. There was a non-significant trend that sperm sired a higher percentage (64.8 $\pm$ 13.1%) of the larvae, when they had the same genotype as the individual whose eggs were used at the receptor locus and a different genotype at the ligand locus. This is interesting because it suggests that contrary to what was found in sea urchins, matching at the male locus does not affect paternity in this species (Palumbi 1999, Levitan and Stapper 2010). Instead, my results suggest that matching at the female locus might increase the number of larvae an individual may sire. If increased sample size shows that sperm that match eggs at the female locus have a slight competitive advantage, I would hypothesize that in wild populations an excess of offspring with homozygote receptors would be produced, particularly as the level of sperm competition increases and eggs can exert more choice (Sherman et al. 2015, Levitan 2018).

Ultimately, the power to distinguish between differences in binding abilities in this experiment was low, so I was unable to confirm if there was a true difference in binding

affinities between different receptor-ligand parings. However, there are some hints suggesting differences in binding abilities may exist. The change in rank order of mating success between choice and no-choice assays follows patterns predicted if differences in compatibility between GRP ligand variants did exist. Additionally, there are some hints that matching between expressed and unexpressed receptor genotypes may play a role in determining affinities. Thus, further investigation to see whether the patterns suggested by the data are actually occurring is warranted.

Table 3.1: Table showing the results of the three generalized linear models fitted to each of the three male GRP genotypes, to determine if patterns in paternity could be attributed to the GRP genotype of the challenger sperm, egg, or the interaction between the two. Male and female identities were included in the full model as random factors. Shown here are the fixed effects and interactions presented in a type II ANOVA table using a Wald Chi-square test of significance.

TT Males

Factor	DF	$\chi 2$	*P*
Challenger Male GRP Genotype	1	0.988	0.32015
Female GRP Genotype	2	3.693	0.15777
Sperm Ratio	**1**	**5.912**	**0.01504**
Challenger Male: Female GRP Genotype	2	0.306	0.85797

TM Males

Factor	DF	$\chi 2$	*P*
Challenger Male GRP Genotype	1	0.112	0.738
Female GRP Genotype	2	0.334	0.846
Sperm Ratio	**1**	**7.174**	**0.007**
Challenger Male: Female GRP Genotype	2	1.276	0.528

MM Males

Factor	DF	$\chi 2$	*P*
Challenger Male GRP Genotype	1	0.056	0.812
Female GRP Genotype	2	3.179	0.204
Sperm Ratio	1	0.008	0.930
Challenger Male: Female GRP Genotype	2	0.290	0.865

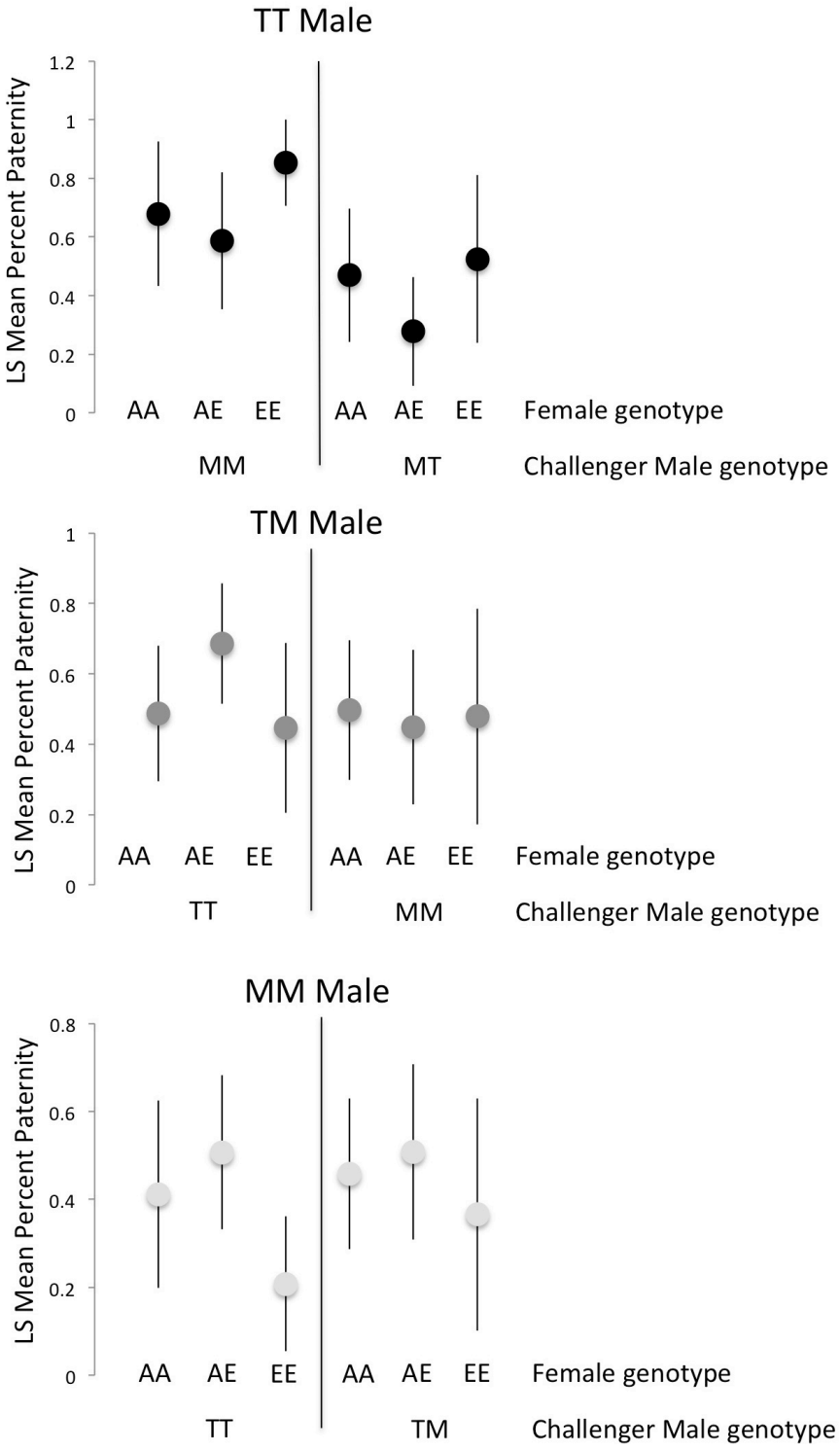

Figure 3.1: Least-squared mean percent paternity for each of the three male genotypes based on the other sperm's genotype in the two male mixture (challenger male genotype) and the genotype of the egg's receptor protein (female genotype). Error bars are $\pm$ SE.

Table 3.2: Table showing the results of a generalized linear model to determine if patterns in paternity could be attributed to the egg and sperm sharing the same alleles at either the male locus or the female locus. Individuals were considered to match at a locus if the genotypes were the same. Female identity and male identity nested inside cross were included in the full model as random factors. Shown here are the fixed effects and interactions presented in a type II ANOVA table using a Wald Chi-square test of significance.

Factor	DF	$\chi 2$	*P*
Female GRP Genotypes Match	1	1.028	0.311
Male GRP Genotypes Match	1	0.106	0.745
Sperm Ratio	**1**	**14.178**	**< 0.001**
Female Genotypes Match: Male Genotypes Match	1	0.480	0.489

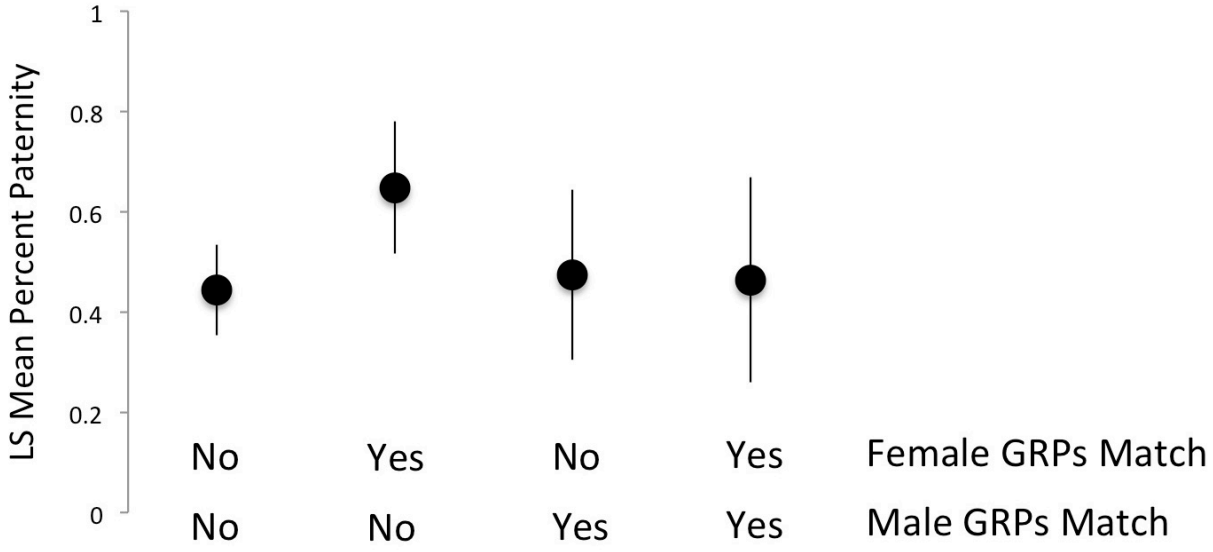

Figure 3.2: Least-squared mean percent paternity for males based on whether the male's unexpressed receptor genotype matched the female's receptor genotype (female GRP match), and if the male's ligand genotype matched the female's unexpressed ligand genotype (male GRP match).

CHAPTER 4

EXAMINING LINKS BETWEEN GAMETE RECOGNITION PROTEIN VARIATION AND CHEMOATTRACTION-BASED SPERM BEHAVIOR

4.1 Introduction

Egg chemoattractants have long been known to help increase fertilization success by changing sperm swimming behavior and increase the effective target size of an egg via their diffusive properties (Miller 1982, Bolton and Havenhand 1996, Jantzen et al. 2001, Riffell et al. 2004, Kaupp et al. 2008). In addition to increasing the target size of an egg, chemoattractants can provide information about the egg's identity to the sperm prior to sperm-egg binding; which although reversible, may damage the sperm and prevent it from binding to a more suitable partner (Lambert 1986, Yamaguchi et al. 2011). Differences in chemoattractants between species can allow for sperm to differentiate between conspecific and heterospecific eggs and preventing hybridization (Riffell et al. 2004, Yeates et al. 2013, Yoshida et al. 2013). Within a species, studies have found that sperm can use chemoattractants to differentiate between eggs in order to prevent selfing in a species that exhibits high inbreeding depression (Kawamura et al. 1987, Murabe and Hoshi 2002, Kosman et al. 2017). Because of their potential ability to offer chemical cues to allow sperm to differentiate between eggs in a population, it has been hypothesized that chemoattractants may be used to moderate sperm competition by promoting fusion with more compatible eggs within a population (Evans et al. 2012, Lymbery et al. 2017).

Only a few chemoattractants have been positively identified and in some species separate sperm-activating factors that either increase sperm's ability to orient or their motility and velocity have been identified (Ward et al. 1985, Yoshida et al. 2002, Kaupp et al. 2008, Yoshida et al. 2013), suggesting that a mixture of chemicals may be involved in chemotaxis and chemokinesis. Additionally, individual eggs may differ in the chemical composition of the attractants that they produce, which can affect the ability of sperm to detect the eggs (Hussain et al. 2017). The variance in chemical make-up of chemoattractants between individual eggs and variance in sperm responses to those eggs can be the basis for sexual selection. Sperm may have a competitive edge if they are more 'responsive' to eggs that they are more compatible with

(Lambert 1982, Evans et al. 2012, Evans and Sherman 2013). This could result in a functional link between chemotactic behaviors and compatibility.

Studies have shown that when given the choice, sperm will swim towards eggs that they fertilize in greater numbers (Evans et al. 2012, Oliver and Evans 2014, Hussain et al. 2016, Lymbery et al. 2017). However, in many cases it is difficult to distinguish whether sperm fertilize the eggs in greater numbers because they are more compatible or because they respond better to the eggs' chemoattractants. The goal of this study is to examine how variation in compatibility may affect chemoattractant-induced sperm behaviors, by comparing differences in sperm kinesis and orientation based on the genotypes of the gamete recognition proteins (GRPs) in the tunicate *Ciona robusta*. Linking genetic differences in GRPs to differences in sperms' reactions to egg chemoattractants would provide direct evidence for a functional link between chemoattractant-induced sperm behaviors and compatibility, as genetic variance in GRPs are known to affect compatibility in several species (Levitan and Ferrell 2006, Moy and Vacquier 2008).

In *C. robusta*, chemoattractants play a large role in the activation and motility of sperm, as they are virtually non-motile in the absence of these chemicals (Miller 1982, Yoshida et al. 1993, Bolton and Havenhand 1996). The eggs release a sperm-activating and attracting sulfate steroid from the vegetal pole, which can influence both sperm directionality and swimming speed (Yoshida et al. 1993, 2002). Additionally, sperm swimming behavior is known to be negatively affected by increasing genetic-relatedness between the sperm and egg (Kawamura et al. 1987, Saito et al. 2012, Kosman et al. 2017), suggesting that sperm of this species already utilize chemoattractants to avoid 'non-compatible' eggs within a population. The GRP genes affecting compatibility have been identified in this species. Both the male GRP gene (CiUrabin) and its binding partner, the receptor protein gene CiVC57, are known to be highly variable (Yamada et al. 2009, Yamaguchi et al. 2011). Previous research has also suggested this variation can affect fertilization success (Chapter 2). Thus, *C. robusta* is an ideal candidate to investigate whether differences in sperm performance as mediated by chemoattractants can be linked with genetic variation in GRP, which mediate compatibility between egg and sperm (Palumbi 1999, Levitan and Ferrell 2006).

4.2 Methods

4.2.1 Gamete Collection and DNA Extraction

Gametes were obtained from adult individuals of *C. robusta* collected from Quivera Basin in San Diego, CA. Individuals were collected from winter of 2016 through spring of 2017. Eggs were removed from the oviduct and rinsed with fresh seawater using a 60 um mesh as a precaution to remove any possible allosperm from the eggs. The egg concentration per mL was estimated using the average egg count of three 25 µL sub-samples from the stock egg solution. Sperm were pipetted directly from the spermiduct and kept undiluted until use. Sperm concentration of the stock sperm was estimated using a hemocytometer. For the duration of the experiment, sperm and eggs were kept cool on ice, or in a temperature controlled room with an ambient temperature of 19°C. Tissue from the siphon of each individual was collected and preserved in 95% ethanol for DNA extraction.

For DNA extraction, preserved tissue was digested in CTAB and proteinase K in a 64°C hot water bath for 12-14 hours. DNA was purified using magnetic beads. For each individual, the male ligand and female receptor GRP genes were sequenced. For CiVC57 (the receptor gene), a 372 bp region was amplified to target the non-synonymous mutation of interest located shown to affect fertilization success (CiVC57Exon7F: 5' – TTCTAGGCATGCCCTGGTGATTCT-3' and CiVC57Exon7R: 5'-CCATAGTGTGAACCCGCCTTTACT-3'). For CiUrabin (the ligand gene), a 594 bp region was amplified using primers developed to target the non-synonymous mutation of interest known to affect fertilization success (CiUrabinCF: 5'-GTAGTTCCATCTGCGAGTAACA-3' and CiUrabinFR: 5'-ACATAAGTGCGGAGAGTGTAAT-3'). The SNP of interest for the male and female genes was recorded for each individual as the male and female GRP genotype for use in data analysis.

4.2.2 Chemoattractant-Mediated Sperm Choice

To determine whether chemoattractant-mediated sperm choice is influenced by variation in GRPs, sperm choice was assessed using a dichotomous chamber consisting of two wells connected by a shallow chamber made from thick plexiglass blocks (Figure 4.1). The entire chamber held about 4 mL of seawater and the two wells were 3 cm deep and 1cm in diameter,

separated by a 2.5 cm long depression that was 0.5 cm deep. Eggs from different individuals were added in each well at a concentration of 300 eggs per mL and were allowed to sit in the chamber for 60 minutes prior to sperm addition to create a chemoattractant gradient. 20 μL of undiluted sperm was added to the center of the chamber. Approximately 300 μL of seawater was collected from ~ 0.5 cm above the bottom of each well 15 minutes after sperm addition.

The number of sperm recovered from each well was estimated by counting the number of sperm in a 0.004 mm^3 area using a hemocytometer and taking the average of three of these subsamples as the number recovered for that well. The percentage of sperm recovered for each egg was calculated by dividing the number of sperm recovered from that well by the total number of sperm recovered from both wells. By utilizing the percentage of sperm recovered rather than the absolute number of sperm recovered, I was able to compare across genotypes without needing to correct for potential differences in the absolute number of sperm recovered due to initial sperm concentration difference between males. To preclude the possibility of unequal diffusion, potential biases due to collection artifacts, or a non-chemotactic directional swimming bias skewing the results, each combination of two females and one male were tested twice, with the eggs switched to opposite wells in the second chamber. 70 unique blocks consisting of one male and two females were performed. Between tests all dichotomous chambers were rinsed with hot fresh water and allowed to dry for 48 hours or more, to remove any lingering chemoattractants.

To examine whether more sperm were recovered from a focal female's well based on the ligand GRP genotype of the individual whose sperm was used, or the receptor GRP genotype of the second (challenger) individual whose eggs were used in the opposite well, or interactions between the two, a generalized linear model with a Poisson link function was fitted to each of the three female receptor genotypes. A separate model was fitted for each female receptor genotype, as comparisons between the percentages of sperm recovered could only be made between two of the three possible receptor pairings; as the third pairing lacked that specific genotype (i.e. AA females could only be examined in two of the three receptor pairings: AA vs AE and AA vs EE, but not AE vs EE). For each model fitted to the receptor genotype, the ligand genotype for the individual whose sperm was used, the challenger female's receptor genotype, and their interaction were included as fixed effects. Male and female identity was included as a random effect, to account for the fact that each triad was replicated twice.

As previous studies have only examined compatibility based on whether the female had the same ligand genotype as the male whose sperm the female was crossed with (Palumbi 1999, Levitan and Ferrell 2006), I also examined whether having the same genotype at the ligand or receptor locus affected sperm behavior. To determine if sperm choose eggs based on genotype matching rather than specific GRP genotype, a single generalized linear mixed effect model was fitted to the data. Whether males and females matched at the ligand (Y/N), and whether they matched at the receptor locus (Y/N), and their potential interaction were included as fixed effects. For this model, male identity was included as a random effect, but the random effect of female identity was nested within chamber identity to account for the fact that the percent of sperm recovered from one well was dependent upon the percentage of sperm recovered in the opposite well.

4.2.3 Investigating Changes in Swimming Behavior Based on GRP Identity

To determine if sperm velocity or motility was different based on GRP genotype of either the individual whose eggs were used or the individual whose sperm was used, recordings of sperm were analyzed using a computer assisted sperm analysis (CASA) program in Image J (ver. 1.43). Sperm were recorded in seawater obtained from the stock solution of an individual's eggs after an hour of incubation time, which allowed for chemoattractants to seep into the water (hereafter called egg water). Each individual's sperm was filmed in the egg water of two females and the average curvilinear velocity and percent motility was estimated from three recordings taken per female. Sperm were filmed at 80 fps using a Fuji Finepix HS30. For each recording, 15 seconds were analyzed using CASA (ImageJ ver. 1.43; Schneider et al. 2012). Sperm from 70 different individuals were used in this experiment.

Two linear mixed-effects models were fitted to determine if there was a difference in either swimming velocity or motility based on variation in gamete recognition proteins. For the both models, male GRP genotype and female GRP genotype were included as fixed effects, and male and female identity were included as random effects to account for the fact that each individual used as a male was filmed with two different females egg water, while each female's egg water was used with two different males. Additionally, egg concentration of the stock solution was included as a covariate to account for possible differences in swimming kinetics due to possible differences in chemoattractant concentration.

Two additional linear mixed-effect models were used to examine whether changes in either swimming speed or motility could be predicted based on whether the genotype of the individual whose eggs were used was the same as the genotype of the individual whose sperm was used was examined, i.e. whether they matched. These analyses allowed me to compare the results to other studies, which have only examined compatibility in the context of whether the (unexpressed) ligand genotype in the female used in the cross matched with the ligand genotype of the male (Palumbi 1999, Levitan and Ferrell 2006). Whether the genotypes were the same at the sperm ligand locus (Y/N), or whether they were the same at the egg receptor locus (Y/N), and the interactions between the two were included as fixed effects, male and female identity were included as random effects, and egg concentration was included as a covariate.

4.3 Results

4.3.1 Chemotaxis-Mediated Egg Choice

For females that were homozygous for glutamic acid (EE), there was no significant effect of challenger genotype at the female locus ($p = 0.237$), or sperm genotype at the male locus ($p = 0.272$) on the difference in sperm recovered (Table 4.1). However, there was a marginally significant interaction between the two ($p = 0.035$), with fewer sperm recovered from well with eggs from females who were EE when the challenger was heterozygous at the female locus and the sperm was heterozygous at the male locus. A lower percentage ($30.6 \pm 4.5\%$) of the sperm recovered from a well with a female that was homozygous for glutamic acid (EE), when the challenger female was heterozygous at the receptor locus and the sperm was heterozygous at the ligand locus (Figure 4.2). Conversely, about half of the sperm recovered ($53.5 \pm 7.1\%$) was recovered from EE wells when the challenger female was heterozygous at the receptor locus but the individual whose sperm was used was homozygous for Methionine (MM), and similar amounts ($48.5 \pm 4.4\%$) were recovered when the individual whose sperm was used was homozygous for Threonine (TT, Figure 4.2). This suggests that sperm from individuals that are heterozygous at the ligand tend to aggregate around eggs from individuals that are heterozygous at the receptor when given a choice between them and eggs from individuals that were EE.

There was no effect of the challenger female's genotype at the receptor locus or the male's genotype at the ligand locus for either of the other possible receptor genotypes (AA and

AE, Table 4.1). However, for individuals heterozygous at the receptor locus (AE), more sperm (67.8 ± 11.3%) tended to be recovered from the heterozygous well when the challenger was homozygous for glutamic acid (EE) and the individual whose sperm was used was heterozygous at the ligand locus (Figure 4.2). Additionally, less sperm (39.7 ± 6.8%) were recovered from wells with individuals who were heterozygous at the receptor locus when the challenger female was homozygous for glutamic acid (EE) and the sperm was homozygous for Methionine (MM, Figure 4.2). Less sperm (37.5 ± 5.3%) was also recovered from heterozygote egg wells when the challenger female was homozygous for alanine (AA) and the sperm was homozygous for Threonine (Figure 4.2). While not significant, it suggests that when given the choice between eggs from individuals that are homozygous at the receptor and those that are heterozygous, sperm from males who are homozygous at the ligand will aggregate around eggs with homozygous receptors. However, this effect is contingent upon the genotype, as homozygous TT males will tend to aggregate around AA females, but MM males aggregate around EE females.

There was an effect of GRP matching on in the percentage of sperm recovered from a well, based on whether the individual whose eggs were used had the same receptor genotype as the individual whose sperm was used (Table 4.2: $p = 0.011$). Significantly less sperm was recovered from wells where the eggs were from individuals that did not have the same genotype as the individuals whose sperm was used at the receptor locus (LS mean of 42.2 ± 2.0%), than when they did have the same receptor genotype (49.9 ± 4.5%, Figure 4.3). This suggests that sperm will aggregate around eggs that share the same receptor genotype as them.

4.3.2 Investigating Changes in Swimming Behavior Based on GRP Identity

There was a no significant effect of either the ligand genotype or the receptor genotype on sperm velocity (Table 4.3: $p = 0.077$ and $p= 0.874$, respectively). There was also no effect of egg or sperm GRP genotype on motility of the sperm, nor was there a significant interaction (Table 4.3: $p > 0.05$ in all cases). However, the interaction between male and female GRP genotypes on percent motility was borderline significant, as the best performing male-female pairing could increase motility by 20% when compared with the worst performing pair (Table 4.3: $p = 0.054$, Figure 4.4).

There was no effect of matching at the male locus or at the female locus (Table 4.4: $p = 0.865$ and $p = 0.475$, respectively), nor was there a significant interaction on sperm velocity (Table 4.4: $p = 0.165$). Similarly, there was no effect of genotype matching at either locus on percent sperm motility (Table 4.4: $p > 0.05$, in both cases), nor did an interaction affect percent motility (Table 4.4: $p = 0.420$).

4.4 Discussion

It has been proposed that chemoattractants could moderate sperm competition and sexual conflict by providing information about compatibility via a chemical signal prior to sperm-egg collision and binding (Evans et al. 2012, Oliver and Evans 2014, Hussain et al. 2016, Lymbery et al. 2017). This study is the first to actually link differences in sperm behavior with genetic differences in the proteins that mediate compatibility. The genotype of the individuals' whose eggs the sperm could choose between and the genotype of the individual whose sperm were used influenced which well a greater percentage of sperm was recovered from, suggesting that sperm will aggregate around certain eggs based on their respective genotypes. However, there did not appear to be an effect of genetic variability on sperm kinetics, as neither swimming speed nor the percentage of sperm that were mobile after exposure to egg chemoattractants changed based on GRP genotype.

Previous studies have found that sperm who were better able to orient themselves towards eggs tended to fertilize a higher number of eggs (Evans et al. 2012, Oliver and Evans 2014, Hussain et al. 2016), although they were unable to differentiate whether the greater fertilization success was due to increased compatibility between those eggs and sperm or the sperm's ability to orient themselves better towards eggs in general. In this study, a comparison of orientation based on genetic variation in gamete recognition proteins allowed for a relatively direct examination of whether sperm 'chose' which eggs to swim towards based on compatibility. Twice as many sperm were recovered from wells that had eggs from females who were heterozygous at the receptor locus (AE), when sperm from individuals that were heterozygous at the male locus (TM) were allowed to choose between them and eggs from females who were homozygous for glutamic acid (EE), although this effect was only marginally significant. Previous fertilization assays have suggested that individuals who are heterozygous at

the receptor locus may have a higher binding affinity with sperm from individuals who are heterozygous at the ligand (Chapters 2 and 3). While I do not know for certain whether individuals heterozygous at the receptor locus have higher binding affinity with individuals who are heterozygous at the ligand locus, the patterns seen in choice and no-choice fertilization assays matched those seen in compatible protein pairings found in sea urchins, where compatibility is known (Levitan and Ferrell 2006, Levitan and Stapper 2010, Tomaiuolo and Levitan 2010).

Sperm were also able to orient themselves based on whether the (unexpressed) receptor locus of individuals' whose sperm was used matched the (expressed) receptor locus of individuals' whose eggs were used (Yamada et al. 2009). Other studies have found that matching between GRP ligand genotypes can be used to predict compatibility between males (where the ligand is expressed) and females (where it is not) in sea urchins (Palumbi 1999, Levitan and Stapper 2010). In this species, it is matching at the receptor locus and not the ligand locus that may predict compatibility, as there was a (non-significant) trend for individuals who matched at the receptor locus to sire ~1.3x as many larvae (Chapter 3). This study found that ~1.7x more sperm were recovered from wells when the receptor genotype of individual whose sperm was used was the same as the receptor genotype of the individual whose eggs were used, suggesting that sperm will aggregate around eggs that could carry the same allele at the receptor locus as them. This could give sperm that have matching receptor genotypes a competitive advantage, and it may result in an excess of offspring that are homozygous at the receptor proteins when sperm competition occurs.

While differences in directional swimming based on variation in GRPs were observed using dichotomous chambers, there was no evidence of variation in compatibility causing a difference in either swimming velocity or motility. In other species, there has been an effect of chemoattractants on both sperm orientation and velocity (Riffell et al. 2004, Oliver and Evans 2014, Lymbery et al. 2017), although not in *Ciona robusta* (Kawamura et al. 1987, Kosman et al. 2017). It is possible that different chemoattractants than those that affect sperm orientation govern sperm velocity and motility in *Ciona*. However, currently only a single chemical chemoattractant has been identified in this species, and is known to affect both directionality and speed (Yoshida et al. 1993, 2002). It still may be possible that more chemicals are involved that have yet to be identified; this study used egg-conditioned water, which contained all chemical

cues produced by an egg rather than the pure SAAF sulfate-steroid extract. A more in-depth look at the chemical cues produced by eggs and their effects on sperm behavior is needed to understand the mechanisms potentially linking chemotaxis, chemokinesis, and compatibility.

Ultimately, these results suggest that chemoattractants may provide sperm with information on compatibility, as well as species identity. Additionally, they support the idea that sperm will orient themselves towards eggs based on what they may be more compatible with when given the choice (Evans et al. 2012, Oliver and Evans 2014, Hussain et al. 2016, Lymbery et al. 2017). This can have two important consequences; it can reduce sperm competition at the egg by providing a competitive advantage to more compatible sperm prior to collision and primary binding, and it can promote assortative mating between compatible gametes, increasing the functional linkage between the gamete traits governing compatibility and chemotaxis. The mechanism behind this functional linkage between compatibility and chemotaxis remains to be determined. It is possible that genetic linkage between male and female GRPs variants and chemoattractants produced may have arisen, especially given the consequences of choosing a more compatible egg on subsequent fertilization success and offspring fitness (Palumbi 1999, Oliver and Evans 2014).

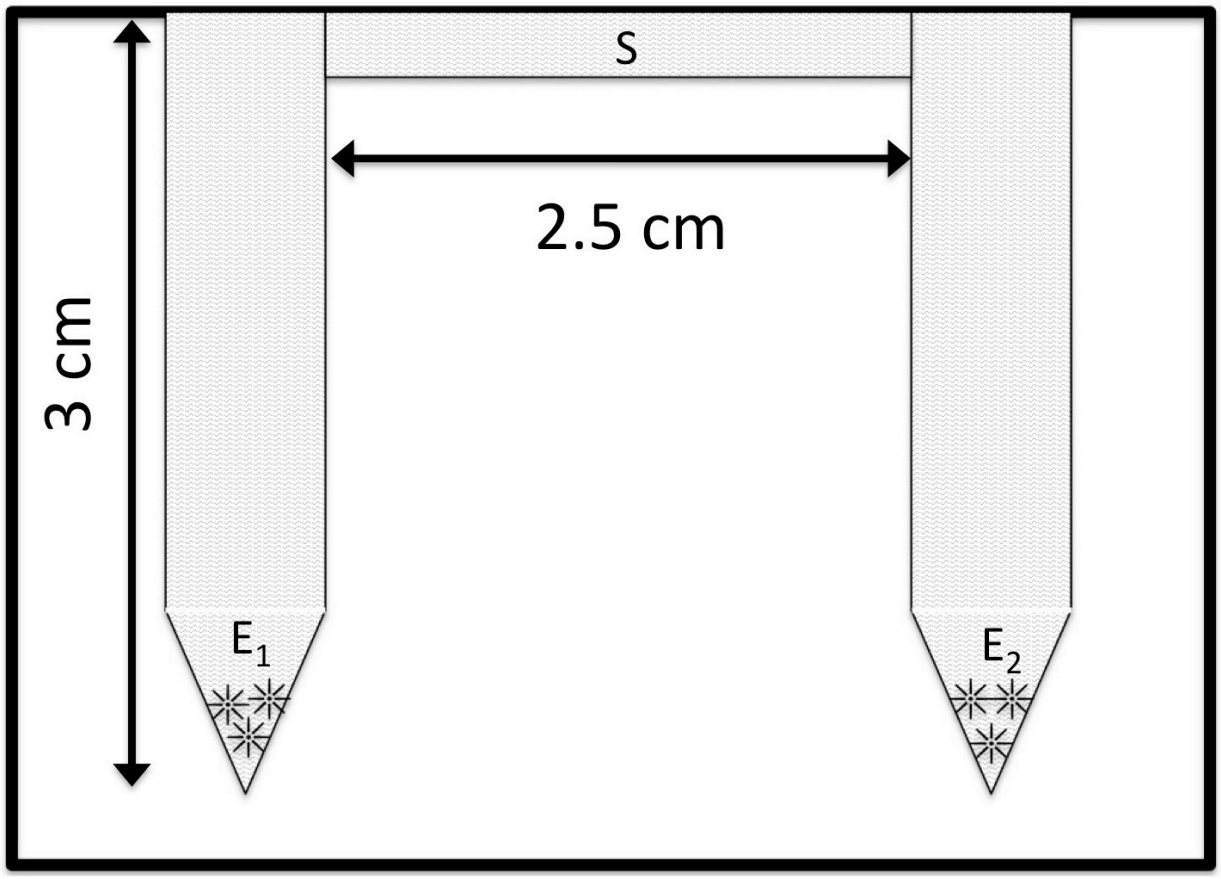

Figure 4.1: Diagram of a dichotomous chamber used to examine chemoattractant-mediated egg choice. Eggs from an individual were placed in each of the 3 cm long 1 cm in diameter wells (E). The wells were separated by a 2.5 cm long groove that was ~0.5 cm deep. 20 ul of dry sperm from an individual was placed in the center groove (S), and 300 ul of seawater was collected from near the base of each well 15 minutes after sperm addition.

Table 4.1: Results from generalized linear mixed-effect models fitted to examine whether percentage of sperm recovered from a focal female's well in a dichotomous chamber was affected by the ligand genotype of the individual whose sperm was used, the genotype of the challenger female at the receptor locus, or an interaction between the two. Female identity and male identity were included in the full model as random factors. Shown are the fixed effects and interactions presented in a type II ANOVA table using a Wald Chi-square test of significance.

AA Females

Factor	DF	$\chi 2$	*P*
Challenger Female GRP Genotype	1	0.454	0.500
Male GRP Genotype	2	1.139	0.566
Challenger Female: Male GRP Genotype	2	1.195	0.550

AE Female

Factor	DF	$\chi 2$	*P*
Challenger Female GRP Genotype	1	1.877	0.171
Male GRP Genotype	2	3.423	0.181
Challenger Female: Male GRP Genotype	2	4.237	0.120

EE Females

Factor	DF	$\chi 2$	*P*
Challenger Female GRP Genotype	1	1.396	0.237
Male GRP Genotype	2	2.602	0.272
Challenger Female: Male GRP Genotype	**2**	**6.727**	**0.035**

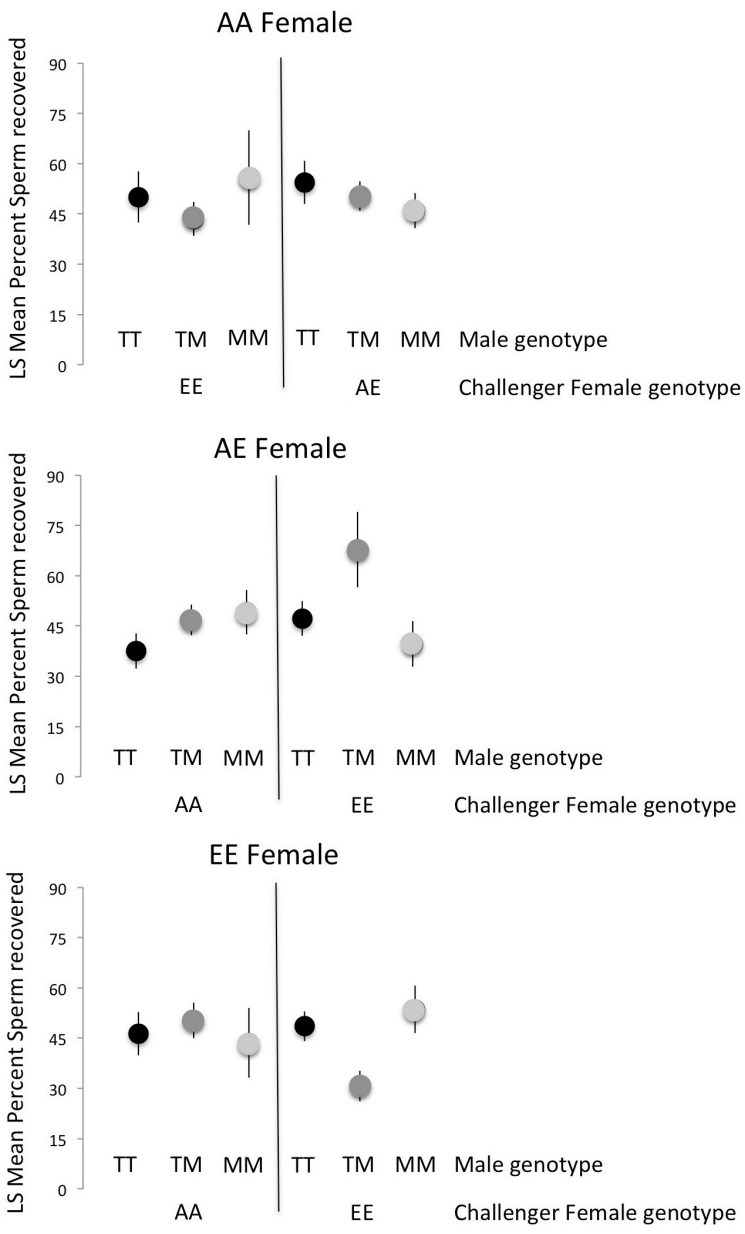

Figure 4.2: Least-squared mean of the percentage of sperm recovered from the well of the dichotomous chamber for each receptor genotype possible, based on the receptor genotype of the challenger female in the opposite well and the ligand genotype of the individual whose sperm was allowed to choose between wells. Error bars are $\pm$ SE.

Table 4.2: Results from generalized linear mixed-effect models fitted to examine whether individuals having the same genotype at the male ligand locus, or the female receptor locus, affected percentage of sperm recovered from a well. Male identity was included as a random effect, as was female identity, which was nested within chamber identity to account for the fact that the wells of the dichotomous chamber were not independent. Shown here are the fixed effects and interactions presented in a type II ANOVA table using a Wald Chi-square test of significance.

Factor	DF	$\chi 2$	*P*
Female GRP Genotypes Match	**1**	**6.466**	**0.011**
Male GRP Genotypes Match	1	0.160	0.689
Female Genotypes Match: Male Genotypes Match	1	0.063	0.802

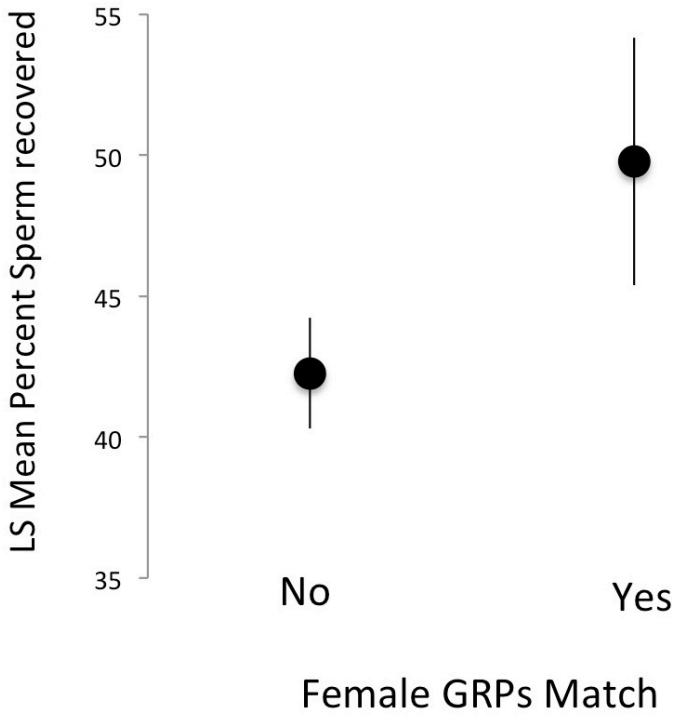

Figure 4.3: Least-squared mean percentage of sperm recovered from a well where the females whose eggs were used had the same receptor genotype as the individual whose sperm was used (right, yes), or whether they did not match (left, no). Error bars are $\pm$ SE.

Table 4.3: Linear mixed-effect models exploring the effect of GRP genotype on sperm swimming speed and percent motility. Male GRP genotype and female GRP genotype were included as fixed effects, egg concentration as a covariate, while male and female identities were included as random effects. Shown are the fixed effects and interactions presented in a type II ANOVA table using a Wald Chi-square test of significance.

Factor	DF	χ^2	*P*
Sperm Velocity			
Male GRP Genotype of Sperm	2	5.123	0.077
Female GRP Genotype of Eggs	2	0.269	0.874
Egg Concentration	1	2.231	0.135
Male x Female GRP Interaction	4	2.099	0.718
Percent Motility			
Male GRP Genotype of Sperm	2	1.554	0.460
Female GRP Genotype of Eggs	2	0.298	0.861
Egg Concentration	**1**	**5.488**	**0.019**
Male x Female GRP Interaction	4	9.310	0.054

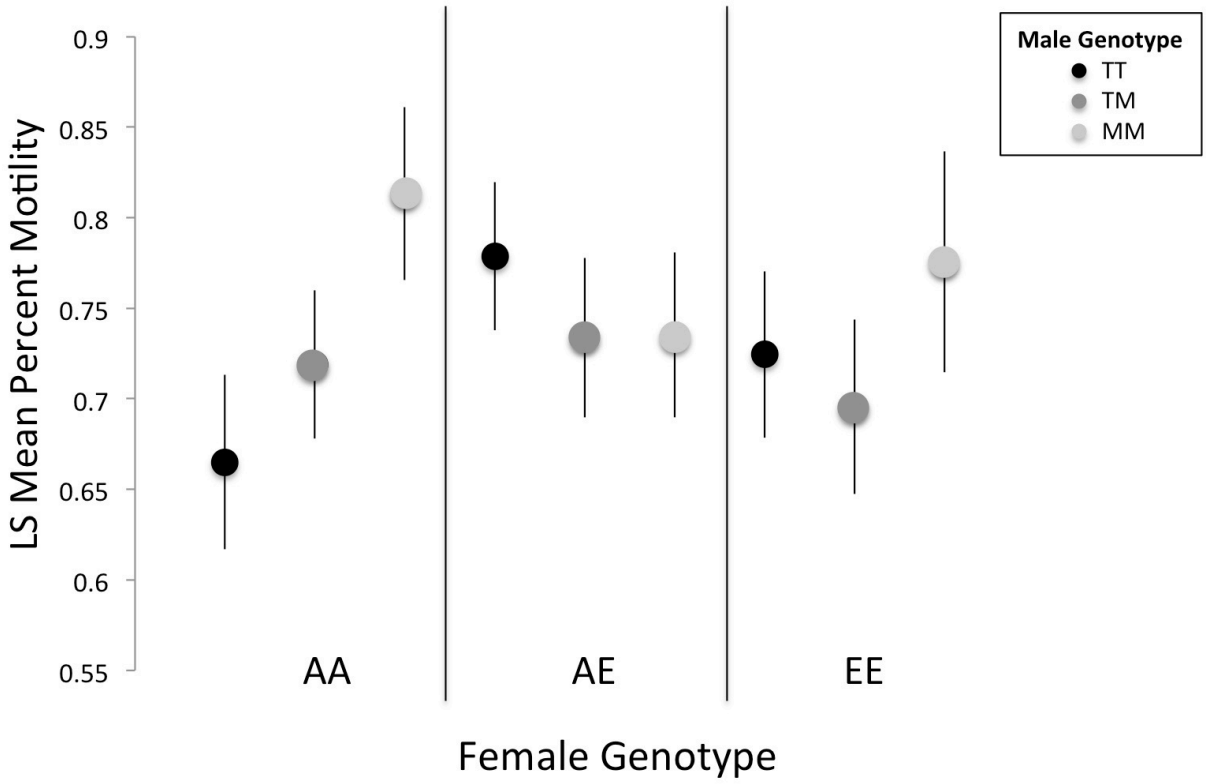

Figure 4.4: LS mean percentage of motile sperm based on the sperm's GRP genotype at the male locus and the female GRP genotype of the female whose egg chemoattractants they were exposed to. Error bars are ± SE.

Table 4.4: Linear mixed-effect models exploring the effect of genotype matching on sperm swimming speed and percent motility. Whether the egg and sperm matched at the male and female locus were included as fixed effects, egg concentration as a covariate, while male and female identities were included as random factors in the full model. Shown are the fixed effects and interactions presented in a type II ANOVA table using a Wald Chi-square test of significance.

Factor	DF	χ^2	*P*
Sperm Velocity			
Matching at Male Locus	1	0.029	0.865
Matching at Female Locus	1	0.511	0.475
Egg Concentration	1	**4.560**	**0.033**
Male x Female Matching Interaction	1	1.926	0.165
Percent Motility			
Matching at Male Locus	1	0.389	0.533
Matching at Female Locus	1	2.611	0.106
Egg Concentration	**1**	**8.538**	**0.003**
Male x Female Matching Interaction	1	0.650	0.420

CHAPTER 5

THE EFFECTS OF POPULATION DENSITY ON CHANGES IN GAMETE TRAITS AND TRAIT ASSOCIATIONS IN A WILD POPULATION

5.1 Introduction

Shifts in population abundances can have a large effect on reproduction, particularly for broadcast spawners, whose fertilization success is dependent upon the frequency of gamete interactions (Levitan 1996, Styan 1998, Crimaldi and Zimmer 2014). Laboratory experiments have shown that strength and direction of selection on traits that affect sperm-egg collision and fusion rates can change based on the sperm environment, in order to maximize reproductive output for that environment (Levitan 2000, Farley and Levitan 2001, Levitan 2012, Evans and Sherman 2013, Lymbery et al. 2018). Interspecific comparisons have confirmed patterns seen in the laboratory, namely species that spawn at high densities tend to have smaller, less compatible eggs and faster sperm compared to those who spawn at lower densities (Levitan 2002, Levitan et al. 2007). The egg traits favored at high sperm availability are expected to reduce polyspermy (egg death by multiple insemination), while increased sperm swimming speed is expected to increase the odds of the sperm winning during sperm competition (Levitan 2000, Farley and Levitan 2001, Levitan 2012, Lymbery et al. 2018). At low sperm environments gamete traits that increase collision or fusion rates, like large target sizes, long-lived sperm, and sperm-egg proteins that are more compatible are favored (Levitan 1996, Crean and Marshall 2008, Levitan 2008, Levitan 2012).

While many studies have examined how the sperm environment can affect the evolution of gamete traits that affect collision rates, few have directly linked changes in gamete trait values to changes in population densities, and those that have generally depend on artificial manipulation of population densities or spawning behaviors (e.g., Levitan 1996, Crean and Marshall 2008, Levitan 2008). Empirical evidence linking changes in traits that govern sperm egg-fusion rates with changing population densities is even more rare. Only two studies to date have even attempted to determine how genetic variation in gamete recognition proteins (GRPs, which mediates sperm-egg compatibility) changes with population density, despite the fact that

genetic variance in these proteins may be maintained through density-dependent processes (Levitan and Ferrell 2006, Tomaiuolo and Levitan 2010, Levitan 2012). Of the two studies examining how changes in population density may affect genetic variation in proteins that determine fusion rates (Levitan 2012, Gilg et al. 2016), only one of the two found evidence of a density-based change in GRP variation (Levitan 2012). In sea urchins, a steady population increase throughout the region due to a decline in a major predator may have helped create a fairly distinct difference in spawning conditions that could be identified over time, resulting in a clear increase in a less-compatible ligand variant with increasing population size, matching laboratory predictions (Tomaiuolo and Levitan 2010, Levitan 2012).

In both studies examining how genetic variation in GRPs changes with population density, the effect of population density on only the sperm ligand was examined. However, theory suggests assortative mating between different GRP ligand and receptor pairs may occur at high sperm densities, such that associations between proteins should become more evident as populations increase (Gavrilets and Waxman 2002, Tomaiuolo and Levitan 2010). Similarly, studies looking at traits that affect collision rates, have rarely looked at whether association between traits occur based on sperm environment, despite the fact that assortative mating between gametes based on specific traits combinations may occur more often at high sperm availabilities when sperm competition and/or cryptic egg choice occurs (Sherman et al. 2015, Levitan 2018).

I aim to examine how gamete trait values and associations between traits change with natural population fluctuations. This will be accomplished by tracking the settler numbers (as a proxy for population density) of *Ciona robusta* and examining whether changes in the values of collision rate traits correlate with settler density. I also will examine whether there is evidence of assortative mating with increasing settler density based on GRP genotype, by examining whether deviations in genotype frequencies from expected values under random mating increase with increasing settler density. Additionally, I will determine if there is an interaction between settler density and collision rate traits based on GRP genotype, as might be expected if fusion and collision rates traits become associated via assortative mating.

I expect that collision rate trait values should match predictions made from laboratory results; as population sizes increase, traits that reduce polyspermy, like increasing accessory structure size to slow sperm and decreasing ovum size will be evident in eggs, while traits that

increase the probability of fertilization success under sperm competition, like increases in velocity and/or motility, will be found in sperm (Levitan and Irvine 2001, Podolsky 2001, Crean and Marshall 2015, Lymbery et al. 2018). However, I hypothesize that the relationship between settler density and changes in gamete trait values that affect collision rates may be modified by traits that influence fusion rates (e.g., less compatible egg receptors may become associated with faster sperm, while more compatible egg receptors may become associated with slower sperm). These associations may be likely to occur because at higher sperm availability, multiple different trait combinations can yield equivalent fertilization success. Those combinations that maximize fertilization success should become more prevalent as sperm competition, polyspermy, and cryptic female choice become more common (Sherman et al. 2015, Levitan 2018, Chapter 2).

Ciona robusta population densities can fluctuate widely from over 3,000 to 5 individuals per m^2 over the course of a year (Caputi et al. 2015). Larval period in *C. robusta* is also relatively brief (~18 hours from hatching to metamorphosis), so the number of settlers in a given area most likely reflects the reproductive output of that area (Yamaguchi 1975, Carver et al. 2006). While the growth rate and adult life span is dependent upon temperature, on average individuals can reach sexual maturity within one to two months and live for approximately three to 6 months in warmer waters (Carver et al. 2006). After reaching sexual maturity, individuals can spawn every 2-3 days (Yamaguchi 1975).

In this species, both the male GRP ligand (CiUrabin) and its female receptor (CiVC57), are known to be genetically variable (Yamada et al. 2009, Yamaguchi et al. 2011). Interactions between the male and female proteins, and interactions with gamete traits that affect collision rates are known to affect fertilization success (Chapter 2). Previous laboratory results suggested that the interactions between collision rate and fusion rate traits are relatively important determining fertilization success across many sperm environments (Chapter 2). For this experiment, I asked two main questions: Can changes in settler density (as a proxy for population density and sperm environment) affect associations between GRPs, and can changes in trait values of collision rate traits in response to settler density be modified by associations with fusion rate traits (GRPs)?

5.2 Methods

5.2.1 Deviations in GRP Genotype Frequencies from Random Mating Based on Settler Density

Settlement racks were deployed in four different locations in Quivera Basin in San Diego, CA for two years, from February 2015 to February of 2017 (Figure 5.1). At each location, a single rack consisting of four circular plates approximately 9 cm in diameter was deployed. Every 5 weeks, the plates from each rack were removed and replaced with fresh plates. The number of settlers per 9 m^2 was recorded for each five-week time period, by examining each of the four plates for every rack under a dissecting scope and counting the number of settlers for all 16 plates. For each five-week time period, approximately 10 individuals per rack were selected and preserved in 95% ethanol for later GRP sequencing of the male ligand and female receptor genes. When possible, five individuals from larger size classes ($\leq$ 2 cm) and five individuals from smaller sizes classes (> 2 cm) per rack were selected in order to ensure that settlers from the entire time period the plates were deployed were chosen for sequencing. This resulted in settler GRP genotypes and counts for a total of 20 time periods (every 5 weeks for 2 years).

To determine if there was a general signature of selection on either the ligand or the receptor over the duration of the experiment, a linear correlation of the observed frequency for one of the two alleles for each locus (receptor and ligand) with time was performed. To examine the possibility that assortative mating based on genetic variation in GRPs was more likely to occur at higher densities, a regression of the deviation from Hardy-Weinberg expectations based on settler densities was performed for each locus. The deviation from Hardy-Weinberg expectations was calculated by subtracting the frequency of homozygotes expected under random mating for a time period from the observed frequency of homozygotes for that time period. Deviations from Hardy-Weinberg for homozygosity were calculated for both the male and female locus, and both were regressed against settler number, to examine whether the amount of deviation from the expected frequency under random mating increased with settler numbers.

In addition to examining the deviations from Hardy-Weinberg ratios for each locus separately, the expected frequency of individuals for a 'multi-locus' genotype (both male ligand and female receptor GRP genotype from a single individual) was calculated based on Hardy-

Weinberg expectations, to determine if associations between genotypes at the male and female locus were more likely to occur at increasing settler densities. The multi-locus genotypes were corrected for any deviations from HW in the individual loci when calculating the expected multi-locus frequencies. The deviation from the expected multi-locus genotype was regressed against settler number, to examine whether certain male-female genotype pairings were found more often than would be expected from randomly combining ligand and receptor genotypes based on settler density. As there were a total of nine different possible multi-locus genotypes, nine regressions were performed. To control for a false discovery rate of 5%, Benjamini and Hochberg adjusted critical values were used.

5.2.2 Changes in Collision-Rate Gamete Traits Based on Settler Density and Interactions with Compatibility

In order to determine how the gamete trait values for traits involved in collision may change based on population densities and associations with GRPs, the gamete traits of adult tunicates were measured during the same two-year time frame as settler density. For 14 of the 20 time periods, approximately a week after plates were exchanged, adult tunicates were collected from the same area in Quivera Basin, San Diego, CA. Eggs were removed from the oviduct and sperm was pipetted directly from the spermiduct for gamete trait measurement. Tissue from the siphon of every individual adult was preserved in 95% ethanol for later sequencing, in order to determine whether there were differences among individuals in their collision traits based on GRP genotype, as might be expected if an association between different combinations of traits occurred.

For the adults collected, both sperm and egg gamete traits were measured, and their male and female GRP genotypes were recorded (see section 5.2.3). Average path curvilinear velocity (VCL), as an estimate of sperm swimming velocity, and percent motility (the percentage of sperm that were actually motile) was estimated for each individual from a 15 second recording using a computer assisted sperm analysis program in ImageJ (CASA). Sperm were recorded at a concentration of 10^7 cells per mL with a Fujifilm Finepix HS30exr camera mounted on a Leitz microscope, in water that had been conditioned with a mixture of eggs from several individuals. For each individual the average ovum diameter and follicle cell length was estimated from the

measurements of the ovum diameter and follicle cell length of 15 eggs, using the computer-imaging program, ImageJ (ver. 1.43; Schneider et al., 2012).

To determine how the collision-rate traits changed over settler densities and identify possible temporary trait associations between collision traits and compatibility, a general linear model (GLM) was performed for each trait (ovum diameter, follicle cell length, sperm velocity, and sperm motility), with average lagged settler density, male GRP genotype, and female GRP genotype as fixed effects. The average lagged settler density was calculated by averaging settler density over the two time periods prior to adult collection. Average lagged settler density was used in these models as it was unclear exactly what settler densities the adults whose gamete traits were measured were spawned at. Two time periods, or 10 weeks, was considered the most likely settling period of sexually mature adults used in this experiment. As the sample sizes of some of the 9 possible male by female GRP pairs were low, the three-way interaction between density, male GRP genotype, and female GRP genotype was excluded from the model, and only two-way interactions were examined.

5.2.3 Sequencing Gamete Recognition Proteins from Settler and Adult Tunicates

Tissue collected from each adult and from larger settlers was digested in CTAB and proteinase K in a 64°C hot water bath for 12-14 hours for DNA extraction. DNA was purified using magnetic beads, and stored at -20°C. In cases where the settlers selected for sequencing were smaller than 4 mm, the DNA extraction was performed using a Tween and proteinase K cocktail. From previous studies (chapters 2-4), a single nucleotide polymorphism in the receptor gene and one in the ligand gene that were known to affect fertilization success were targeted for sequencing.

For every individual, the female GRP receptor gene was sequenced using primers designed to target a 372 bp region that contained the SNP known to affect fertilization success (CiVC57Exon7F: 5’ –TTCTAGGCATGCCCTGGTGATTCT-3’ and CiVC57Exon7R: 5’-CCATAGTGTGAACCCGCCTTTACT-3’). This SNP was recorded as the GRP genotype for the female receptor locus. Similarly, all individuals also had their male GRP genotypes sequenced using primers designed to amplify a 594 bp region of CiUrabin that contained the SNP known to affect fertilization success (CiUrabinCF: 5’-GTAGTTCCATCTGCGAGTAACA-3’ and CiUrabinFR: 5’-

ACATAAGTGCGGAGAGTGTAAT-3'). This SNP was recorded as the GRP genotype for the male ligand locus.

5.3 Results

5.3.1 Deviations in GRP Genotype Frequencies from Random Mating Based on Settler Density

Throughout the two-year period settler numbers fluctuated seasonally (Figure 5.2). Periods of high settler numbers generally occurred mid to late spring in both years, while a secondary peak in settler numbers occurred sometime after summer; late winter in 2015 and late fall in 2016. There was no significant linear change in allele frequency over time for the male GRP allele (Figure 5.3A: $p = 0.618$, $R^2 = -0.041$), however there was a slight but significant linear increase in the receptor allele coding for glutamic acid (E) over the course of the study (Figure 5.3B: $p = 0.035$, $R^2 = 0.182$).

There was a significant correlation between deviations from Hardy-Weinberg expected homozygosity with settler density for both the male and female GRP genotypes (Figure 5.4). There was a significant decrease in male GRP homozygosity with settler density (Figure 5.4A: $p = 0.013$, $R^2 = 0.258$), while the opposite pattern was seen in the female protein (Figure 5.4B: $p < 0.001$, $R^2 = 0.558$). This suggests that sperm were more likely to fertilize eggs that matched at the female locus and did not match at the male locus.

There was no significant correlation between deviations from expected values for the multi-locus GRP genotypes and settler density for any of the nine genotypes after correcting for multiple comparisons (Table 5.1: $p > 0.05$ for all). However, a close to significant increase of individuals who were homozygous at the receptor for glutamic acid (EE) and homozygous at the ligand for methionine (MM) from expected values with increasing settler density was observed (Figure 5.5: $p = 0.055$, $R^2 = 0.313$), suggesting that sperm that carried the E allele at the receptor locus and M allele at the ligand were slightly more likely to fertilize eggs also carrying the E receptor allele and M ligand allele.

5.3.2 Changes in Collision-Rate Gamete Traits Based on Settler Density and Interactions with Compatibility

There was a significant effect of the average lagged settler density (the settler density averaged over two time periods prior to adult collection) on sperm velocity (Table 5.2). As average lagged settler density increased so did the swimming speed of sperm that those adults produced (Table 5.2: $p = 0.006$, $\chi^2 = 7.606$, Figure 5.6A). A similar pattern was seen for sperm motility, as adults tended to produce sperm more motile when the average lagged settler density for the time period they most likely settled at was higher (Table 5.2: $p = 0.001$, $\chi^2 = 10.745$, Figure 5.6B). Additionally, there was a significant interaction between average lagged settler density and female GRP for sperm motility (Table 5.2: $p = 0.018$, $\chi^2 = 8.060$). Individuals who were homozygous for either receptor protein (AA or EE) tended to produce more motile sperm as the settler numbers for the time period that they most likely settled increased, while heterozygous individual did not (Figure 5.7).

For egg traits, there was a significant effect of average lagged settler density on ovum diameter, with smaller ova being produced as the settler density for the time period that they most likely settled at increased (Table 5.2: $p < 0.010$, $\chi^2 = 6.610$, Figure 5.8). There was no effect of average lagged settler density, GRP genotype, or interactions between the two, on average follicle lengths produced (Table 5.2: $p > 0.05$ all).

5.4 Discussion

As populations increase, there can be a shift in the direction of selection on gamete traits that affect fertilization success, in order to maximize fertilization success in the new sperm environment (Levitan 2002, Luttikhuizen et al. 2011, Levitan 2012, Lymbery et al. 2018). For fusion rate traits, evidence suggests that GRP variants that are more resistant to polyspermy should increase in frequency as the probability of polyspermy increases in the absence of sperm competition (Levitan and Ferrell 2006, Levitan 2012). However, in the presence of sperm competition, eggs may be better able to discriminate among sperm, leading to an increase in assortative mating between compatible receptor and ligand pairs (Sherman et al. 2015, Levitan 2018). I found evidence for assortative mating in both the male and female GRP proteins as settler numbers increased. There was an increase in the proportion of individuals that were

homozygous at the receptor protein than would be expected if only random mating was occurring at high settler densities, and the opposite pattern occurred at the ligand protein.

As the ligand became increasingly heterozygous, it is unlikely that the patterns of increasing homozygosity at receptor locus were due to inbreeding. Instead, it seems that sperm were more likely to fertilize eggs carrying an allele that matched the sperm's allele at the receptor protein. This pattern matches non-significant trends from previous studies that suggested that sperm from individuals who matched at the receptor protein tended to garner a larger paternity share than individuals that did not match (Chapter 3). Additionally, results examining sperm behavior have suggested that when given a choice, sperm will aggregate around the eggs of individuals who match them at the receptor protein (Chapter 4). Taken together, the laboratory results suggest that sperm have a competitive advantage with eggs from individuals who have the same genotype at the receptor protein. This competitive advantage could explain why more individuals with homozygous receptors were observed in the wild population as sperm availability and sperm competition increased.

It is less clear as to why individuals became increasingly heterozygous at the ligand locus as settler density increased. There is less evidence that sperm might have a competitive advantage when they do not match the ligand genotype of the individuals whose eggs were used, although there was a slight tendency for individuals who did not match at the ligand to garner a higher share of the paternity when they matched at the receptor protein (Chapter 3). Another possible explanation for why more heterozygous ligands were found as population densities increased, is that heterozygotes may have a slight advantage in fertilizing individuals who are homozygous at the receptor on average. Heterozygote ligands perform just as well as the homozygote ligands with either homozygote receptor, but there are slight differences in performance between individuals that are homozygous at the ligand based on which homozygous receptor they are paired with (Palumbi 1999, Chapter 3). This would allow for ligands to have a greater share of the paternity in the population as a whole, as a type of 'generalist' ligand.

However, even if heterozygous sperm can garner a higher paternity on average across all receptors, it would not necessarily result in an increased number of offspring that were heterozygous. For more offspring that are heterozygous at the ligand to be produced than expected under random mating, sperm must be able to identify and fuse with eggs that are not carrying the same allele as themselves at the ligand locus. However, there seems to be no

evidence that sperm preferentially aggregate around eggs that do not match at the ligand locus (Chapter 4). Another possibility is that settlers that are heterozygous at the male GRP locus are more likely to survive than those that are not. But it is difficult to postulate why there would be a difference in offspring survivorship based on ligand genotype, and why that difference would be more evident at higher settler densities. Further study is needed to determine whether heterozygous ligands act as a superior 'generalist' ligand, or affect post-fertilization survivorship to understand why this pattern occurs.

For collision rate traits, it was expected that as settler density (as a proxy for sperm availability) increased, the traits that decreased polyspermy would be favored in eggs, while traits that increased the probability of sperm-egg collision would be favored in sperm if sperm competition occurred (Levitan 2000, Farley and Levitan 2001, Johnson et al. 2013, Crean and Marshall 2015). I found that as the settler density for the time period the adults most likely settled at increased, sperm swimming speed and motility increased while average ovum diameter produced decreased. Smaller eggs are thought to reduce polyspermy by decreasing target size and reducing collision rates (Farley and Levitan 2001, Crean and Marshall 2008, Chapter 2). For sperm, increasing sperm swimming speeds and motility can increase collision rates, which although can cause polyspermy, will allow individuals to win in direct competition for an egg (Levitan 2000, Johnson et al. 2013, Lymbery et al. 2018).

It is possible that these results are due to plasticity rather than an actual shift in trait values due to selection on those traits, as phenotypic plasticity in egg and sperm traits in response to density has been shown in another species of solitary tunicate (Crean and Marshall 2008). However, it seems unlikely that the differences in sperm motility caused by the interaction between settler density and female genotype can be explained via phenotypic plasticity alone, unless some genotypes are more plastic than others. Instead, this may represent an association between traits; some genotypes that have a difference in compatibility may become associated with sperm traits that maximize fertilization success via assortative mating.

In short, as mate density increases, more sperm are available for eggs to sample, so the joint mechanisms of sperm competition and cryptic female choice could have a greater role in determining fertilization success and allow for associations between traits to form (Sherman et al. 2015, Levitan 2018, Chapter 2). My results revealed clear shifts in gamete trait values in a natural population of *C. robusta* in the directions predicted by laboratory and theoretical work

(Levitan 2012, Evans and Sherman 2013, Johnson et al. 2013). The strength of the associations between GRP alleles in both the receptor and ligand locus increased as settler number increased, most likely due to increased assortative mating between gametes. And while the exact nature of the affinities between different protein variants is unknown, pattern seen between sperm motility and the female GRP locus, suggest that collision rate traits and traits that affect compatibility may also become associated. My results showing relatively rapid shifts in trait values in response to shifts in abundance also illustrate how variance in gamete traits may be maintained: first through shifting selection pressures due to changes in the sperm environment, and secondly through interactions with other traits, which allow for different trait combinations to have equivalent fertilization success. Finally, they suggest that assortative mating can play a large role in forming trait associations particularly at higher sperm availabilities.

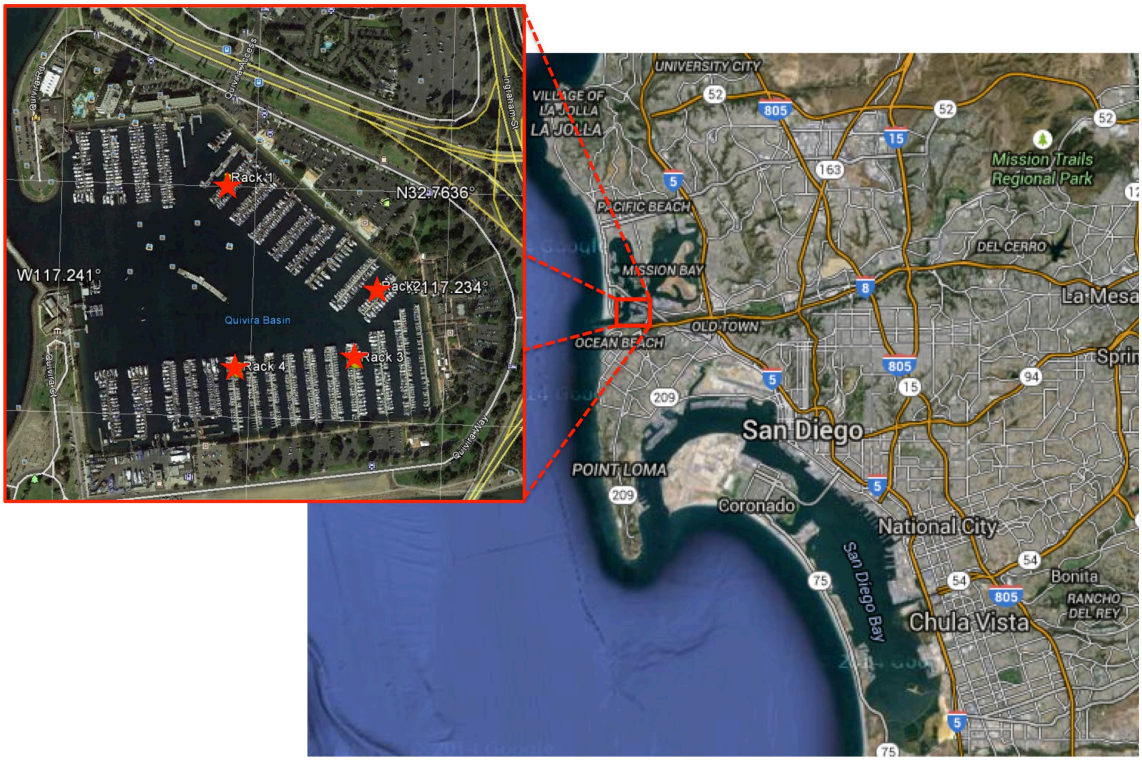

Figure 5.1: Map of the San Diego coast with insert showing a close up of Quivira basin in San Diego, CA. Stars represent locations where a settlement rack was deployed. Map data © 2015 Google.

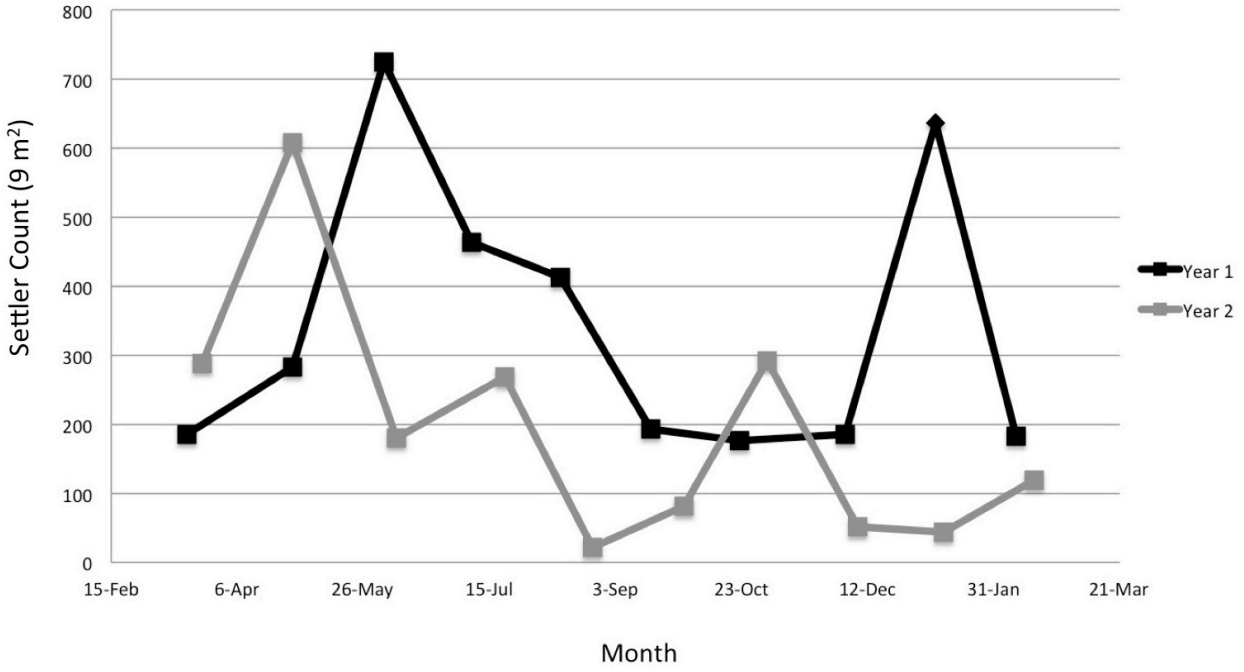

Figure 5.2: Number of settlers found on the four racks (with four dishes per rack) placed in Quivira Basin in San Diego, CA over a two-year time period. The black line is from March 2015 to February 2016, while the grey line is numbers from March 2016 to February 2017.

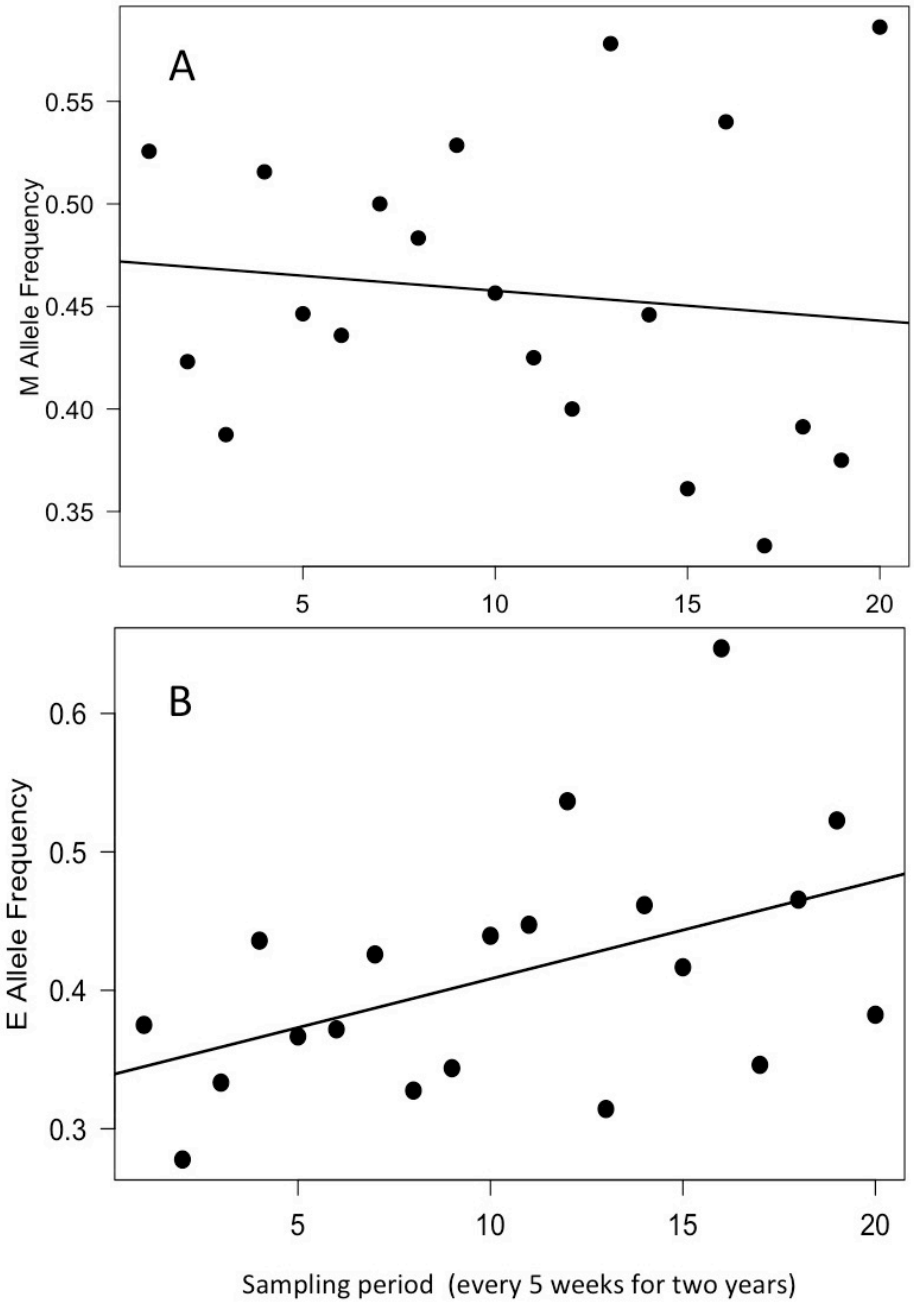

Figure 5.3: Correlations between SNP frequency coding for methionine (M, top) in the male ligand protein and the SNP coding for glutamic acid (E, bottom) in the female receptor protein over the 20 sampling periods of the experiment (5 weeks for 2 years). There was a significant increase in the E allele over the course of the study ($p = 0.035$, $R^2 = 0.182$, $b = 0.007$), but no significant relationship between the M allele and sampling period ($p = 0.618$, $R^2 = -0.041$).

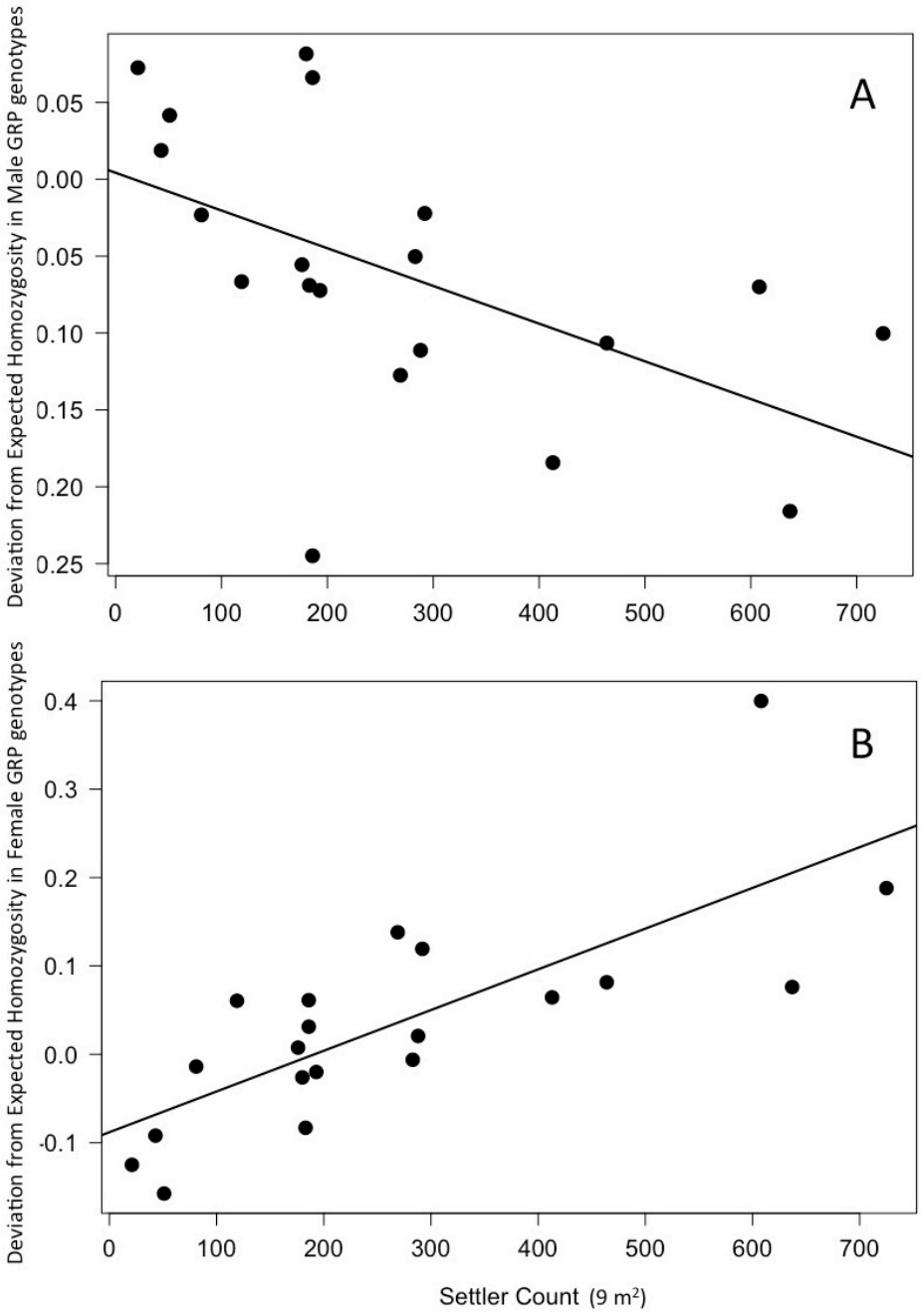

Figure 5.4: Correlations between the deviation from Hardy-Weinberg expected values of homozygosity in the male GRP genotypes (A) and female GRP genotypes (B) and settler densities over the two-year experimental period. There was a significant decrease in homozygosity with settler density in male GRP genotypes ($p = 0.013$, $R^2 = 0.258$, $b = -0.0003$) and a significant increase in homozygosity in female genotypes ($p < 0.001$, $R^2 = 0.558$, $b = 0.0005$), with increasing settler densities.

Table 5.1: Slopes, R^2, p and Benjamini-Hochberg corrected P-values for the 9 multi-locus regressions, examining whether deviation in the expected multi-locus (female and male) GRP genotype correlated with settler density. For the multi-locus genotype, the 1st two letters designate the genotype for the receptor, while the two letters after the period designate the genotype for the ligand.

Multi-locus Genotype	b	R^2	P	P corrected
AA.TT	0.00002	-0.029	0.501	0.563
AA.TM	0.00003	-0.037	0.577	0.577
AA.MM	-0.00003	-0.028	0.493	0.563
AE.TT	-0.00005	0.024	0.240	0.540
AE.TM	0.00009	0.105	0.089	0.266
AE.MM	-0.00004	-0.024	0.469	0.563
EE.TT	0.00003	-0.029	0.500	0.563
EE.TM	-0.00010	0.181	0.035	0.157
EE.MM	0.00007	0.313	0.006	0.055

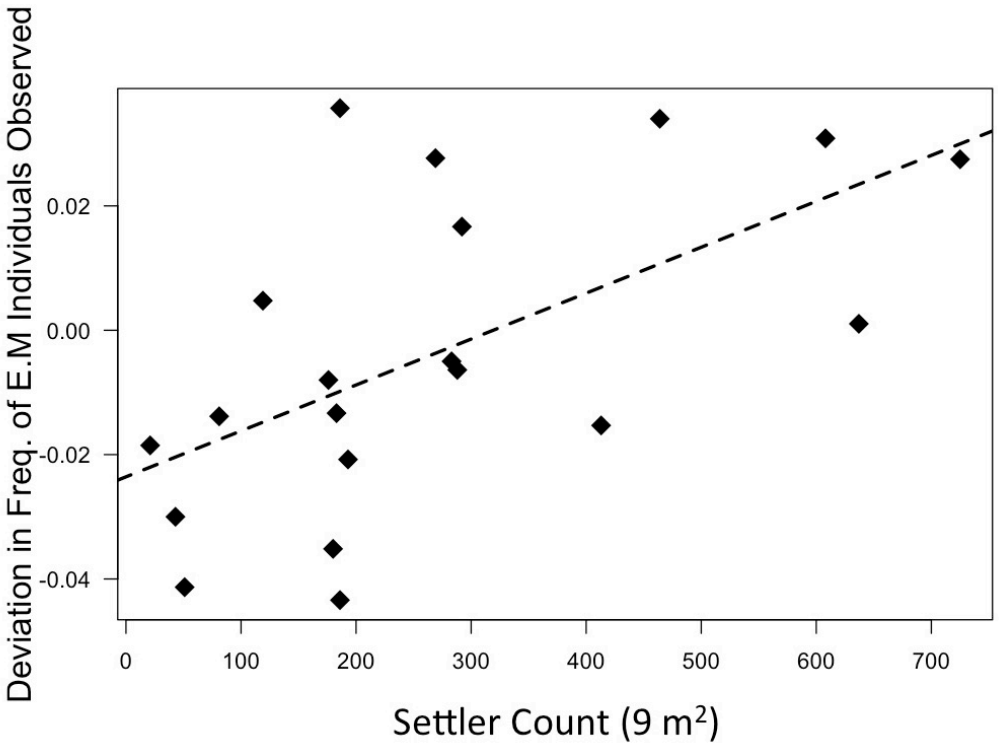

Figure 5.5: Correlation between the deviation from Hardy-Weinberg expected values of individuals with the multilocus genotype EE.MM and settler densities. After correction for multiple comparisons, the relationship between the higher than expected (under random mating) frequency of individuals who were homozygous at the receptor for glutamic acid (EE) and homozygous at the ligand for Methionine (MM) and settler density was deemed non-significant ($p = 0.055$, $R^2 = 0.313$, $b = 0.0007$).

Table 5.2: Model results for the general linear model exploring how average settler density (averaged over the two time periods prior to adult collection), male GRP genotype, and female GRP genotype may interact to affect collision rate traits. Shown here are the fixed effects and interactions presented in a type II ANOVA table using a Wald Chi-square test of significance.

Factor	Df	χ^2	P
Sperm Velocity			
Avg. Settler density	**1**	**7.606**	**0.006**
Male GRP Genotype	2	1.878	0.391
Female GRP Genotype	2	0.815	0.665
Avg. Settler density: Male GRP Genotype	2	5.031	0.081
Avg. Settler density: Female GRP Genotype	2	2.986	0.225
Sperm Motility			
Avg. Settler density	**1**	**10.745**	**0.001**
Male GRP Genotype	2	1.945	0.378
Female GRP Genotype	2	0.138	0.934
Avg. Settler density: Male GRP Genotype	2	4.772	0.092
Avg. Settler density: Female GRP Genotype	**2**	**8.060**	**0.018**
Avg. Ovum Diameter			
Avg. Settler density	**1**	**6.610**	**0.010**
Male GRP Genotype	2	1.186	0.553
Female GRP Genotype	2	2.554	0.279
Avg. Settler density: Male GRP Genotype	2	1.098	0.578
Avg. Settler density: Female GRP Genotype	2	5.710	0.058
Avg. Follicle Cell Length			
Avg. Settler density	1	1.599	0.206
Male GRP Genotype	2	2.294	0.318
Female GRP Genotype	2	2.143	0.343
Avg. Settler density: Male GRP Genotype	2	0.289	0.865
Avg. Settler density: Female GRP Genotype	2	2.933	0.231

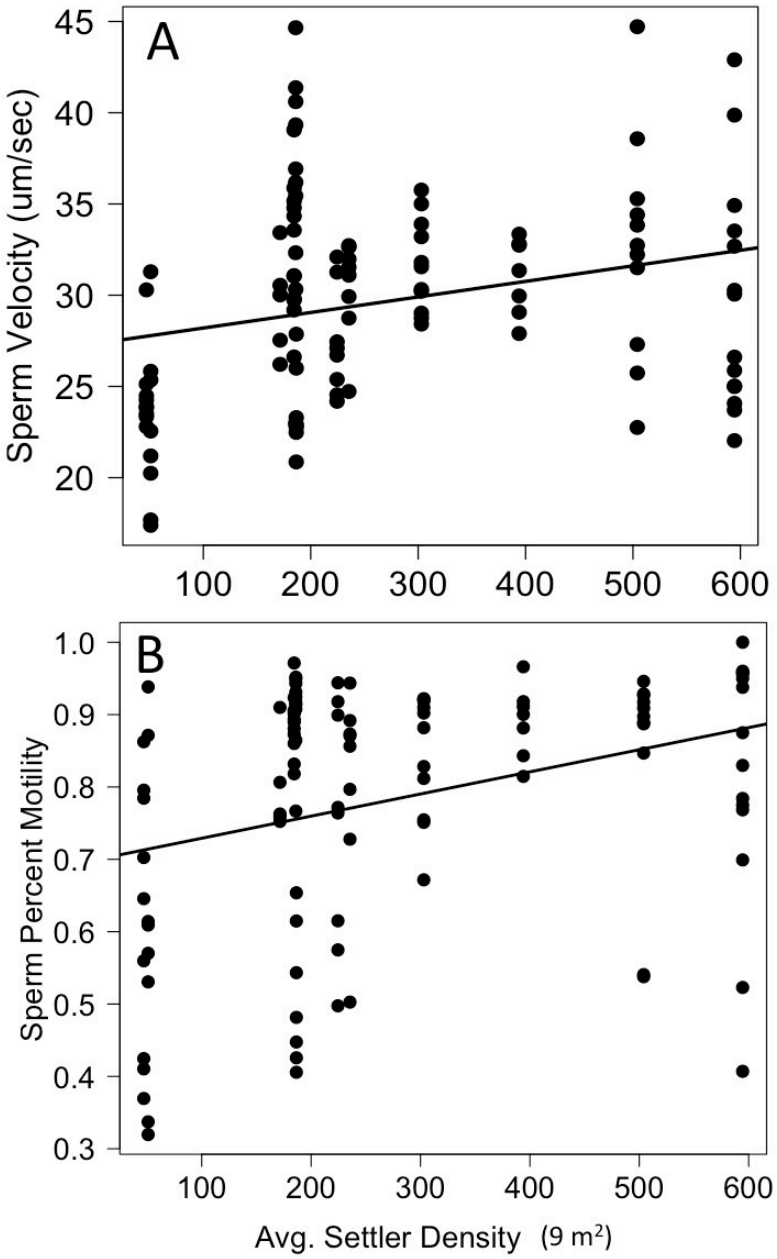

Figure 5.6: Correlation between sperm velocity (A) and sperm motility (B) and the average number of settlers seen in the two time periods prior to adult collection (10 weeks).

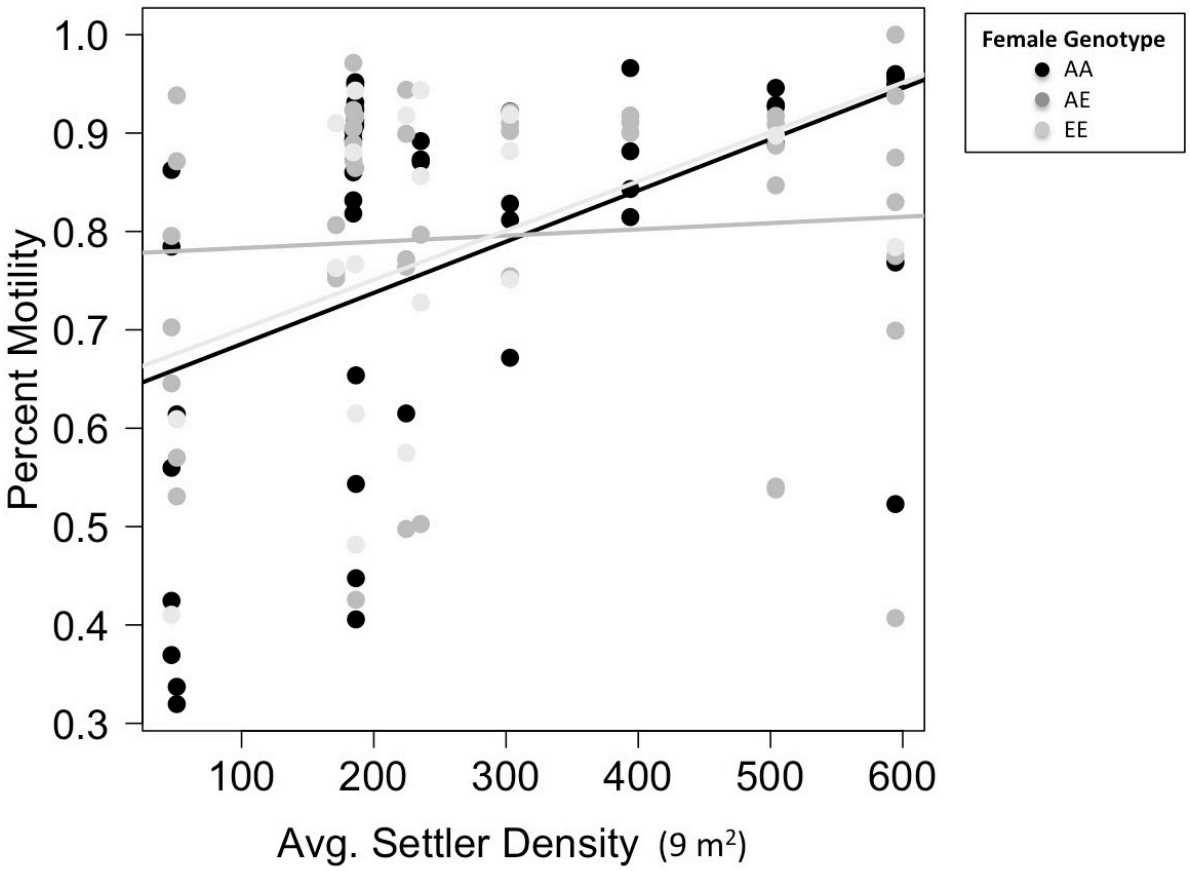

Figure 5.7: Correlation between percent motility of sperm produced and the average number of settlers seen in the two time periods (10 weeks) prior to adult collection based on adult female GRP genotype. Individuals who were homozygous at the receptor for alanine (AA) are depicted in black, heterozygote (AE) individuals in grey, and homozygous for glutamic acid (EE) individual are light grey.

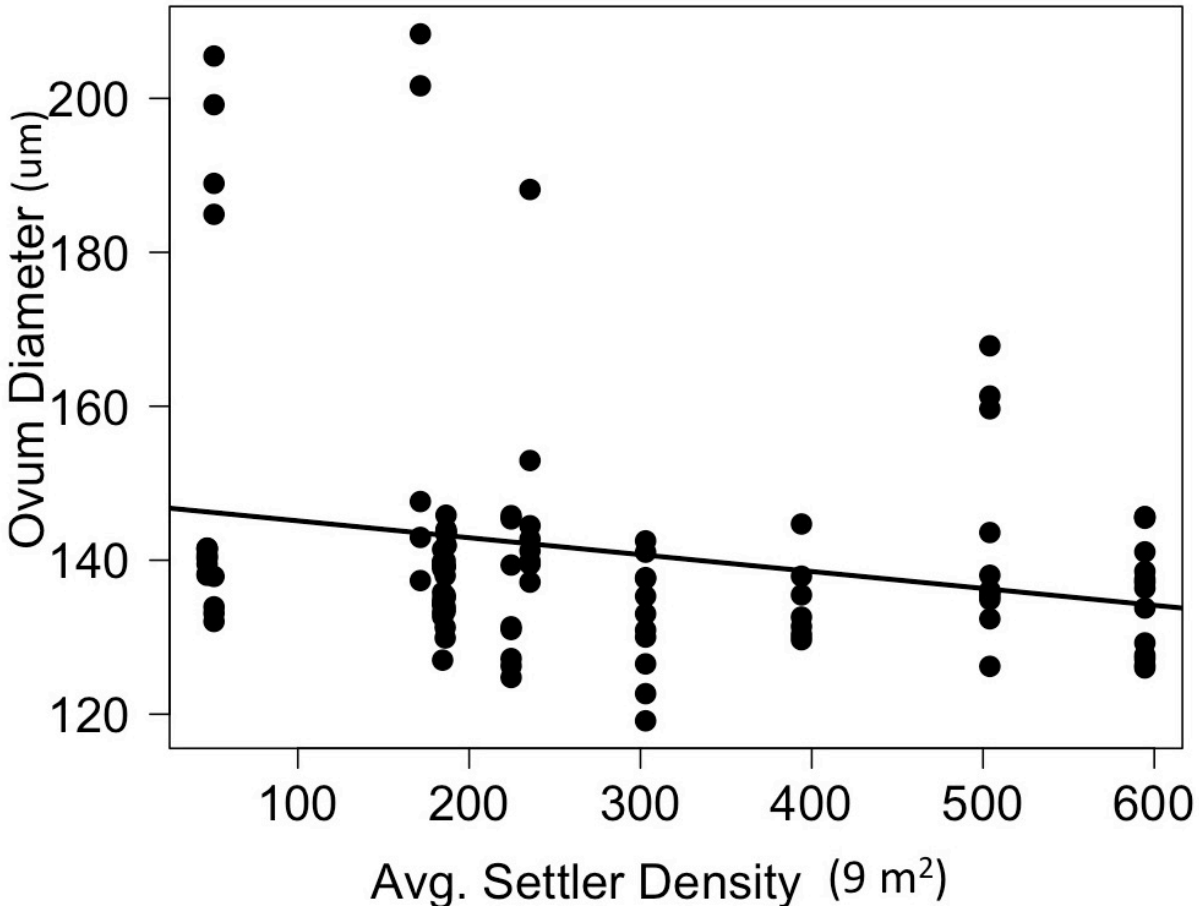

Figure 5.8: Correlation between the average ovum diameter produced and the average number of settlers seen in the two time periods (10 weeks) prior to adult collection.

CHAPTER 6

CONCLUSIONS

Fertilization is a complex process made up of numerous traits interacting that can be influenced by many environmental and biological factors, among which is the availability of sperm (Levitan 1996, Franke et al. 2002, Crimaldi and Zimmer 2014). My goal was to examine how interactions between traits, particularly those that affected different processes in fertilization (i.e. collision and fusion rates), might influence fertilization in different sperm environments. I had hypothesized that at low sperm densities collision rate would play a larger role in determining fertilization success than compatibility, but that compatibility would play a larger role as sperm availability increased and eggs were exposed to more sperm, which they could then discriminate between (Sherman et al. 2015, Levitan 2018). Instead, my results suggested that interactions between traits were important in determining fertilization success in most sperm environments and offered a mechanism for how assortative mating between gametes could occur in nature.

Chemoattractants can mediate sperm competition by providing sperm with information about sperm-egg compatibility prior to attempted binding (Frank 2000, Evans et al. 2012, Hussain et al. 2017). This work is the first time differences in sperm swimming behavior have been linked with genetic differences in GRPs (which mediate compatibility). I found that more sperm were recovered from wells with eggs from individuals who had the same female GRP genotype as them (Chapter 4). This tendency for sperm to aggregate around eggs from individuals who have the same genotype as them at the receptor locus may give those sperm an advantage during sperm competition. This would result that an increasing number of offspring that are homozygous at the receptor being produced as sperm competition increases. This pattern was exactly what was seen in wild populations; as settler density increased, an increasing proportion of those settlers were homozygous at the receptor protein, more than would be expected under random mating (Chapter 5). Additionally, laboratory results also hinted that sperm from individuals that had the same genotype at the receptor locus as the individual whose eggs they were exposed to could garner a higher share of paternity (Chapter 3).

While my results suggest that functional links exist between genetic variance in genes mediating compatibility and chemotaxis, it is still difficult to parse out how much of an individual's fertilization success may be due to compatibility and how much is due to the advantage gained by being able to better orient towards a specific egg type. It is possible that individuals that share the same genotype at the receptor locus are not truly more compatible but gain a higher paternity in the field and in the lab because the sperm are better able to orient themselves towards those eggs (Hussain et al. 2016). Further examination is needed to determine the relative contributions of compatibility and chemotaxis on fertilization success, as well as to determine the mechanism behind the functional link between compatibility and chemoattractants found here. However, my results do suggest that chemoattractants can be used to mediate sperm competition and promote assortative mating between gametes based on their GRP genotypes.

Assortative mating between individuals who share the same GRP genotypes has been seen in sea urchins, although it was between individuals that share the same ligand rather than receptor genotype (Palumbi 1999, Levitan and Stapper 2010). In sea urchins it was hypothesized that linkage disequilibrium between the male and female protein may exist due to assortative mating as the unexpressed ligand genotype in female urchins could be used to predict fertilization success with males based on their (expressed) ligand genotype (Palumbi 1999, Levitan and Ferrell 2006, Levitan and Stapper 2010). Some genetic evidence exists which suggests that linkage disequilibrium between receptor and ligand genes does exist (Stapper et al. 2015), however direct examinations of how genetic variation in receptor proteins and interactions between receptor and ligand proteins can affect fertilization success have not been completed until now.

I was unable to detect differences in compatibility between specific ligand-receptor pairings in laboratory studies (Chapters 2 and 3). However, the same pattern of increased fertilization success based on GRP matching at the receptor locus was seen in natural populations (Chapter 5) and hinted at in laboratory-based crosses (Chapter 3), suggesting that assortative mating between compatible individuals may be occurring. In the field, deviations of observed homozygosity from expected genotype values under random mating increased with increasing settler density, resulting in an increased number of offspring that were produced that were homozygous at the receptor locus and heterozygous at the ligand locus. This suggests two

things; one assortative mating was more likely to occur when more sperm were present in the water column. Second, like in urchins, matching between expressed and unexpressed genotypes can have an important effect on fertilization success, again suggesting that linkage between male and female proteins may exist. It is possible that a different marker than the one that I used for the receptor protein would reveal linkage between different male and female protein pairs and show greater difference in affinities between them.

Regardless of the exact nature of compatibility between different GRP variants, my results showed that genetic variation in GRPs could affect fertilization success. The difference in fertilization success between high performing and low performing male GRPs could be as high as 34% in environments where sperm availability was high (Chapter 2). Although differences between male ligands were found to affect fertilization success at high sperm environments, my results suggests that the joint actions of traits that affect collision and fusion rates are most important in determining fertilization success (Chapter 2). It also suggests that the interactions between traits can be an important mechanism for maintaining variation within traits, as different combinations of traits could be selected for, particularly as sperm availability increases (Chapter 2). And while studies have shown that context-dependent selection based on sperm environment exists (Levitan and Ferrell 2006, Crean and Marshall 2008, Johnson et al. 2013), this is one of the few times it has been examined in the context of both sperm environment and other trait values (Chapters 2 and 5). My results suggest that a trait's response to increasing sperm availability can be modified based on the values of other traits found within an individual (Chapter 5). This suggests that associations between traits can arise, again due to assortative mating between gametes.

Overall my dissertation shows that interactions between traits that affect different fertilization process, like fusion and collision, can play an important role in fertilization success, and thus may shape gamete trait evolution. Associations between traits, like the functional link between GRP variation and chemoattractants, can offer a mechanism for assortative mating between different gametes. And while changes in collision rate traits are generally in the direction predicted based on sperm environment (Levitan and Irvine 2001, Podolsky 2001, Crean and Marshall 2008, Lymbery et al. 2018), they can be modified based on other trait values, suggesting that associations between traits produced by an individual can arise. Taken together,

this suggest that associations between traits created and maintained via assortative mating, particularly at higher sperm availabilities, may help to maintain variation within a trait.

APPENDIX A

SUPPLEMENTARY INFORMATION FOR CHAPTER 2

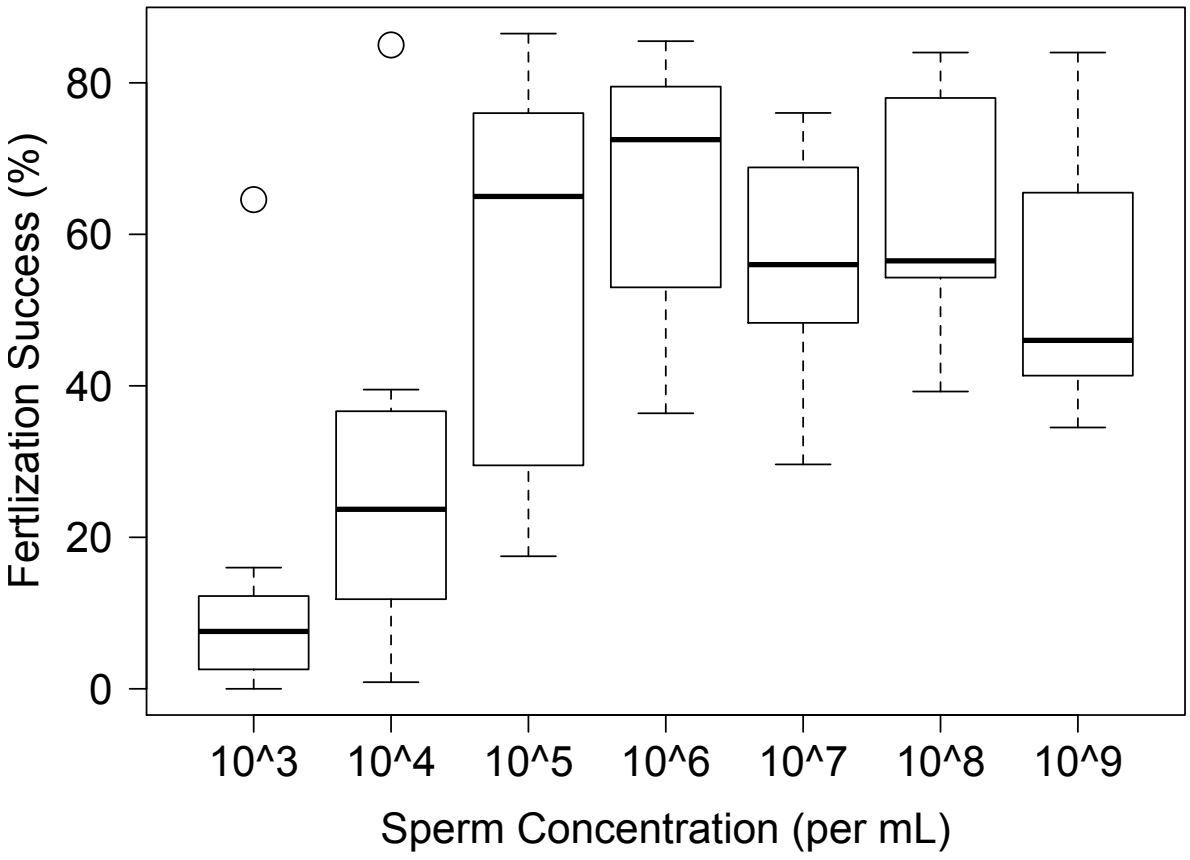

Figure A.1: Fertilization curve for *Ciona robusta* created by fertilizing a pool of eggs at multiple sperm concentrations and counting the number of fertilized eggs. At least seven crosses were performed at each sperm concentration, and sperm pooled from several males were used.

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BIOGRAPHICAL SKETCH

Ellen Kosman was born in Rochester, NY and began studying biology in Buffalo Sate College, before obtaining her Bachelor of Science degree in biology at San Francisco State University in 2005. She then moved to California State University at Long Beach, where she studied how variation in maternal investment in Bryzoans affected various aspects of offspring morphology and behavior. She earned her MS in Biology from CSU Long Beach in 2008. After a brief hiatus, where she worked at Cabrillo Marine Aquarium as an educator and aquarist specializing in the care of planktonic and larval organisms, she came to Florida State University in 2011. While at Florida State University, she studied how sperm availability could affect the evolution of gamete traits, combining her interests in evolution and ecology. She defended her dissertation in 2018.

Publications:

Kosman, E.T., B. Hipp, and D.R. Levitan. 2017. Chemoattractant-mediated preference of non-self eggs in *Ciona robustas* sperm. *Biological Bulletin*. 233:183-189

Kosman, E.T. and D.R. Levitan. 2014. Sperm competition and the evolution of gametic compatibility in externally fertilizing taxa. *Molecular Human Reproduction*. 20:1190-1197 (Invited Review)

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Presentations:

Kosman, E. and D. Levitan. 2017. Evidence of temporal linkage between settler density and gamete compatibility in a tunicate population. Benthic Ecology Meeting, Myrtle Beach, SC

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Kosman, E. and D. Levitan. 2015. Interactions between molecular and physiological gamete traits on fertilization success under sperm-limiting and polyspermy conditions. Society for Integrative and Comparative Biology, West Palm Beach, FL

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Kosman, E.T. and S. Cohen. 2005. Nuclear and mitochondrial DNA variation between onshore and offshore populations of the American lobster (*Homarus americanus*) between Long Island and the Gulf of Maine. West Coast Biological Sciences Undergraduate Research Conference (poster)

Scholarships & Grants:

2017	Robert B. Short Zoology Scholarship (FSU)
2017	Owenby Travel Award (FSU)
2015	Gramling Research Award in Marine Biology (FSU)
2014	Gramling Research Award in Marine Biology (FSU)
2012	Committee on Faculty Research Support Award (FSU, joint application prepared with advisor)
2012	Coastal Marine Graduate Student Scholarship (FSU CML)
2006	Grant in Aid of Research (SICB)
2005	Marine Biology Educational Scholarship (SCTC)
2005	Research Experience for Undergraduates Scholarship (NSF)
2004	Undergraduate Mentoring in Environmental Biology Scholarship (NSF)