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Establishing the Biological and Chemical Processes Regulating Fertilization Success in the Red Abalone, *Haliotis rufescens*

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Biology

by

Jeffrey Allan Riffell

2004

INFORMATION TO USERS

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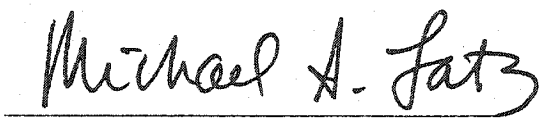

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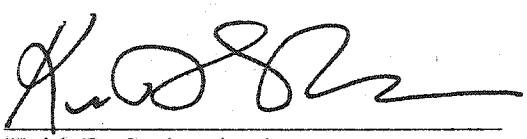

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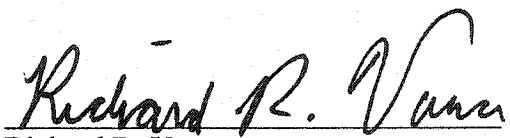

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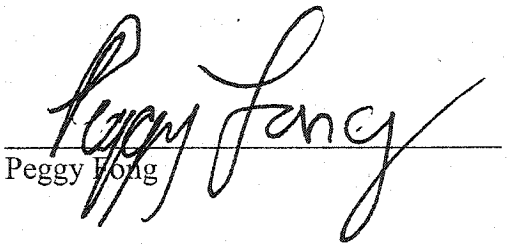

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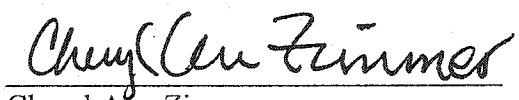

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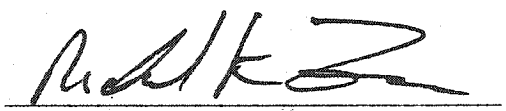

Richard K. Zimmer, Committee Chair

University of California, Los Angeles

2004

To my parents - Jim and Marty,

for providing the foundations of love and inspiration.

In memory of Mia Tegner.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGMENTS

Mia J. Tegner was a leading researcher and tireless advocate for the conservation of endangered abalone populations, and first introduced me to the joys (and pain) of working with abalone. This dissertation would not have been possible without her, and is dedicated to her memory.

The dissertation author is the primary researcher and author of all chapters. Chapter I is a version of Riffell, J.A., P.J. Krug, and R.K. Zimmer. 2002. Fertilization in the sea: The chemical identity of an abalone sperm attractant. *Journal of Experimental Biology 205*: 1439-1450. Chapter II is a version of Riffell, J.A., P.J. Krug, and R.K. Zimmer. The ecological and evolutionary consequences of sperm chemoattraction for fertilization success. *Proceedings of the National Academy of Sciences, USA 101(13)*: 4501-4506. For both chapters, the contributions of Patrick J. Krug and Richard K. Zimmer (coauthors) were inestimable in improving the quality of the manuscripts with their groundbreaking ideas and hard work. I would also like to acknowledge the help of Luis Vilchis, Kristin Riser, Paul K. Dayton, Victor D. Vacquier, Michael I. Latz, and Cheryl Ann Zimmer for graciously contributing ideas, laboratory space and facilities during the experimental and writing phases of these chapters. A version of Chapter III will be submitted for publication in the journal *American Naturalist*. The contribution of Richard K Zimmer (coauthor on this study) to this chapter has been invaluable. I would also like to thank Cheryl Ann Zimmer, Richard R. Vance, Paul K. Dayton and Michael I. Latz with help on the statistics and editing of this chapter.

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During my dissertation, all matters financial and academic were facilitated by the excellent support staff at the UCLA Department of Ecology and Evolutionary Biology (EEB). Significant efforts by Jocelyn Yamadera ensured that I was able to get my degree on time, and receive all the necessary departmental signatures while I was in Arizona. Sorry about that Jocelyn. The EEB business office kept a constant supply of resources and equipment flowing into this project. Rosalina Steiner and Dan Horowitz saved me many times. Finally, the faculty and graduate students in EEB made my time at UCLA both fun and exciting.

This study required a significant amount of time (~ 4 years) using the facilities and laboratories at Scripps Institution of Oceanography (SIO). I am forever indebted to Paul K. Dayton and Michael I. Latz for allowing me to run amok in their labs, answering my inane questions, and allowing me to learn natural history, marine ecology, and small scale biological-physical processes from the best. I can't say enough about these guys.

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tutored me in ecology and hydrodynamics, and has done her best to cure my numerous problems with the English language. I thank her for her persistence. Mike Latz has generously provided resources, ideas, and support throughout this project. In fact, he was the impetus for much of my work at SIO, and thus for the success of my research. Two colleagues, although not official committee members, provided helpful advice, guidance, and encouragement during this project: Vic Vacquier was extremely generous with his time and helped me to understand the evolutionary implications for gamete recognition factors; the generosity, wisdom, and vision of Paul Dayton helped me tremendously, and serves as a model for my academic career.

Richard Zimmer, my advisor, is an incredible man. He has mentored me in science and non-science, and has supported me when I needed it the most. Through his guidance, I have experienced things that I never could have imagined. I have been able to ask, and answer, questions that I would never have dreamed would be possible. His view and knowledge of chemical communication, behavior, and ecology is inspiring. His stories are hilarious. I am very proud to have been his student.

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complete without the unselfish contributions and love of my fiancée Kiley. She is my inspiration, and I cannot imagine a more perfect lifelong companion.

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PUBLICATIONS

Riffell, J.A., P.J. Krug, and R.K. Zimmer. 2002. Fertilization in the sea: The chemical identity of an abalone sperm attractant. *Journal of Experimental Biology 205*: 1439-1450.

Spehr, M., G. Gusselman, A. Poplawski, J.A. Riffell, R.K. Zimmer, and H. Hatt. A novel testicular odor receptor controls human sperm chemotaxis. *Science 299*: 2054-2058.

Riffell, J.A., P.J. Krug, and R.K. Zimmer. Sex and the single cell: The consequences of sperm chemoattraction for fertilization success. *Proceedings of the National Academy of Sciences, USA 101(13)*: 4501-4506.

Spehr, M., Schwahn, K., Riffell, J.A., Barbour, J., Zimmer, R.K., Neauhaus, E.M., Hatt, H. Olfactory receptor-mediated chemotaxis in human sperm: key role of particulate adenylate cyclase. *Journal of Biological Chemistry 279(40)*.

ABSTRACT OF THE DISSERTATION

Establishing the Biological and Chemical Processes

Regulating Fertilization Success in the Red Abalone, *Haliotis rufescens*

by

Jeffrey Allan Riffell

Doctor of Philosophy in Biology

University of California, Los Angeles, 2004

Professor Richard K. Zimmer, Chair

Chemical communication between sperm and eggs is ubiquitous in organisms with widely divergent reproductive strategies. Sperm attractants are found in marine taxa that broadcast their gametes into the sea, as well as terrestrial organisms with internal fertilization, such as humans. Chemosensory-mediated behavior thus is a key component in sperm-egg interactions and fertilization. Despite nearly a century of research, the mechanisms controlling fertilization remains one of the least understood biological processes. For example, waterborne egg-factors that attract sperm are thought to be ecologically meaningful for increasing gamete encounters, or evolutionary significant by maintaining species barriers, but the contributions remains unknown. In this study, I (i) developed analytical and behavioral techniques to establish the reproductive consequences of sperm attractants, and (ii) examined the biological processes controlling

fertilization success in the red abalone, *Haliotis rufescens*. Using RP and size-exclusion chromatographic techniques, the amino acid L-tryptophan, was found to be the natural sperm attractant. The production of tryptophan from live abalone eggs elicited sperm chemotaxis, effectively doubling the egg size. When the enzyme tryptophanase, which selectively degrades tryptophan, was added to the seawater around eggs, sperm no longer were able to locate the eggs, gamete encounters dropped, and fertilization levels dramatically lowered. Thus, the natural tryptophan gradient is critical for gamete encounters. To establish the evolutionary consequences of sperm attractants, red and green (*Haliotis fulgens*) abalone gametes were crossed, and fertilization assays conducted. Although long theorized as barriers to hybridization, species-specific sperm attractants were only minor contributors to reproductive isolation. When the influence of sperm and egg concentration, sperm-to-egg ratio, and gamete contact times on reproductive success was established, egg concentration had the same effect as sperm concentration on fertilization rates, and eggs, not sperm, were the limiting factor controlling abalone fecundity. These results contrast widely with the theory that sperm is the primary determinant for fertilization success. Given the fact that eggs, rather then sperm, effectively control reproduction, chemoattractants may have evolved thru sperm competition.

Chapter I

Fertilization in the sea: The chemical identity of an abalone sperm attractant

Abstract

Chemical communication between sperm and egg is a key factor mediating sexual reproduction. Dissolved signal molecules that cause sperm to orient and accelerate towards an egg could play pivotal roles in fertilization success, but such compounds are largely undescribed. This investigation considered the behavioral responses of red abalone (*Haliotis rufescens*) sperm to soluble factors released into seawater by conspecific eggs. Sperm in proximity to isolated live eggs swam significantly faster and oriented towards the egg surface. Bioassay-guided fractionation was employed to isolate the chemoattractant, yielding a single pure, fully active compound after reversed-phase and size-exclusion HPLC. Chemical characterization by NMR indicated that the free amino acid L-tryptophan was the natural sperm attractant in *H. rufescens*. L-tryptophan was released by eggs at concentrations that triggered both activation and chemotaxis in sperm, exhibiting significant activity at levels as low as 10^{-8} *M*. The D-isomer of tryptophan was inactive, showing that the sperm response was stereospecific. Serotonin, a potent neuromodulator and tryptophan metabolite, had no effect on sperm swim speeds or orientation. In

experimental treatments involving either an elevated, uniform concentration of tryptophan (10^{-7} *M*) or the addition of tryptophanase, an enzyme that selectively digests tryptophan, sperm failed to navigate towards live eggs. A natural gradient of L-tryptophan was therefore necessary and sufficient to promote recruitment of sperm to the surface of eggs in red abalone.

Introduction

Chemical signals play a crucial role in many aspects of fertilization, including activation and attraction of sperm, induction of the acrosome reaction by egg jelly, binding of sperm to the egg envelope, and oocyte maturation (Ward and Kopf, 1993; Foltz and Lennarz, 1993; Vacquier, 1998; McCarter et al., 1999). Evidence that sperm and eggs communicate at a distance has been gathered from taxa with highly divergent reproductive strategies (Miller, 1985; Ward and Kopf, 1993; Eisenbach, 1999; Miller et al., 2001). Sperm activation and chemotaxis have been demonstrated in marine animals that broadcast gametes into the sea, such as corals and sea urchins, and from terrestrial organisms with internal fertilization, including humans (Miller, 1985; Eisenbach, 1999). Chemically mediated behavior may thus be a key component regulating sperm-egg interactions, whether occurring in the turbulent ocean environment or within a mammalian reproductive tract. Although long recognized as potentially critical, specific functions of dissolved chemical signals in fertilization are unknown, especially under natural environmental conditions. Signaling between eggs and sperm may be important evolutionarily for maintaining species barriers, and significant ecologically for increasing

gamete encounters, but the role of sperm attractants in mediating these processes has not been determined.

Examples from diverse taxa have suggested a role for egg-derived chemoattractants, compounds that activate conspecific sperm (increasing swim speed) and trigger chemotaxis towards the egg (Miller, 1977, 1985, 1997). Such attractants are believed to increase the probability of contact between sperm and egg, and may enhance fertilization success in aquatic environments. Despite the potential importance of dissolved signal molecules, few sperm attractants have been isolated and chemically characterized. A series of peptides, described from sea urchin egg jellies, increased sperm respiration and/or motility at nanomolar levels (Ward et al., 1985; Suzuki and Yoshino, 1992). Peptides with similar activity were isolated from eggs of other echinoderms (Miller and Vogt, 1996). These peptides are broadly active for species within a given order, but it is unclear whether they are effective at physiological pH (Ward and Kopf, 1993). Furthermore, only one such peptide triggered chemotaxis (Ward et al., 1985; Suzuki and Yoshino, 1992). A mixture of fatty alcohols was reported to induce sperm chemotaxis in the mass-spawning coral *Montipora digitata*, while a similar role was attributed to the diterpene (-)-*epi*-thunbergol from eggs of the coral *Lobophytum crassum* (Coll et al., 1994, 1995). None of the compounds from coral eggs were water-soluble, however, and the activity of the diterpene was not stereospecific. Moreover, these compounds were active only at high concentrations (10^{-4} *M*).

Although current theory holds that membrane-bound proteins promote gamete recognition, soluble egg compounds that attract only conspecific sperm might act as pre-

zygotic factors maintaining species integrity and increasing fertilization rates (Harrison et al., 1984; Vacquier, 1998). Surface proteins involved in sperm-egg interactions have been better characterized for abalone (genus *Haliotis*) than for any other organism, and demonstrate strong selection for species-specific gamete recognition (Vacquier, 1998). This system therefore represented a logical starting point to investigate the importance of soluble egg factors and chemosensory-mediated sperm behavior in fertilization and evolution. Further, natural populations of abalone have been heavily exploited and adult densities are at historic lows (Tegner et al., 1989, 1996; Davis et al., 1996; Alstett et al., 1996). If free-spawned sperm and eggs are diluted before gametes can make contact, fertilization success may represent a bottleneck to population recovery in the field. Understanding how sperm sensory systems and egg signals mediate fertilization thus has important implications for conservation efforts to preserve and restore endangered populations of vulnerable species.

Materials and Methods

Abalone collections, maintenance and spawning

This study was performed at Scripps Institution of Oceanography, University of California at San Diego. Mature *Haliotis rufescens* (red abalone) from Cultured Abalone, Inc. (Goleta, CA) were held in aquaria of running seawater (15 °C). The animals were fed fresh kelp, *Macrocystis pyrifera*, collected twice weekly from the Point Loma area. Ripe adults were identified by gonadal growth beyond the shell (Hahn, 1989), and the sexes were separated and fed *ad libitum* for 2 wks prior to spawning induction (Morse et

al., 1977). Individuals were placed singly in chambers and the seawater pH was raised to 9 by adding 6.5 ml of 2 *M* Tris-base (tris-hydroxymethyl-aminomethane) per liter, followed by 4 ml of 6% H_2O_2 per liter. After 2.5 h exposure, the chamber was emptied, rinsed and refilled with 0.45-μm filtered seawater (FSW). Spawning occurred within 2 - 4 h of H_2O_2 exposure, with males releasing gametes 10 - 30 min before females. A typical male (12 cm length) spawned 20 - 60 ml of sperm solution (10^9 sperm/ml), whereas a female of similar size spawned 10 - 20 ml of egg suspension (10^5 eggs/ml). Gametes were collected above the excurrent tremata and placed in centrifuge tubes (sperm) or beakers (eggs) filled with FSW until used. Experiments employed only fresh eggs and sperm, beginning 10 min after spawning and continuing for 30 min thereafter. Additionally, fertilization assays were conducted in combination with each experiment to establish the competency of harvested gametes (see methods in Gee and Zimmer-Faust, 1997). Material from each of the spawns was viable, producing 83-99% fertilization when sperm and eggs were mixed.

Measurements of sperm activation and chemotaxis

Fertilization may be mediated by waterborne chemical attractants that cause sperm to orient and swim faster. Our goal was to determine if sperm recognize signal molecules released from conspecific eggs at a distance, and change their swimming behavior to increase the probability of successful contact. The behavioral responses of sperm to isolated eggs and chemical solutions were measured using: (1) Computer-Assisted Video

Motion Analysis (CAVMA) to detect changes in swimming speeds (sperm activation) and (2) a flat capillary assay for chemotaxis near a source of diffusing material. In each experiment, data from bioassays were statistically analyzed by 1-way ANOVA, using a post-hoc Scheffé test for unplanned comparisons of means when the *F* ratio was significant (Day and Quinn, 1989).

Sperm activation – Because abalone release sperm into the ocean, fertilization occurs where walls (i.e., microscope slides) are absent. Several theoretical treatments predict that drag forces significantly affect flagellar motion within ~10 sperm body lengths of a slide surface (Gray and Hancock, 1955; Reynolds, 1965; Katz, 1974; Katz and Blake, 1975; Lighthill, 1975). Near a wall, propulsive forces generated by flagellae are larger and drive a cell forward at a faster speed (Gee and Zimmer-Faust, 1997). To minimize potential artifacts, we assayed sperm motility in a 10 mm (length) × 10 mm (width) × 5 mm (depth) Plexiglas chamber held at 15 °C. Images of sperm (at 2.5×10^3 cells/ml) swimming were recorded on magnetic tape using a video camera (NEC model TI 23A) mounted on an Olympus IX70 compound light microscope at a 90× magnification. The camera had a 100 μm depth of field and focused on a region ~ 2 mm, or about 70 sperm body lengths, away from the nearest chamber wall.

Video images of sperm were digitized at 30 frames/sec and processed over 10 sec intervals using a CAVMA system (Motion Analysis Corp. model VP 320, ExpertVision, and custom software) interfaced with a Sun SPARC 2 computer workstation (Gee and Zimmer-Faust, 1997). Speed and direction of swimming were determined on a frame-by-frame basis, then averaged over each entire path. To avoid mistaking vertically for

horizontally moving cells, short paths ($\leq$ 10 frames) that consisted of computer images with sperm changing more than 20% in apparent size were discarded. All other paths were included in analyses.

Sperm chemotaxis – The migration of cells towards a region of elevated chemical concentration is called chemo-attraction, also known as positive chemotaxis. In this study, chambers with four separate compartments (Palleroni, 1976) were used for bioassays of chemotaxis thus modifying the capillary method of Adler (1966, 1973). Each compartment consisted of two wells connected by a channel. A six-microliter, flat micro-capillary tube (Drummond Scientific Co.) containing either a test or control solution was inserted into the channel with its ends touching fresh sperm suspensions (10^6 cells/ml) within the two wells. If attracted, significantly more sperm were expected to migrate towards the inside of the capillary with test solution than the control. Eight replicate capillaries of each test or control solution were used in each experiment; microelectrode measurements in and outside of the capillaries showed no change in oxygen levels during trials. After 15 min of incubation at 15 °C, the contents of each capillary were transferred to the well of a toxoplasmosis slide, heat fixed, and stained with 0.1% acridine orange for 1 min. Cell numbers were then determined by direct counting with an Olympus model BH2 microscope (at 67× magnification) equipped for phase-contrast and epifluorescence applications (Lopez-de-Victoria and Lovell, 1994).

Sperm behavior around individual live eggs

An initial experiment was performed to determine if the natural release of factors from a single live egg could affect the motility of nearby sperm. A freshly spawned, live egg was placed in an observation chamber, and sperm (2.5×10^3 cells/ml) were gently pipetted into the chamber 10 min after addition of the egg. This was the shortest delay permitted by experimental constraints (preparation of sperm solution, locating and centering the egg in the field of view), and ensured that sperm were added to all treatments in an equivalent manner. Sperm behavior within 300 μm of the egg surface was videotaped for 30 sec. Alternatively, as a control, a brine shrimp egg was placed into a chamber, sperm from the same male was added, and behavior was again recorded. Seven replicate trials were each conducted for abalone and brine shrimp eggs. Video images were digitized and analyzed through CAVMA to quantify sperm speed and orientation. Swim speeds of sperm near abalone versus brine shrimp eggs were compared with an unpaired 2-tailed *t*-test. Circular statistics were applied to describe sperm navigation around individual eggs. Both the vector mean length (*r*) and swim direction were determined for each treatment, with angles measured relative to the egg surface (0°). Mean direction was compared to a uniform circular distribution using a unimodal Rayleigh's test (Zar, 1984).

Release of sperm attractant from eggs into surrounding sea water

We next determined how rapidly eggs could release sperm attractants into the surrounding medium. Solutions of egg-conditioned sea water (ESW) were prepared by incubating freshly spawned red abalone eggs (5×10^4 eggs) in 1.0 ml FSW in sterile tubes, using 3 replicates per time point. Control solutions were prepared in parallel using 1.0 ml

FSW in replicate tubes. Eggs were incubated at 15 °C for the shortest times possible (2 min in one set of treatments and 5 min in a second set). After incubation, eggs were removed using a mesh filter and all solutions were then filtered to 0.22 μm to remove particulates. An aliquot of freshly spawned sperm solution was placed in a chamber filled with FSW (400 μl) for observation at a final concentration of 2.5×10^3 sperm/ml. Each conditioned sea water solution was assayed by gently injecting a 5 μl aliquot through a microsyringe into the chamber, and recording activity of sperm over 10 sec within 300 μm of the syringe tip. Sperm swimming behavior was analyzed by CAVMA as described above; the order of treatments was randomized during bioassays.

Isolation of sperm attractant by bioassay-guided fractionation

The sperm attractant from red abalone eggs was isolated from egg-conditioned seawater (ESW). This solution was prepared by bathing 10^3 eggs/ml in FSW for 18 hr at 15 °C; the prolonged incubation was used to concentrate attractant without damaging the eggs. A reversed-phase C_{18} cartridge (10 ml volume) was washed with methanol and equilibrated in water. An ESW solution (200 ml) was loaded onto the column, followed by a wash with MilliQ water (40 ml) to elute all salts and highly polar compounds. Bound material was then eluted from the column using a stepwise gradient from 25 to 100% methanol in water, in 25% increments (40 ml each). Each fraction was dried under vacuum and re-suspended in the original volume of FSW prior to bioassays. Bioactivity was quantified using both the flat-capillary assay for chemotaxis and CAVMA for sperm activation.

The bioactive fraction from the C_{18} Sep-Pak cartridge was further purified by reversed-phase (RP) HPLC on a Phenomenex C_{18} column (250 × 10 mm, 5 μm particle size). Elution was isocratic at 1 ml/min with 5% solvent B for 5 min, followed by a linear increase in solvent B from 5 to 50% from 5 to 35 min (Solvent A, 0.1% TFA in water; Solvent B, 0.1% TFA in methanol). Peaks were monitored using a diode array detector (Beckman) measuring absorbance at 220 and 254 nm. Fractions (0.5 ml) were collected throughout the run using an automated fraction collector (Gilson). Aliquots (40 μl) were taken from fractions corresponding to one major and three minor peaks observed at 220 nm, and were dried to remove all solvents and then re-suspended in 20 ml FSW for bioassays.

Final purification of the bioactive peak employed size-exclusion HPLC, using a Bio-Sep SEC-S2000 column (300 × 7.8 mm) eluting at 1 ml/min in water. The column was calibrated with an HPLC peptide standard mixture (Sigma), and the effect of $\log_{10}$-transformed molecular weight on retention time was determined by a Model 1 regression; the approximate size of unknown compounds eluting from the column could then be calculated using the regression function. Fractions were collected throughout the run, and those corresponding to peaks detected at 220 nm were pooled, dried, and re-suspended in FSW. The major peak and regions of the chromatogram corresponding to minor peaks were bioassayed against freshly spawned sperm.

Structure elucidation of the natural sperm attractant

Complete structure elucidation of the natural product was performed by nuclear magnetic resonance spectroscopy (NMR) on a Varian Inova NMR operating at 500 MHz for ^{1}H detection (Glycobiology Core Facility, UC San Diego). The attractant was pre-exchanged twice in D_2O to remove exchangeable hydroxyl protons prior to spectral acquisition. All NMR experiments were performed in "100%" D_2O at 25 °C. One-dimensional ^{1}H NMR spectra and 2D COSY spectra were acquired using a ^{1}H Nano-NMR probe on the small amount of sample (50 μg) initially available. Heteronuclear 2D experiments were performed on 400 μg of the pure attractant, using a Shigemi NMR tube and a 5-mm inverse detection probe operating at 500 MHz. Carbon chemical shifts, relative to an internal acetate standard, and the structure of the compound were assigned through HSQC and HMBC 2D NMR experiments.

Verification of chemoattractant structure

To confirm the identity of the natural product, the retention time of the purified attractant was compared with a synthetic standard by analytical HPLC. Prior to chromatography, attractant or standard solutions were treated with *o*-phthalaldehyde (OPA), which reacts with primary amines to generate fluorescent derivatives. To prepare a reagent solution, OPA (50 mg) was dissolved in absolute methanol (1.25 ml), and 2-mercaptoethanol (50 μl) and 0.4 *M* sodium borate buffer (11.2 ml, pH 9.5) were added and mixed. A solution of attractant or a synthetic standard in HPLC-grade water was then mixed with the OPA reagent in a 4:1 ratio and reacted at room temperature for 1 min prior

to injection by an autosampler (Beckman model 508). Fluorescent derivatives were separated by RP HPLC on an Ultrasphere ODS column (4.6 mm × 25 cm, 5 μm particle size) equilibrated in Solvent A. One min after injection, a linear gradient from 0 to 20% Solvent B in Solvent A was run for 15 min, increasing to 50% Solvent B in 9 min, and finally to 100% Solvent B in 15 min (Solvent A- 1:19:80 tetrahydrofuran:methanol:0.05*M* sodium acetate; Solvent B- 80:20 methanol:0.05*M* sodium acetate). Solvent mixtures were pH 6.8 and elution was at a flow rate of 1 ml/min. Post-column detection used a fluorometer (Jasco Instruments) monitoring at 453 nm with an excitation wavelength of 332 nm; peaks were integrated by Gold Nouveau software (Beckman Coulter). The method was calibrated by injection of standards to determine the expected retention time and for automatic quantification of sample runs. Co-injection of the natural attractant with a synthetic standard was used to demonstrate elution of a single pure peak.

Activity of sperm chemoattractant and effects of enzymatic treatment

To ensure that trials were performed at physiologically relevant concentrations, dose-response curves were generated for the pure attractant to identify the ED_{50} (i.e., the dose of half-maximal response; Tallarida and Murray, 1981). Attractant solutions were tested on fresh sperm in activation and chemotaxis bioassays at concentrations ranging from 10^{-5} to 10^{-9} *M* with FSW and red abalone ESW as negative and positive controls. Relationships between the dose of the solution (log_{10}-transformed) and sperm swim speed or cell density were estimated and compared by Model 1 regression and ANCOVA (Sokal and Rohlf, 1981). To provide information on structure-activity relationships and

the specificity of sperm response, structurally related agents were obtained from Sigma at the highest degree of purity, and their activity was compared with that of the natural attractant.

To test the effects of enzymatic degradation on attractant solutions, the enzyme tryptophanase (0.1 mg/ml) was incubated with 100 μM pyridoxal-5'-phosphate in FSW (pH = 7.9) at 37 °C for 1 h to ensure maximum formation of the holoenzyme (Phillips, 1991). Assays were initiated by addition of a given substrate solution (50 ml) to the enzyme mixture (1 ml), and incubated at 37 °C. After 10 min, the mixture was boiled to denature the enzyme and quench the reaction. Untreated ESW served as a positive control; boiled ESW was used as a control for the deactivation step of the enzymatic reaction. As a control for added protein content in the enzyme treatment, enzyme was added to ESW and immediately denatured by boiling the solution, before appreciable catalysis could occur. The negative control was FSW.

Requirement of the identified sperm attractant for navigation around individual live eggs

A final experiment was performed to test if sperm orientation depended on a gradient formed by diffusion of the attractant from the egg surface. A freshly spawned, live egg was placed in a chamber containing 400 μl FSW alone (control), or in one of three treatments. In the first treatment, eggs were placed in a solution of the tryptophanase enzyme, activated as described above and used at the same final concentration (2 μg/ml). The enzyme digests tryptophan in the medium around the egg, thus preventing the establishment of a gradient. In the second treatment, an egg was

placed in a solution of 10^{-7} *M* tryptophan. This condition tested for sperm behavior in the presence of a uniform concentration of the attractant, sufficient to overwhelm any gradient formed by diffusion from the egg. In the final treatment, an egg was placed in a solution of 10^{-7} *M* tyrosine, to control for any nonspecific effects of aromatic amino acid concentration on sperm swimming. Five replicate trials were conducted per test or control treatment.

Sperm (2.5×10^3 cells/ml) were gently pipetted into the chamber 10 min after addition of an egg to a solution, the shortest delay which allowed for locating the egg and uniformly adding sperm to each treatment. Sperm behavior within 300 μm of the egg surface was videotaped for 30 sec once fluid came to rest. Video images were digitized and analyzed through CAVMA to quantify sperm orientation to each egg. A unimodal Rayleigh's test was then applied to compare the vector mean direction with a uniform circular distribution (Zar, 1984). Trials were also run to establish any effects of treatment solutions on gamete viability. Fertilization assays were conducted as described above, but with eggs and sperm exposed to each of the four treatments (4 replicates per treatment); all treated gametes produced 93–100% fertilization.

Results

Sperm response to soluble egg factors

An initial experiment was performed to determine if sperm of the red abalone could recognize and respond to conspecific eggs at a distance. Behavior was quantified for sperm in proximity to an isolated abalone egg using computer-assisted video motion analysis

(CAVMA). There was a significant increase in sperm orientation towards the surface of an abalone egg (Figure 1-1, and results of a Rayleigh's test: mean angle = 2°, $r = 0.86$, $z = 59.3$, $p < 0.001$). In contrast, sperm orientation to brine shrimp eggs was at random (Fig. 1b, and results of a Rayleigh's test: mean angle = 60°, $r = 0.08$, $z = 0.2$, $p > 0.50$). Furthermore, sperm swam significantly faster near a live abalone egg than near a brine shrimp egg (unpaired 2-tailed *t*-test: $t = 2.14$, $p < 0.05$). Together, these results established that the natural release of compounds from abalone eggs affects sperm swimming speed and also sperm orientation.

Accumulation of the sperm attractant in sea water around freshly spawned eggs

The release of a sperm attractant from live eggs was predicted to condition sea water over time, resulting in a solution that would affect sperm motility. Freshly spawned eggs were therefore incubated in sea water for brief intervals, and sperm response to the resulting egg-conditioned sea water (ESW) was assayed. After 2 min, eggs had released enough attractant into the surrounding medium to cause a significant increase in sperm swimming speed, compared to FSW controls (Figure 1-2, and results of a 1-way ANOVA: $F_{2,184} = 31.1$, $p < 0.0001$; Scheffé test, $p < 0.0001$). Swim speeds were further elevated in ESW prepared for 5 min, compared to 2-min ESW (Figure 1-2; Scheffé test, $p < 0.005$) or FSW (Scheffé test, $p < 0.0001$). The attractant was thus rapidly released from fresh eggs, and accumulated in seawater in a time-dependent manner.

Isolation of red abalone sperm chemoattractant by bioassay-guided fractionation

The sperm attractant was isolated from bulk preparations of ESW by bioassay-guided fractionation. Solutions of ESW were initially loaded onto a C_{18} Sep-Pak cartridge, desalted with water, and eluted with a gradient of 0-100% CH_3OH. When column fractions were diluted back to the original volume of sea water and assayed, only material eluting in 25% CH_3OH significantly increased sperm swimming speeds compared with FSW controls (1-way ANOVA: $F_{6,312} = 13.2$, $p < 0.0001$; Scheffé test, $p < 0.005$). The 25% CH_3OH fraction induced the same swimming speed as unfractionated ESW (Scheffé test, $p > 0.99$).

The active fraction was concentrated and then partially purified by reversed-phase (RP) HPLC on a C_{18} column, eluting with a gradient of 5 to 50% CH_3OH in water/0.1% TFA. One major and three minor peaks were observed at 220 nm (Figure 1-3). All activity was contained in the major peak; this fraction induced the same level of activity as ESW, indicating that bioactivity was not affected by the chromatography. Fractions corresponding to the major peak had a significant effect on sperm swim speed, compared with the other peaks and FSW controls (Figure 1-3B, and results of a 1-way ANOVA: $F_{5,93} = 3.8$, $p < 0.005$). Mean swim speeds for FSW controls and inactive fractions ranged from 25-37 μm/s, while sperm exposed to ESW or the active fraction swam at 75-79 μm/s. The bioactive peak also induced significant attraction of sperm in the flat capillary assay (Figure 1-3C). The active peak attracted the same number of sperm to the capillary as unfractionated ESW (Scheffé test, $p > 0.87$), and no other fraction attracted significantly more sperm than FSW controls (Scheffé test, $p > 0.98$).

Final purification by size-exclusion HPLC yielded a single pure compound (Figure 1-4A). Only the major peak significantly elevated sperm swim speeds, compared with FSW controls (Figure 1-4B, and results of a 1-way ANOVA: $F_{5,138} = 15.8$, $p < 0.0001$; Scheffé test, $p < 0.0001$). The three minor peaks that eluted at earlier times from the column did not induce higher speeds than FSW. The major peak also significantly increased sperm recruitment to the capillary in chemotaxis assays, doubling sperm density in comparison to FSW controls and the three minor fractions (Figure 1-4C, and results of a 1-way ANOVA: $F_{5,93} = 8.4$, $p < 0.0001$; Scheffé test, $p < 0.05$). When diluted with the equivalent volume of sea water, the active peak did not differ from unfractionated ESW in either speed or flat-capillary assays (Scheffé test, $p > 0.93$). The column was calibrated with a peptide mixture of known molecular weights, obtaining a standard curve of $\log_{10}$-transformed molecular weight versus retention time. The approximate molecular weight of the attractant was estimated to be 200 Da based on its time of elution from the sizing column.

Structure elucidation of the natural sperm attractant

Preliminary analysis used a nano-NMR probe to obtain data on a small quantity of the attractant. The ^{1}H NMR spectra indicated that the molecule contained an aromatic heterocycle and was a single, pure compound after isolation by size-exclusion HPLC. Purification was scaled up 10-fold to provide sufficient material for analysis by 2D heteronuclear NMR experiments. Full assignment of all proton and carbon resonances was completed through HSQC and HMBC experiments. All chemical shifts and

observed 2D correlations were consistent with published data for the amino acid tryptophan (Pretsch et al., 1989).

Comparison with synthetic standard

Amino acids react with OPA (*o*-phthalaldehyde) to yield fluorescent derivatives that can be separated and quantified by analytical HPLC with a sub-nanomolar detection limit. An aliquot of the attractant was derivatized and compared with a synthetic tryptophan standard by RP HPLC. The derivatized attractant yielded a single peak with the same retention time as the co-injected synthetic standard (Figure 1-5), confirming that the natural attractant purified from ESW was tryptophan.

Dose-response curve to isolated sperm chemoattractant

Sperm responded to synthetic L-tryptophan in a dose-dependant manner, exhibiting elevated swim speeds and demonstrating chemotaxis in capillary assays. At $10^{-6} - 10^{-7}$ *M*, L-tryptophan increased sperm swim speed and density to the same degree as unfractionated ESW, relative to FSW controls (1-way ANOVA: $F_{5,195} = 7.6$, $p < 0.0001$; Scheffé test, $p < 0.05$). Levels of tryptophan in ESW solutions were approximately $1 - 2 \times 10^{-7}$ *M*. When plotted as $\log_{10}$-transformed data, the dose of tryptophan had a significant effect on sperm behavior (Figure 1-6, and results of a Model 1 regression of dose, x, on (a) swim speed, y: $y = 6x + 115$; $p < 0.0001$, and (b) cell density, y: $y = 8.5x + 126$; $p < 0.0001$). Doses above 10^{-6} *M* did not produce further increases in speed or density. The ED_{50} was 3×10^{-8} *M* for both swimming speed and

cell density, indicating that the change in dosage had a comparable effect on both aspects of sperm behavior.

Comparison of bioactivity of D- and L-tryptophan and serotonin

The D and L enantiomers of tryptophan have identical chemical properties in an achiral environment, such as an aqueous solution, and hence cannot be chemically distinguished by NMR or RP HPLC. If the observed bioactivity was due to any nonspecific effects of the tryptophan molecule, both D- and L-tryptophan would be expected to affect sperm behavior. To confirm that the attractant was the naturally occurring L-tryptophan, both isomers were tested independently in sperm activation and chemotaxis bioassays. A 10^{-7} *M* solution of L-tryptophan was identical in activity to unfractionated ESW (Figure 1-7), both in swimming speed (Scheffé test, $p > 0.97$) and chemotaxis assays ($p > 0.69$). However, a 10^{-7} *M* solution of the unnatural isomer D-tryptophan was inactive (Figure 1-7), inducing no more activity than FSW controls in swimming speed (1-way ANOVA: $F_{4,185} = 12.2$, $p < 0.0001$; Scheffé test, $p > 0.99$) and chemotaxis assays ($p > 0.69$). Similarly, the neuromodulator serotonin, a biosynthetic derivative of tryptophan, had no significant effect on abalone sperm when assayed at the same concentration (Figure 1-7; swimming speed, $p > 0.83$; chemotaxis, $p > 0.51$).

To establish that no component of ESW other than tryptophan was responsible for activity, solutions of ESW were treated with the enzyme tryptophanase. This enzyme selectively cleaves the indole ring from the tryptophan molecule. Treatment with tryptophanase destroyed all bioactivity of an ESW solution (Figure 1-8). Enzymatic

treatment of ESW reduced sperm swimming speeds (1-way ANOVA: $F_{4,141} = 12.9$, $p < 0.0001$) and cell densities (1-way ANOVA: $F_{4,95} = 21.1$, $p < 0.0001$) significantly below the corresponding untreated controls (Scheffé test, $p < 0.05$). Boiling alone did not affect the activity of ESW solutions. A positive control of ESW with tryptophanase added and immediately deactivated by boiling remained fully effective, demonstrating that the presence of denatured enzyme did not affect sperm behavior. Treatment of a 10^{-7} *M* tryptophan solution with the enzyme produced the same pattern of results (data not shown). All attractant activity therefore resided solely in L-tryptophan released from red abalone eggs.

Sperm navigation depends on a gradient of L-tryptophan around eggs

A final experiment was performed to test if abolishing the natural gradient of tryptophan around individual eggs would prevent navigation by sperm. Individual eggs were placed in assay chambers filled with FSW or a test solution, and after a brief interval freshly spawned sperm were added and imaged as they swam near the egg surface (Figure 1-9). In positive controls using a live egg in FSW, a significant proportion of sperm oriented and swam towards the egg (Figure 1-9A, and results of a Rayleigh's test: mean angle = 4°, $r = 0.75$, $z = 18.5$, $p < 0.0001$). However, when eggs were incubated in the presence of the enzyme tryptophanase, sperm were unable to orient towards the egg and swam at random (Figure 1-9B, and results of a Rayleigh's test: mean angle = 37°, $r = 0.27$, $z = 2.3$, $p > 0.10$). Enzymatic digestion prevented the establishment of a tryptophan gradient, and therefore inhibited sperm chemotaxis.

Controls showed that enzyme treatment had no effect on sperm viability, and there were no other apparent effects on cell motility.

To further test if sperm require a chemical gradient for navigation, eggs were placed in a 10^{-7} *M* solution of L-tryptophan. Such a uniformly elevated concentration could overwhelm the signal of tryptophan diffusing from an egg, preventing sperm from perceiving a gradient. Sperm movement was at random in 10^{-7} *M* L-tryptophan (Fig. 9C, and results of a Rayleigh's test: mean angle = 27°, $r = 0.11$, $z = 0.5$, $p > 0.50$), confirming that disruption of the natural gradient abolishes sperm orientation. As a control for increasing the concentration of an amino acid in the sea water medium, sperm were also imaged around eggs in a 10^{-7} *M* solution of L-tyrosine (Figure 1-9D). We tested L-tyrosine because it was an aromatic molecule similar to tryptophan, and as an L-amino acid could potentially be taken up or metabolized by cells. Sperm were significantly oriented towards the egg in 10^{-7} *M* L-tyrosine, as in FSW controls (Figure 1-9D, and results of a Rayleigh's test: mean angle = 3°, $r = 0.66$, $z = 14.2$, $p < 0.0001$).

Discussion

The chemical identity of an abalone sperm attractant

The free amino acid L-tryptophan is the natural chemoattractant for sperm of the red abalone, *Haliotis rufescens*. This metabolite is rapidly released from eggs at concentrations sufficient to induce changes both in speed and direction of sperm swimming. The L-enantiomer of tryptophan was bioactive at a concentration as low as 10^{-8} *M*, but the D-stereoisomer had no effect on sperm behavior. This finding eliminates

the possibility that sperm responded to nonspecific effects of the tryptophan molecule, such as the hydrophobicity of the indole side chain; the two enantiomers are chemically identical, except for interactions with a chiral environment such as the binding site of a protein. The observed stereospecificity suggests that abalone sperm possess a receptor that binds exclusively to the L-isomer of tryptophan (Schultz et al., 1972; Bender, 1989). Trials using live eggs revealed that the effects of tryptophan on sperm orientation were strikingly context-dependent. The natural gradient around an egg functioned as an aphrodisiac, selectively attracting sperm, whereas a uniformly elevated concentration of tryptophan acted as a contraceptive, by preventing sperm navigation.

The chemoattractant L-tryptophan is a biosynthetic precursor of the neuromodulator serotonin (5-hydroxytryptamine). No previously identified sperm attractant is related to a water-soluble neurotransmitter. This result is intriguing given that serotonin induces an immediate increase in sperm motility in other molluscan species (Kadam and Koide, 1990). There is also evidence of serotonin receptors on molluscan sperm membranes, including 5-HT_1 receptors that result in adenylate cyclase activation, and 5-HT_3 receptors that cause the opening of ion channels (Kadam and Koide, 1990; Bandivekar et al., 1992). We initially hypothesized that the activity of tryptophan might result from uptake across the sperm membrane and enzymatic conversion to serotonin. However, serotonin was inactive when bioassayed against red abalone sperm, indicating that L-tryptophan itself is the active ligand controlling sperm behavior. Sperm activation may result from cAMP-dependant phosphorylation of the α chain of the flagellar protein dynein, but the precise mechanism remains unknown (Stephens and Prior, 1992). Future

investigations of the physiological basis for abalone sperm activation and chemotaxis in response to L-tryptophan are needed. These studies can link the production of an environmental chemical cue with the signal transduction pathway controlling flagellar motion at the molecular level.

Gamete recognition: soluble attractants versus cell-surface proteins

Previous investigations of gamete recognition have emphasized the role of membrane proteins in mediating sperm-egg interactions. Surface-bound proteins can promote species-specific fertilization, and variation in such factors may be sufficient to drive reproductive isolation (Palumbi and Metz, 1991; Palumbi, 1994; Foltz, 1995; Biermann, 1998; Vacquier, 1998). Molecular analysis of molluscs, echinoderms and mammals has revealed extraordinary sequence divergence in gamete-recognition proteins, resulting from positive Darwinian selection for amino acid subsitutions (Swanson and Vacquier, 1995; Vacquier et al., 1997; Hellberg and Vacquier, 2000; Wyckoff et al., 2000; Swanson et al., 2001a,b).

For marine organisms, broadcast spawning presents the possibility of hybrid fertilization among congeneric species (Palumbi, 1994). In the Pacific northeast, there are 7 co-occurring species of abalone with overlapping breeding seasons. Species integrity and reproductive isolation among abalone are believed to result from the rapid evolution of two sperm proteins, lysin and an 18-kDa protein (Vacquier et al., 1997). Lysin creates a hole in the vitelline envelope of conspecific eggs, while the 18-kDa protein is involved in fusion of gamete membranes (Vacquier et al., 1990; Lee et al.,

1995; Swanson and Vacquier, 1995). Lysin and the 18-kDa protein exhibit extreme positive selection and are among the fastest-evolving proteins known (Lee et al., 1995; Vacquier et al., 1997; Vacquier, 1998). In contrast, the egg receptor for lysin (VERL) evolves neutrally by concerted evolution; gradual drift in the structure of egg receptors may thus select for rapid, compensatory divergence of sperm proteins (Swanson and Vacquier, 1998; Swanson et al., 2001a). Sperm may be under similar selective pressure to adapt to changes in egg chemistry, to increase encounter rates and promote recognition of conspecific eggs at a distance, but this hypothesis remains to be experimentally tested.

Waterborne sperm attractants are potentially critical agents mediating fertilization success and driving speciation in the ocean, operating upstream of cell-surface proteins prior to gamete contact (Ward and Kopf, 1993; Vacquier, 1998). To identify the role of soluble egg factors in promoting reproductive isolation, chemically distinct attractants must be identified from eggs of related, overlapping species. Currently, sperm responses to egg extracts are known to be specific at the genus or family level for several taxa (Miller, 1979, 1985, 1997). However, species-specificity of sperm response to factors naturally released by live eggs is not yet demonstrated for any group of congeneric organisms.

Metabolites of tryptophan affect sensory systems controlling diverse phenomena, ranging from insect social behavior to human brain physiology and pathology (Bender, 1989; Harris and Woodring, 1999; Moroni, 1999). For example, decarboxylation and successive modifications to tryptophan produce the neuromodulatory agents tryptamine, serotonin, and melatonin (Sandyk, 1992; Sainio et al., 1996). Alternatively, opening of

the indole ring leads to the kynurenine pathway, producing a series of related aromatic amines (e.g., anthranilic acid, quinolinic acid) with diverse neurophysiological functions (Stone, 1993; Moroni, 1999). In plants, tryptophan is a biosynthetic precursor of the hormone auxin (Bartel, 1997; Zhao et al., 2001) and defensive secondary metabolites termed indole glucosinolates (Chavadej et al., 1994). Small structural changes to tryptophan can thus generate chemically related molecules that vary widely in receptor-binding properties (Berger, 2000; Glennon et al., 2000).

Species-specific signal molecules may well facilitate navigation by abalone sperm towards conspecific eggs. Our initial chemical analysis shows that abalone eggs, and conditioned seawater, indeed contain several tryptophan derivatives (manuscript in prep.). Sperm recognition of a particular tryptophan metabolite, or a unique mixture of structurally related molecules, could trigger activation or orientation only near conspecific eggs, promoting fertilization while preventing hybridization. Alternatively, given its abundance in all living cells, tryptophan might serve as a nonspecific signal that generally increases sperm attraction to eggs. Preliminary results do not support this alternative hypothesis, however. Sperm of red (*Haliotis rufescens*) and green (*H. fulgens*) abalone are attracted to soluble factors released from conspecific but not heterospecific eggs (author's unpublished data). Future efforts should reveal the effects of L-tryptophan on sperm of other abalone, and the chemical identities of sperm attractants from additional species. Elucidation of the sperm attractant for red abalone therefore provides a powerful new tool for guiding future investigations on waterborne chemical signals, gamete interactions and speciation in the sea.

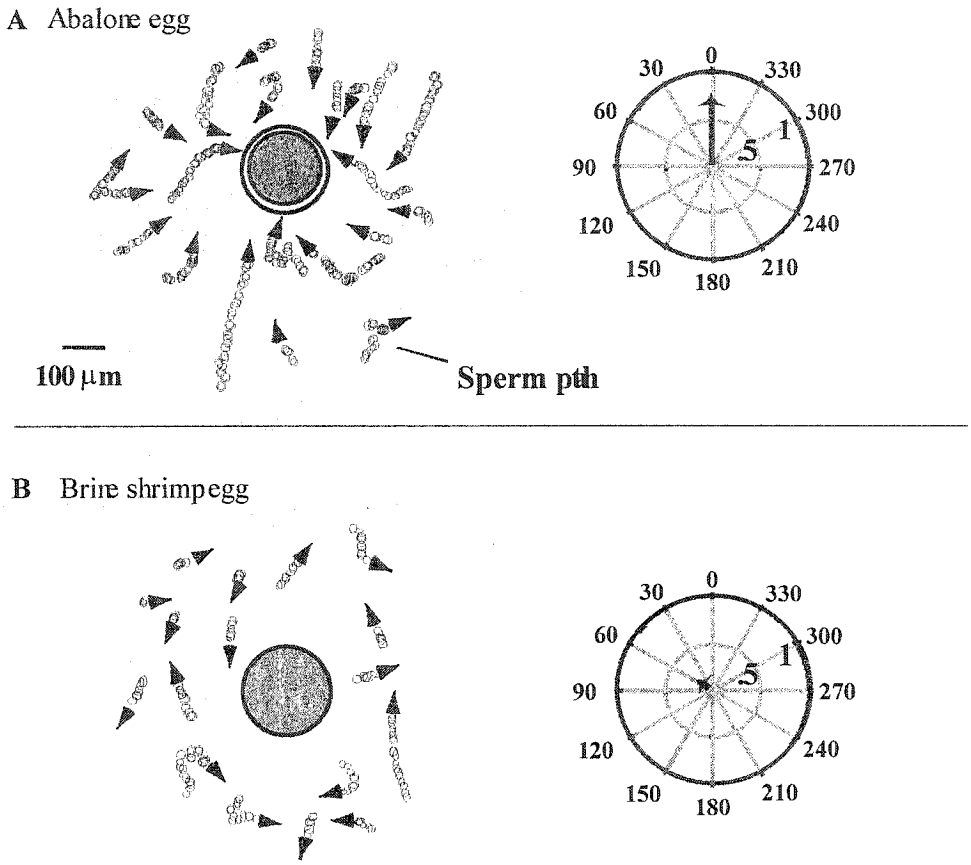

Figure 1-1. Orientation of abalone sperm to individual eggs. Sperm were video-taped for 30 sec in the presence of a red abalone egg, or brine shrimp egg (negative control). Polar plots show the vector mean angle of sperm swimming, relative to the egg surface (0°); the bar represents the vector mean length (*r*). Representative swimming paths of individual sperm are plotted around an abalone or control egg. Dots correspond to video images captured at 0.033 sec intervals, and arrowheads correspond to the directions of travel for individual cells. (A) Behavior of sperm swimming around a live red abalone egg. (B) Sperm swimming around a brine shrimp egg control.

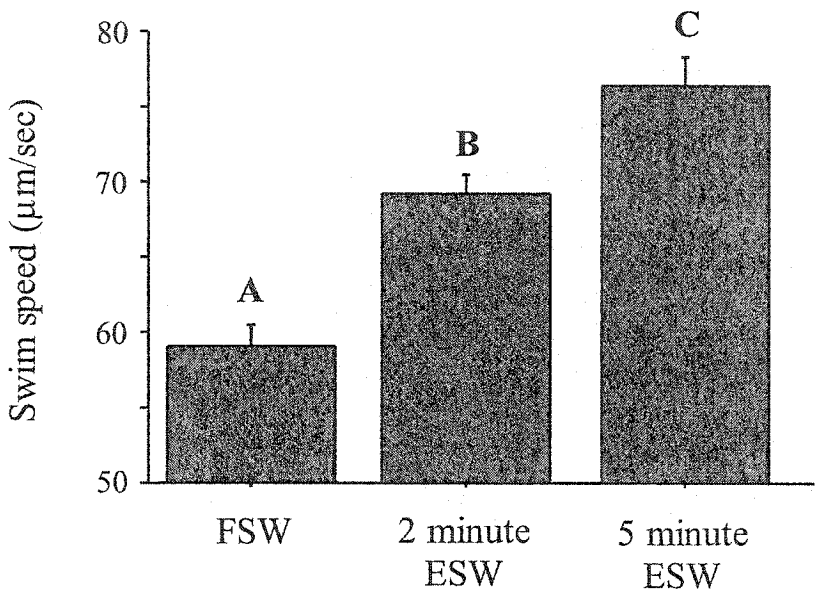

Figure 1-2. Accumulation of the sperm attractant in sea water around live eggs. Freshly spawned eggs were suspended in filtered sea water (FSW) and incubated for 2 min or 5 min. The resulting egg-conditioned sea water (ESW) solutions were filtered to 0.22 µm and immediately bioassayed using fresh sperm. Swimming speed was quantified by Computer-Assisted Video Motion Analysis. Data are plotted as means + SE; means labeled with different letters differed significantly (1-way ANOVA with post-hoc Scheffé test, $p < 0.005$).

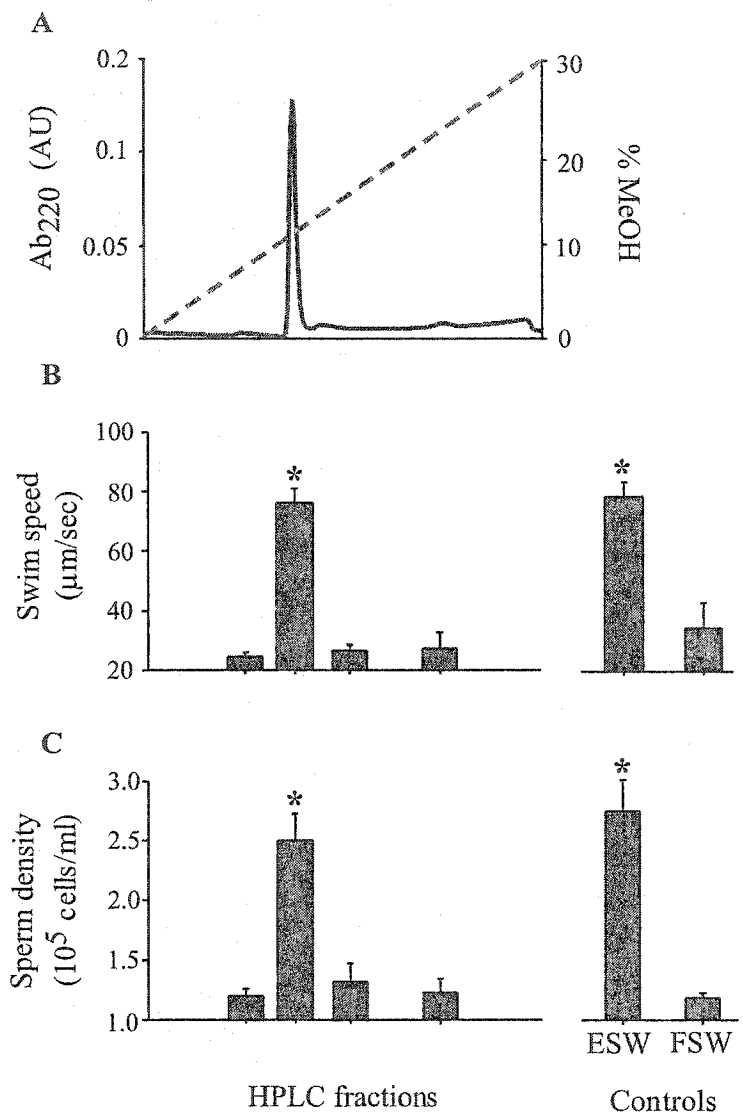

Figure 1-3. Partial purification of the sperm chemoattractant by reversed-phase HPLC. (A) The active fraction from ESW was eluted from a Sep-Pak cartridge in 25% CH_3OH, concentrated, and injected onto a C_{18} column. Compounds were eluted over a 30 min run using a gradient from 5 to 50% CH_3OH in 0.1% TFA. Fractions (0.5 ml) were pooled as indicated, lyophilized, and bioassayed for sperm motility and orientation. (B) Sperm swimming response to isolated fractions. Fractions were diluted to the appropriate starting volume of sea water and tested in the sperm motility assay; swim speeds were measured by CAVMA. Bars are means + SE. Fresh ESW and FSW were used as positive and negative controls, respectively. Means identified with asterisks were significantly greater than negative controls ($p < 0.01$). (C) Chemotactic response of abalone sperm to isolated fractions, represented as cell counts in a flat capillary bioassay. Data are presented as in (B).

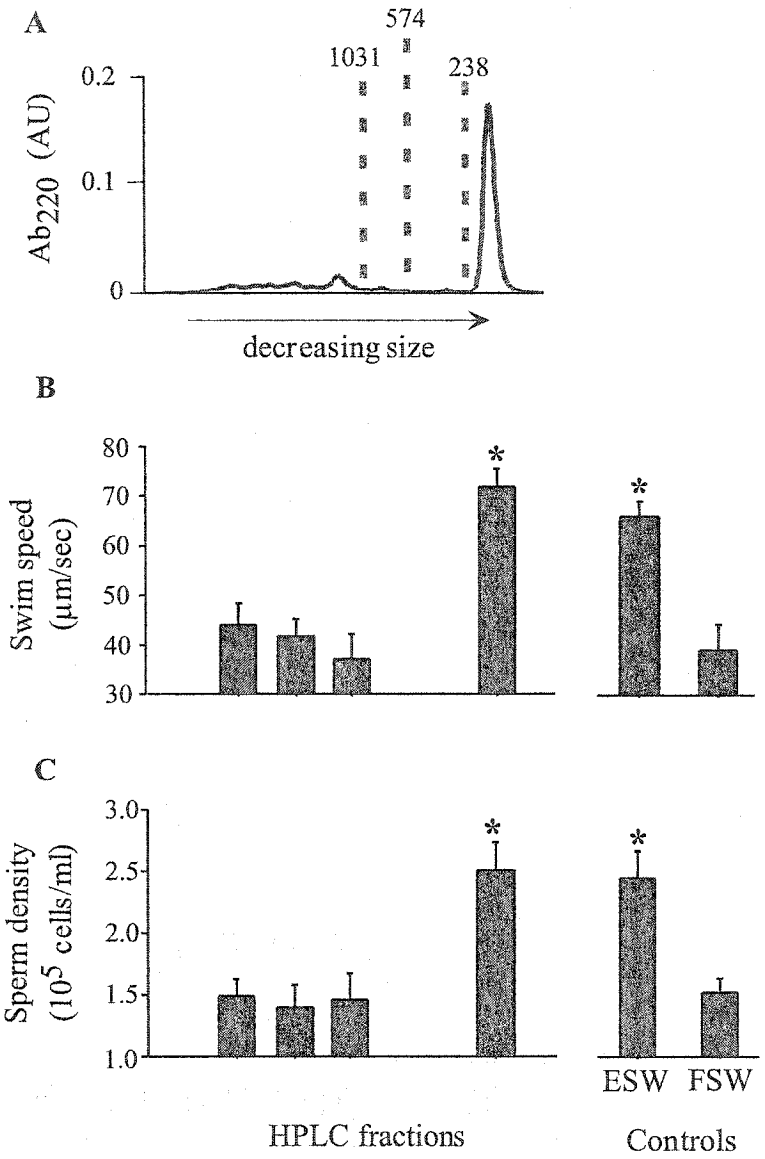

Figure 1-4. Final purification of the sperm chemoattractant by size-exclusion chromatography. (A) The bioactive fraction from RP HPLC was chromatographed on a size-calibrated Bio-Sep S-2000 column, eluting at 1 ml/min in water and monitoring absorbance at 220 nm. Fractions corresponding to detected peaks were pooled, lyophilized, re-suspended in sea water, and bioassayed with freshly spawned sperm. Numbers indicate the elution times of size standards used to calibrate the column. (B) Sperm swimming response to isolated fractions. Bars are means + SE. Fresh ESW and FSW were used as positive and negative controls, respectively. Means identified with asterisks were significantly greater than negative controls ($p < 0.0001$). (C) Chemotactic response of abalone sperm to isolated size fractions. Data are presented as in (B).

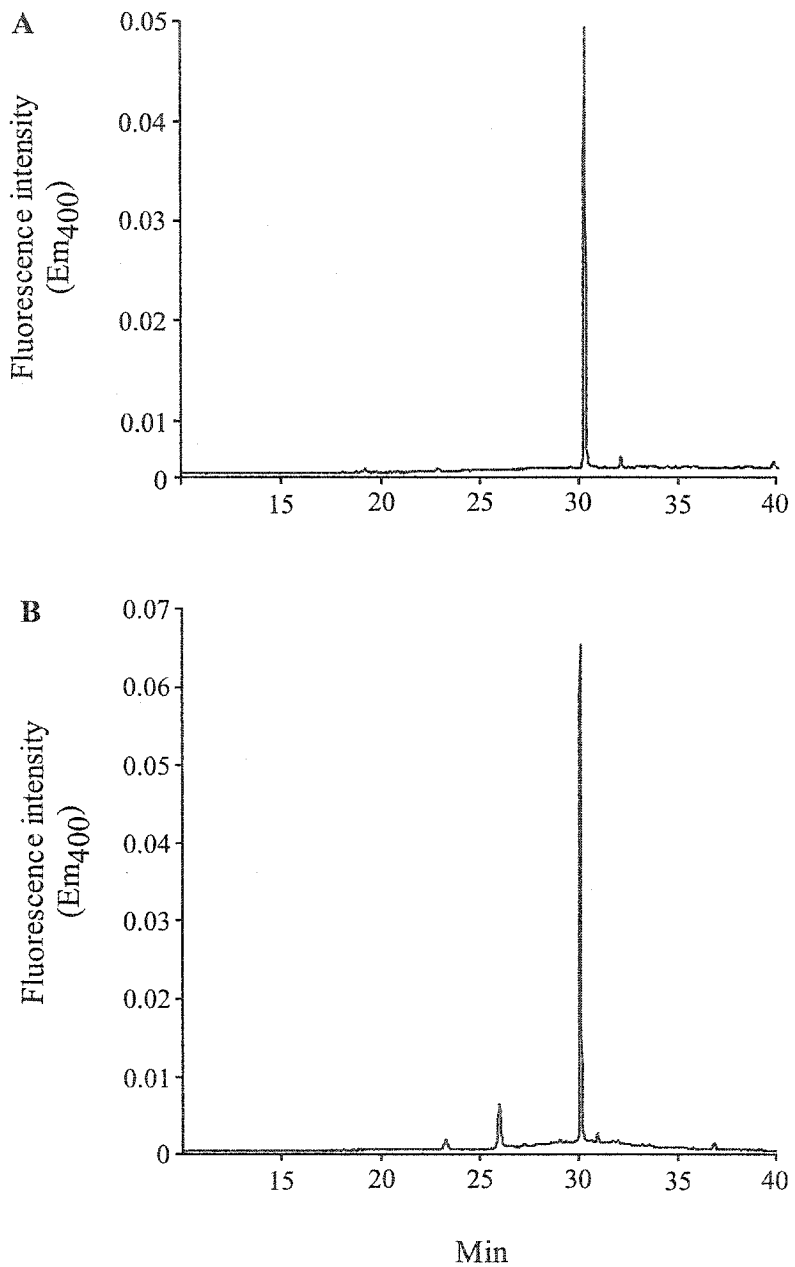

Figure 1-5. Analytical HPLC comparison of the natural attractant and a synthetic tryptophan standard. Samples were reacted with OPA (*o*-phthalaldehyde) prior to injection, producing derivatives that were identified by reversed-phase HPLC with fluorescence detection. (A) An aliquot of the purified natural sperm attractant corresponding to 0.25 n*M*, showing the diagnostic retention time and purity of the sample. (B) Co-injection of the previous sample with 0.25 n*M* of a synthetic standard of L-tryptophan, showing co-elution as a single peak.

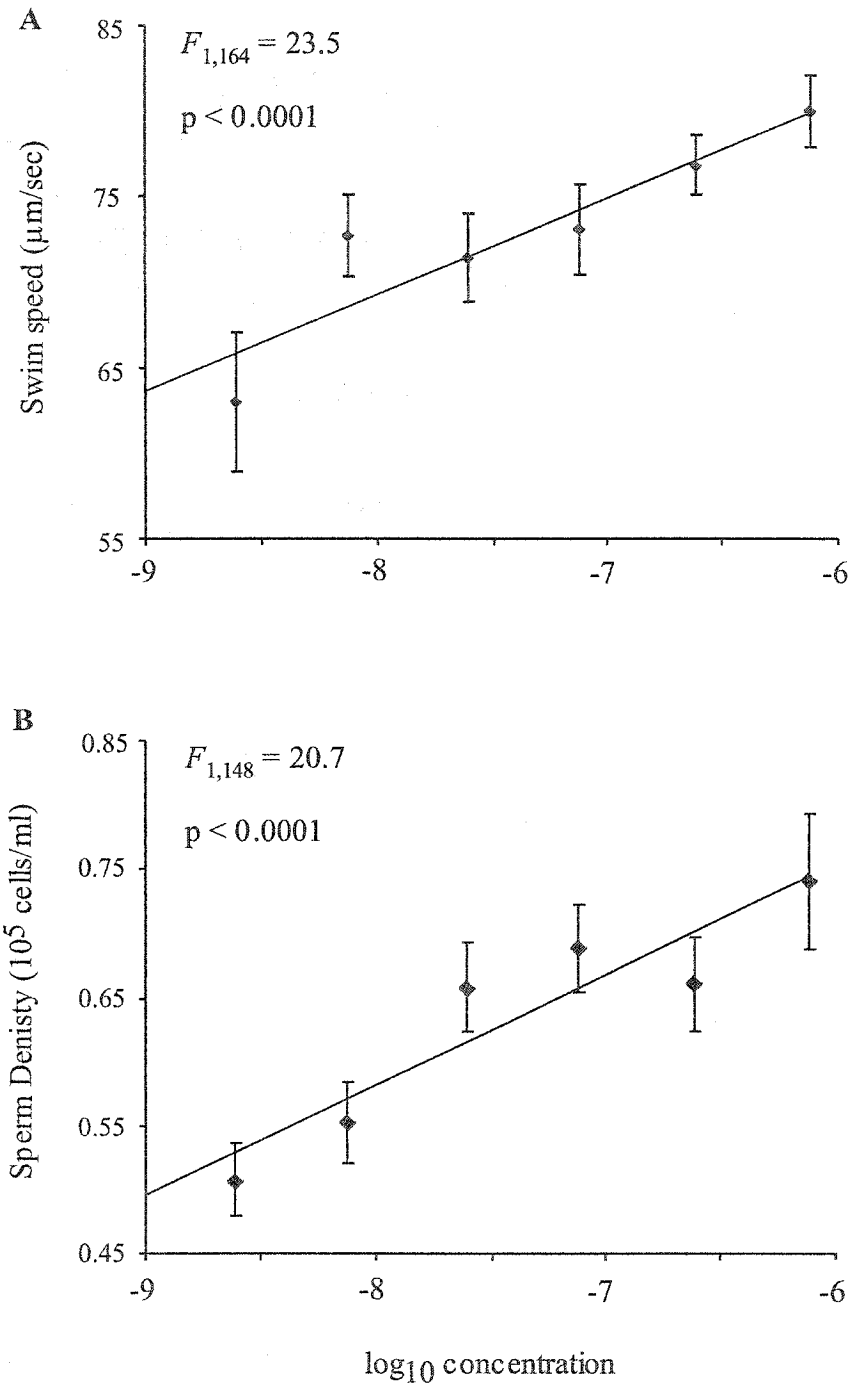

Figure 1-6. Dose-response of red abalone sperm to L-tryptophan. Data are plotted as means ± SE, with the regression line shown as calculated for the raw data. (A) Results of a linear regression of $\log_{10}$-transformed dose on sperm swim speed. (B) Results of a linear regression of $\log_{10}$-transformed dose on sperm density. The ED_{50}, or dose producing the half-maximal response, was calculated from the regressions to be 3×10^{-8} *M* for both swim speed and cell density.

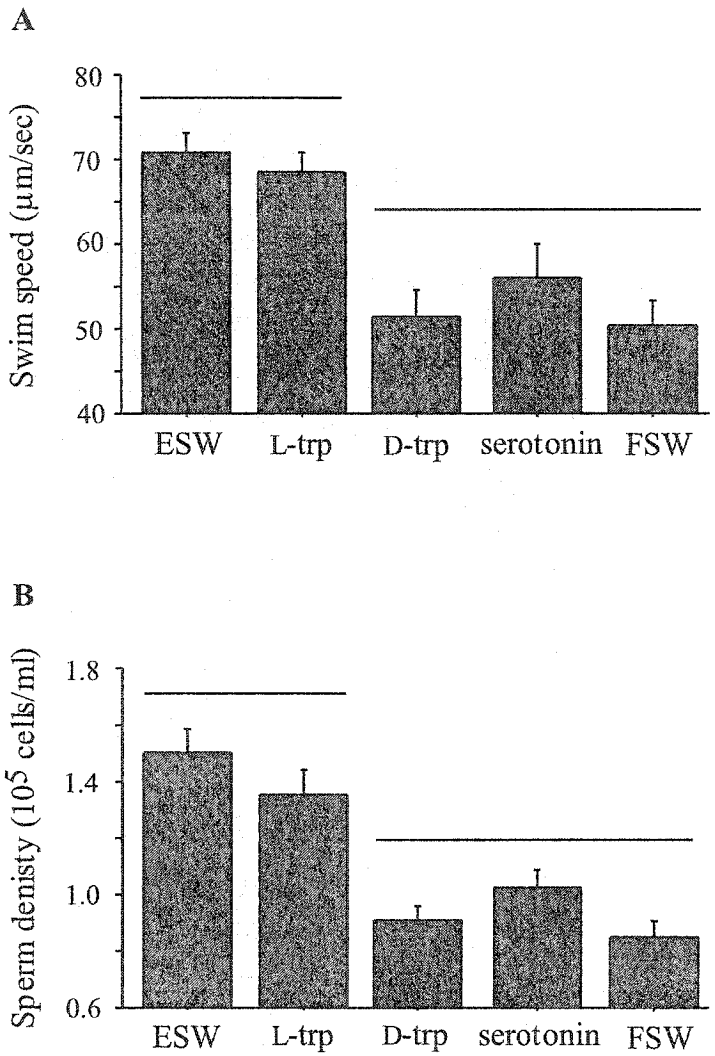

Figure 1-7. Structural specificity of the sperm response to L-tryptophan. Red abalone sperm were assayed for behavioral responses to natural egg-conditioned sea water (ESW), and to synthetic standards tested at 10^{-7} *M* concentration. Both the natural L-enantiomer and the unnatural D-enantiomer of tryptophan were tested to determine the stereospecificity of sperm response. Serotonin, or 5-hydroxytryptamine, is a neuroactive metabolite of L-tryptophan known to activate sperm in other molluscan species. Filtered sea water (FSW) was used as a negative control. Data are plotted as means + SE; means not joined by a horizontal bar differed significantly (1-way ANOVA with post-hoc Scheffé test, $p < 0.05$). (A) Change in sperm swim speed in response to tested solutions. (B) Change in cell density due to sperm chemotaxis.

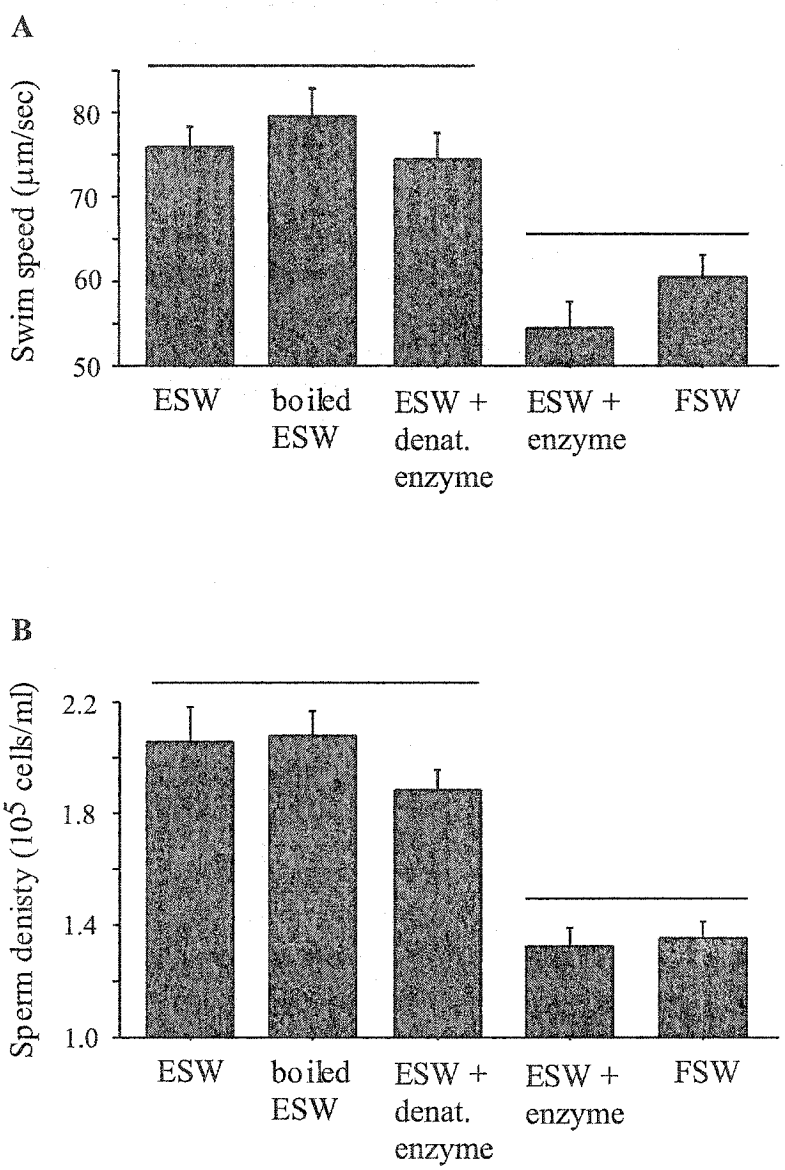

Figure 1-8. Effect of enzymatic treatment with tryptophanase on bioactivity of egg-conditioned sea water (ESW). Active ESW solutions were incubated with the enzyme tryptophanase, which cleaves the indole ring from L-tryptophan; solutions were boiled to quench the reaction after 10 min. In one positive control, ESW solutions were boiled without enzyme, to determine effects of the deactivating step in enzyme treatments. In a second positive control, enzyme was added to ESW and immediately boiled, to ensure that the presence of denatured enzyme did not affect sperm behavior. Negative controls were run in FSW. Data are given as means + SE; means joined by a horizontal bar did not differ significantly (Scheffé test, $p < 0.05$). (A) Change in sperm swims speed in response to tested solutions. (B) Change in cell density due to sperm chemotaxis.

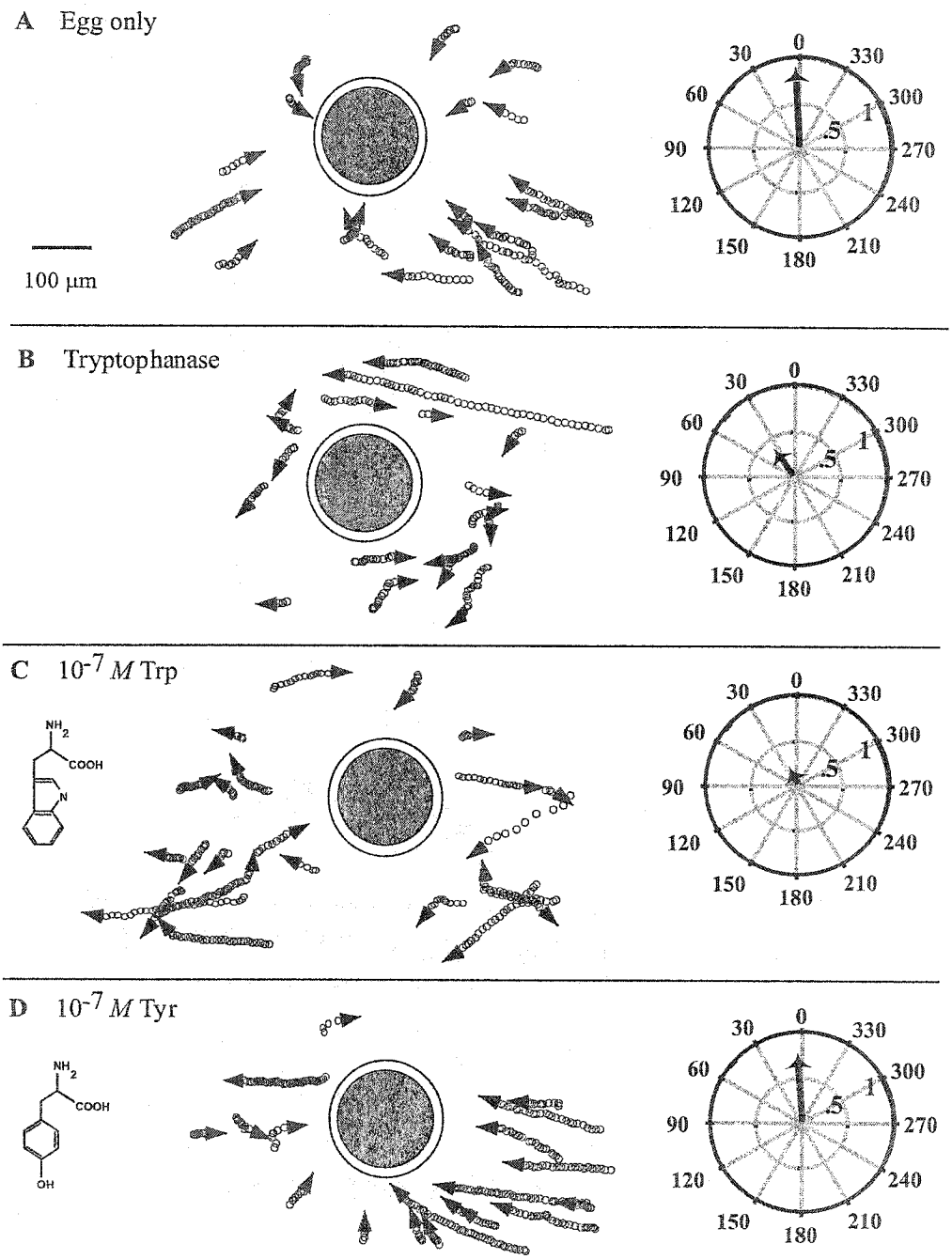

Figure 1-9. Disruption of the tryptophan gradient around live eggs prevents navigation by sperm. Individual red abalone eggs were placed in 400 μl of FSW or the indicated test solution. Polar plots show the vector mean direction of sperm swimming, with angles measured relative to the egg surface (0°). (A) Sperm in FSW. (B) Sperm in the presence of the enzyme tryptophanase. (C) Sperm in a uniform solution of 10^{-7} *M* L-tryptophan. (D) Sperm in a uniform solution of 10^{-7} *M* L-tyrosine.

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Chapter II

Sex and the single cell:

The ecological and evolutionary consequences of sperm chemoattraction

Abstract

Chemical communication between sperm and egg is a critical factor mediating sexual reproduction. Sperm attractants may be significant evolutionarily for maintaining species barriers, and important ecologically for increasing gamete encounters. Still unresolved, however, are the functional consequences of these dissolved signal molecules. Here, we provide the first evidence that sperm chemoattraction directly affects the magnitude of fertilization success. The recent discovery of L-tryptophan as a potent attractant to red abalone (*Haliotis rufescens*) sperm offered the rare opportunity to quantify how navigation affects gamete interactions. Sperm behavioral responses to manipulations of the natural tryptophan gradient around individual eggs revealed that both chemotaxis and chemokinesis significantly promote contacts. Our results showed further that attractant release via diffusion effectively doubles the target size of red abalone eggs which, in turn, significantly increases fertilization success. Although long theorized as potential barriers to hybridization, species-specific sperm attractants in red and green (*H. fulgens*) abalone are not responsible for maintaining reproductive isolation.

For abalone in dense spawning aggregations, chemically mediated navigation may instead promote conspecific gamete interactions and prevent sperm from pointlessly tracking heterospecific eggs. Species-specificity may thus evolve when finding the right target confers a selective advantage on sperm by preventing gamete wastage, even though reproductive isolation fundamentally resides at the level of membrane recognition proteins.

Introduction

Chemical signals between sperm and egg are pervasive among taxa with highly divergent reproductive strategies. Sperm activation and chemotaxis occur in marine animals that broadcast gametes into the sea, as well as in terrestrial organisms, including humans, with internal fertilization (Miller, 1985; Ward and Kopf, 1993; Eisenbach, 1999; Spehr et al., 2003). Chemically mediated behavior thus is a key component of sperm-egg dynamics, whether in the turbulent ocean environment or within a mammalian reproductive tract. Communication between eggs and sperm may be meaningful evolutionarily for maintaining species barriers, and significant ecologically for increasing gamete encounters, but the contributions of sperm attractants to these processes remain undescribed.

Whereas little is known about the functions of dissolved sperm attractants, the role of membrane proteins in fertilization is comparatively well understood (Vacquier, 1998; Swanson and Vacquier, 2002). Molecular analysis of mollusks, echinoderms and mammals has revealed extraordinary sequence divergence in gamete-recognition

proteins, resulting from positive Darwinian selection for amino acid substitutions (Palumbi and Metz, 1991; Palumbi, 1994; Swanson and Vacquier, 1998; Swanson et al., 2001). Variation in surface-bound proteins may promote species-specific fertilization and thus reproductive isolation, especially for marine organisms where broadcast spawning could permit hybridization (Vacquier, 1998; Kresge et al., 2001; Riffell et al., 2002). In the Pacific northeast, there are seven co-occurring species of abalone with overlapping breeding seasons. Species integrity among abalone may result from the rapid evolution of two sperm proteins, lysin and an 18 kDa protein (Swanson and Vacquier, 2002). Lysin creates a hole in the vitelline envelope of conspecific eggs, whereas the 18 kDa protein is involved in fusion of gamete membranes (Foltz, 1995).

Soluble sperm attractants are potentially critical agents mediating fertilization success and driving speciation, operating upstream of cell-surface proteins and prior to gamete contact (Ward et al., 1985; Ward and Kopf, 1993; Vacquier, 1998;). Despite their likely importance, however, few sperm attractants have been isolated and chemically characterized. A sulfonated steroid is responsible for sperm activation and chemotaxis in two ascidian species (Yoshida et al., 2002). In contrast, a series of peptides described from sea urchin egg jellies increases sperm respiration and/or motility at nanomolar levels (Suzuki and Yoshino, 1992; Miller and Vogt, 1996). Peptides with similar functions were isolated from eggs of other echinoderms (Suzuki and Yoshino, 1992). These peptides are broadly active for species within a given family, but it is unclear whether they are effective at physiological pH (Ward and Kopf, 1993). Furthermore,

only one peptide triggers chemotaxis (Suzuki and Yoshino, 1992; Miller and Vogt, 1996).

The paucity of purified attractants has limited efforts to determine the link between sperm chemoattraction and fertilization success. As a consequence, the influence of chemotaxis on fertilization has always been inferred, but never directly tested. Using bioassay-guided fractionation and nuclear magnetic resonance spectroscopy, we recently established that the free-amino acid L-tryptophan is the natural sperm attractant for red abalone (*Haliotis rufescens*) (Riffell et al., 2002). This discovery has permitted experimental study of the relationships between chemical signaling, sperm behavior and fertilization rate. Moreover, it has allowed determinations of the relative contributions of membrane recognition proteins and sperm chemoattractants to reproductive isolation and speciation.

Methods

Abalone collection, maintenance and spawning

Adult males and females were collected from local rocky reefs or procured from Cultured Abalone, Inc. (Goleta, CA, USA), then held in aquaria of running sea water (15 ^{o}C). The animals were fed fresh kelp, *Macrocystis pyrifera*, collected twice weekly. Ripe adults were identified by gonadal growth beyond the shell, and the sexes were separated and fed for 2 weeks prior to spawning induction. Individuals were placed singly in chambers and the sea water pH raised to 9 by adding 6.5 ml of 2 M tris-hydroxymethylaminomethane (Tris-base) per liter, followed by 4 ml of 6% H_2O_2 per liter

(Morse et al., 1977). After 2.5 h exposure, the chamber was emptied, rinsed and refilled with 0.45-□m filtered sea water (FSW). Spawning occurred within 2-4 h of H_2O_2 exposure, with males releasing gametes 10-30 min before females. A typical male (12 cm length) spawned 20-60 ml of sperm solution (10^9 sperm/ml), whereas a female of similar size spawned 10-20 ml of egg suspension (10^5 eggs/ml). Gametes were collected above the excurrent tremata and held in centrifuge tubes (sperm) or beakers (eggs) filled with FSW until use. Experiments involved only fresh eggs and sperm, beginning 10 min after spawning and continuing for 30 min thereafter.

Sperm behavior in response to a natural tryptophan gradient

An initial experiment tested whether a natural chemoattractant gradient surrounding an egg is necessary and sufficient to promote the recruitment of conspecific sperm. A freshly spawned egg of a red abalone was placed in a chamber containing 400 □l FSW alone, or containing one of the following five treatments. (A) A single egg was placed in a solution of tryptophanase (2 □g/ml). This enzyme digests tryptophan in the medium around the egg, thus eliminating the signal. Prior to use, tryptophanase was activated by incubation with 100 □M pyridoxal-5'-phosphate in FSW (pH 7.9) at 37 °C for 1 h to ensure maximum formation of the holoenzyme (Phillips, 1991). (B) An egg was placed in a solution of 10^{-7} M tryptophan. This condition tested for sperm behavior in a uniform concentration of the attractant, sufficient to overwhelm any gradient formed by diffusion from the egg. (C, D) An egg was placed either in a solution of denatured (boiled) tryptophanase or of 10^{-7} M tyrosine. The addition of tyrosine controlled for any

non-specific effects of elevated aromatic amino acid concentration on sperm swimming. (E) Assays were run in FSW, using both sperm and eggs bathed in a tryptophanase solution prepared as above, but rinsed free of enzyme prior to tests.

To begin each trial, red abalone sperm (2.5 X 10^3 cells/ml) were gently pipetted into a chamber 10 min after egg addition, the shortest delay permitted by experimental constraints (preparation of the sperm solution, locating and centering the egg in the field of view). Images of sperm swimming within 250 μm of the egg surface were videotaped for 30 s and recorded on magnetic tape. The camera (NEC model TI 23A) was mounted on an Olympus IX70 compound light microscope at a 90X magnification and had a 100 μm depth of field.

Images were digitized at 30 frames/s using a Computer-Assisted Video Motion Analysis (CAVMA) system (Motion Analysis Corp. model VP 320 and custom software) interfaced with a Sun SPARC2 computer workstation (Lopez-de-Victoria et al., 1995). To avoid problems of parallax, short paths ($\leq$ 10 frames) were discarded that consisted of computer images with sperm changing more than 20% in apparent size. All other paths were included in analyses. Swimming speed of each individual sperm was determined on a frame-by-frame basis, and averaged over each path. Using non-motile sperm as tracers for flow visualization, fluid motion due to convection was insignificant (< 5 □m/s) compared to swimming speeds of live cells. The angle of sperm orientation was measured with respect to an origin (0^o), defined as the shortest tangent between each cell and the egg surface. Circular statistics – in particular, the mean vector (**r**) – was used to describe the average direction of sperm movement at 50 □m intervals from an egg

surface to 250 □m away. A Rayleigh's test was used to compare each mean direction against a uniform circular distribution to determine the significance of cell movement towards the egg surface. Gamete encounter rate was measured as the elapsed time (0.033 s accuracy) for a sperm to contact and attach to an egg.

Tryptophan concentrations in tissues and release rate from eggs

The following adult red abalone tissues were analyzed for tryptophan content: eggs, testes, muscle, stomach, gills, and hemolymph. Testes (0.5 g) were homogenized in 5 ml artificial seawater (N = 3 replicates). For other solid tissues, 1 g was homogenized in 100 ml FSW (N = 5 replicates). Seawater was then filtered to 0.22 μm and frozen prior to analysis. Hemolymph was filtered following extraction from the animals. Tryptophan was quantified by reversed-phase HPLC on an Ultrasphere ODS column (4.6 mm X 25 cm, 5 μm particle size), using a gradient of 5 to 50% CH_3OH in water at a flow rate of 1 ml/min. Peaks were monitored by a spectrofluorometer (Jasco Instruments) at 345 nm using an excitation wavelength of 275 nm, and integrated by Gold Nouveau software (Beckman Coulter).

The rate of tryptophan release was measured to quantify attractant flux from individual eggs. Freshly spawned eggs were concentrated into a dense suspension, and aliquots of 1 ml (3×10^5 eggs) were transferred immediately to beakers containing 25 ml FSW at 15 °C. Eggs were bathed for periods of 0, 2, 15, 30, and 45 min, the maximum duration of viable eggs (N = 5 replicates per time point). At the end of each time point, eggs were removed on a 55 μm mesh and FSW samples were filtered to 0.22 μm and

frozen at –80 °C. Prior to HPLC analysis, thawed samples were desalted and concentrated on C-18 Sep-Pak columns. Columns were first washed with 10 ml CH_3OH to wet the resin, and then equilibrated with 15 ml Nanopure-grade H_2O prior to loading of samples. An equivalent volume of seawater from each time point was loaded onto a fresh column by gravity feed, and washing with 15 ml H_2O then desalted the column. Tryptophan was eluted with 10 ml of 30% CH_3OH in water. The eluant from each column was dried by sequential addition to an individual Eppendorf tube to minimize any transfer loss. Samples were concentrated to 100 µl for injection onto the HPLC column (chromatography conditions as described above). Tryptophan standards were prepared by adding 2.5 or 0.25 nM tryptophan to replicate (n = 3) 25 ml batches of FSW, and processed in parallel with other samples. Standards were used to calibrate HPLC runs and to control for recovery of tryptophan from C-18 columns during sample preparation.

The consequences of sperm chemoattraction for fertilization success

Before chemoattractant effects could be determined, an initial experiment established conditions for fertilization assays. Using a factorial design, crosses were run for a wide range of red abalone egg ($10^0 – 10^5$ gametes/ml) and sperm ($10^1 – 10^8$ gametes/ml) density combinations, over interaction intervals of 5 to 2,400 s. Results showed, first, that percent fertilization was dependent on the ratio of sperm-to-eggs but not on the density of either gamete type. As the ratio of sperm-to-eggs was raised from 1 to 10,000, percent fertilization increased monotonically from 0% to 100%. Second, contact time mattered little. The asymptotic percent fertilization was achieved within 15

s of initial gamete contacts (Riffell, 2004). Hence, fertilization was extremely rapid, even in still water.

Based on these findings, subsequent assays of chemoattractant effects on fertilization success were performed at a single contact time (15 s) and egg density (10^3 eggs/ml). As before, we used six chemical treatments (FSW; tryptophanase; denatured tryptophanase; 10^{-7} M tryptophan; 10^{-7} M tyrosine; sperm and egg exposed to tryptophanase, then rinsed free of enzyme with FSW before assay). Replicate trials were conducted with sperm held at one of five different concentrations (10^3 to 10^7 sperm/ml) for each chemical treatment. In each trial, an aliquot (1 ml) of egg suspension was pipetted into a tube whose bottom had been replaced with a 55 μm mesh screen. These eggs were immersed in a sperm solution for 15 s, while exposed to a chemical treatment. Upon removal, eggs were rinsed thoroughly in clean (0.22 μm-filtered) seawater and held in FSW. Microscopic examinations indicated that the rinse eliminated all sperm from egg surfaces, except those cells attached to the vitelline envelope. After a 3-h FSW incubation – long enough for cleavage to occur (Leighton and Lewis, 1982) – we fixed the eggs in 5% formalin and determined the percentage fertilized.

The consequences of sperm chemoattraction for reproductive isolation and speciation

Current theory predicts that membrane-bound proteins are responsible for gamete recognition, but soluble egg factors might also act as agents mediating fertilization and driving speciation. To evaluate this possibility, we measured (A) swimming speeds and navigation of sperm around isolated eggs, (B) sperm-egg encounter rates, and (C)

percentages of fertilized eggs, after 15 s contact time with sperm at varying densities. In each trial, sperm and eggs were collected from red (*Haliotis rufescens*) and/or green (*H. fulgens*) abalone. The two species cohabit shallow-water rocky reefs of southern California kelp forests, and both species spawn naturally during warm summer months (Cox, 1962; Owen et al., 1977). Thus, there is no geographical or temporal isolation acting as a prezygotic barrier to hybridization. A total of four crosses were performed using gametes in replicate trials for each of the three assays. Experimental methods were identical to those described above. Results were compared between treatments to identify species-specific effects on chemoattraction and fertilization success.

Results

Sperm behavior in response to a natural tryptophan gradient surrounding eggs

As male gametes of red abalone approached within 100 □m of a conspecific egg in FSW, they accelerated significantly and navigated directly towards the egg surface (Figs. 2-1A and 2-2A, B). Control solutions (denatured [boiled] tryptophanase; 10^{-7} M tyrosine; sperm and egg exposed to tryptophanase, then rinsed with FSW before bioassay) were notably without effect on sperm behavior (Figs. 2-1B, C, D and 2-2A, B). Upon addition of tryptophanase (L-tryptophan indole lyase, EC 4.1.99.1) to solution, however, sperm ceased to orient towards an egg or swim faster (Figs. 2-1F and 2-2A, B). In contrast, cells swam significantly faster, but failed to navigate towards egg surfaces when presented with an elevated, uniform, tryptophan concentration (10^{-7} M) (Figs. 2-1E and 2-2A, B). These experiments distinguished effects of chemotaxis (directed

movement with respect to a chemical concentration gradient) from chemokinesis (change in swim speed). Egg-derived tryptophan functions in the dual role of potent chemoattractant and effective swimming stimulant to navigating sperm cells.

Tryptophan concentrations and release rates

HPLC analysis revealed that tryptophan concentrations in adult red abalone tissues were four times higher in the cytoplasm of freshly spawned eggs than in hemolympth, muscle, gills, testes or stomach. It was concentrated in egg cytoplasm, but was absent from the surrounding jelly coat. Tryptophan was released from eggs at a constant rate of ~2.0 X 10^{-4} pmole/egg/min as long as they remained viable for fertilization (Fig. 2-3). This empirically derived rate of release was used in a Fickian diffusion model to estimate signal strength (Crank, 1975; Berg, 1983). Integrating with respect to time (10 min), a minimum effective concentration was calculated by assuming that sperm would respond at a distance of up to 100 □m (*see above*). As calculated here (Fig. 2-3B), the minimum effective dose (~4 X 10^{-9} M) closely matched a threshold value (~10^{-8} M) we determined previously for abalone sperm chemotaxis (using an exogenous source of tryptophan without eggs present).

Gamete encounter rates

Straighter and faster swimming paths need not indicate that chemically mediated behavior increases encounter rates, or ultimately enhances fertilization success. From experiments described above, video images were processed for rates of sperm attachment

to conspecific eggs. Rates were indistinguishable for gametes in filtered seawater, tyrosine and denatured enzyme, and for gametes transiently exposed to enzyme prior to assays (Fig. 2-4). In contrast, attachment rate was depressed to an intermediate level by tryptophan addition and reduced to a minimum by tryptophanase. The relative effect of each chemical treatment on attachment thus could be predicted from the behavioral results. Whereas chemotaxis and chemokinesis each promoted gamete interactions, a combination of these two behaviors maximized sperm-egg encounter and attachment rates.

The consequences of sperm chemoattraction for fertilization success

To quantify the extent to which red abalone sperm chemoattraction affected fertilization success, bioassays were performed at a single contact time of 15 s. This time reflected a short, but realistic, gamete-encounter interval in field habitats (Pennington, 1985; Levitan, 1998; Babcock and Keesing, 1999). For each of the six chemical treatments, a logistic regression described the relationship between percentage of fertilized eggs and sperm-to-egg ratio (Fig. 2-5, F-test: $F \geq 8.61$, df = 1/99, $P < 00001$, all comparisons). When the ratio was too low (1.0 sperm:1.0 egg) or too high (10,000 sperm:1 egg), sperm were either limiting or saturating, respectively. Under these conditions, chemoattraction did not affect fertilization. In contrast, at intermediate ratios (10 – 1000 sperm per egg), fertilization success increased significantly as a function of chemoattraction. To compare across treatments, logistic regression equations were used to calculate effective sperm-to-egg ratios (ER_{50}) fertilizing half of all eggs (Venables and

Ripley, 1999). As calculated, ER_{50s} were almost identical (range; 88.0-95.4 sperm:1.0 egg) for gametes held in filtered seawater (FSW), denatured tryptophanase and 10^{-7} M tyrosine, or transiently exposed to enzyme prior to bioassays. In contrast, ER_{50s} for 10^{-7} M tryptophan and tryptophanase were elevated, significantly, by 1.94-times (169.8 sperm:1.0 egg) and 5.10-times (444.6 sperm:1.0 egg) relative to filtered seawater.

The consequences of sperm chemoattraction for reproductive isolation and speciation

To investigate how chemoattraction contributes to reproductive isolation, we dissected experimentally effects of sperm navigation from processes occurring after gamete contact. Sperm of red (*Haliotis rufescens*) and green (*H. fulgens*) abalone responded to egg factors in a species-specific manner (Fig. 2-6, 2-7 and Table 2-1). When both male and female gametes were drawn from adults of either species, soluble sperm attractants acted as precise signals to promote encounter rates and fertilization (Figs. 2-7 and 2-8). Moreover, swimming speeds of red sperm, but not green sperm, increased significantly in response to a 10^{-7} M tryptophan solution (data not shown). Nonetheless, these results indicate that the pre-zygotic barrier to fertilization between red and green abalone occurs after contact between sperm and egg. Green abalone sperm behaved similarly to red abalone sperm that were enzymatically deprived of their natural tryptophan beacon; neither could orient nor accelerate towards red abalone eggs (Figs. 2-1, 2-2, and 2-6 and Table 2-1). If sperm attraction was the principal prezygotic isolating mechanism, green sperm and navigationally impaired red sperm should have been equally able to fertilize red eggs. However, non-navigating red sperm held a ~ 252-fold

fertilization advantage over green sperm when each was mixed with red abalone eggs (Figs. 2-5 and 2-8). Within this species pair, reproductive isolation therefore must reside downstream of soluble egg factors that affect sperm behavior, most likely at the level of membrane-bound receptors.

Discussion

Sperm attractants and fertilization ecology

Despite a century of research, fertilization remains one of the least understood fundamental biological processes (Vacquier, 1998). Chemical communication between sperm and eggs is purportedly critical in sexual reproduction, but the contribution of soluble egg factors has been elusive. All previous investigations introduced an attractant, via micropipette, to a drop of water containing sperm cells on a microscope slide. Only sperm trapped along the fluid-surface interface were considered in analyses of motility (Miller, 1985; Eisenbach, 1999; Ward et al., 1985; Miller and Vogt, 1996; Yoshida et al., 2002). Such experiments did not take into account important physical phenomena, including viscous wall effects that may influence cell navigation. Moreover, video recordings of sperm behavior were limited to the first minute following pipette placement. Cells were therefore exposed to chemical concentration gradients that varied considerably through time and space. These methodologies render impossible generalizations of sperm behavior for any single set of gradient conditions.

The current study was performed to minimize effects of walls on sperm motility, while maintaining the natural diffusion dynamics of attractant release from live eggs.

Furthermore, sperm behavior was bioassayed 10 min after introducing cells into an experimental chamber. Fluid dynamic theory predicts a steady state in attractant distribution over the observed video time interval (Crank, 1975). For these conditions, egg-derived tryptophan induced both chemotaxis and chemokinesis in red abalone (*Haliotis rufescens*) sperm. Chemotaxis was selectively suppressed by adding exogenous tryptophan, reducing sperm-egg contacts and the number of sperm attached to the egg vitelline envelope. This treatment also increased the number of sperm required to fertilize 50% of all eggs in bioassays. When *both* chemotaxis and kinesis were eliminated by tryptophanase digestion, the deleterious effects on attachment and fertilization *doubled* in magnitude. Thus, chemically mediated navigation and acceleration each contributed equally in promoting fertilization.

Selective pressures must drive the evolution of sperm chemoattraction. Amid shallow-water rocky reefs, male and female abalone broadcast gametes into the ocean, and thus, fertilization occurs externally to the body cavity. Within this turbulent fluid environment, spawned eggs and sperm are rapidly diluted below critical densities for successful fertilization (Pennington, 1985). Sperm may be under intense selective pressure to recognize eggs at a distance, due to competition for limited egg resources or because gamete dilution quickly diminishes mating opportunities. As the probability of contact between male and female gametes is directly proportional to egg radius (Vogel et al., 1982; Levitan 1998; Podolsky 2002), remote chemical communication is an effective means of promoting sperm-egg contacts. Our results indicate that attractant release via

diffusion creates a chemical concentration gradient that doubles the effective target size of red abalone eggs which, in turn, substantially increases fertilization success.

To be a cogent signal molecule for red abalone sperm, tryptophan must perform well in a seawater medium. Dissolved free amino acids (especially glycine, serine, alanine and ornithine) are abundant naturally in coastal ocean waters (Carr, 1988; Coffin, 1989; Decho et al., 1998), but tryptophan stands out by its absence. This property suggests a high signal-to-background noise ratio for tryptophan release, increasing its effectiveness as a beacon compared to related metabolites. Many substances serving as chemical signals in the external seawater environment have parallel roles in internal physiological fluids, where they function as neuroactive agents, such as transmitters and modulators (Carr, 1988). Common metabolites such as tryptophan are likely to be efficient attractants by eliminating costs of natural product biosynthesis.

Sperm attractants and reproductive isolation

Surface proteins involved in sperm-egg interactions are better characterized for abalone (genus *Haliotis*) than for any other taxon, and demonstrate strong selection for species-specific gamete recognition (Swanson and Vacquier, 1998; Hellberg and Vacquier, 2000; Kresge et al., 2001). There are ~60 species of abalone worldwide, many with overlapping breeding seasons and habitats, yet hybrids are rare (Owen et al., 1971; Geiger, 1999). It is unresolved whether chemical communication between sperm and eggs is important in maintaining reproductive isolation among extant abalone species that could potentially hybridize. This issue has never been assessed experimentally for any

organism, largely because it has not been possible to eliminate chemosensory behavior of sperm as a variable. High concentrations of green abalone (*Haliotis fulgens*) sperm are necessary to achieve fertilization of red abalone (*H. rufescens*) eggs. Is this because green abalone sperm do not *navigate* towards red abalone eggs, or because their membrane proteins do not *bind* to cognate receptors on the egg? Either mechanism could potentially block hybridization, but prior to our study, there was no method for determining the relative contributions to fertilization of navigation and membrane-bound proteins.

By enzymatically disrupting the gradient of attractant around live abalone eggs, we compared the fertilization success of non-navigating red versus green abalone sperm. This experiment thus quantified how much of the impeded fertilization was due to interference with sperm chemoattraction. In terms of their ability to fertilize red abalone eggs, non-navigating red sperm were impaired by a factor of 5, whereas non-navigating green sperm were impaired by a factor of ~1,250. Thus, the 250-times difference in fertilization success between non-navigating red and green abalone sperm can only be due to events occurring at or after egg contact.

Currently, abalone populations in southern California are threatened or endangered. Because of a moratorium on all abalone capture from field habitats, heterospecific crosses between additional species could not be performed in the present study. Thus, logistic regressions were used to predict effective sperm-to-egg ratios (ER_{50}), based on fertilization data for heterospecific crosses from previous investigations (Leighton and Lewis, 1982). Red, pink (*H. corrugata*) and green abalone are more

distant relatives, having diverged in the Pacific northeast over a period of 4-20 million years ago. In contrast, red and white (*H. sorenseni*) abalone are closely related and diverged only within the past 1-2 million years (Metz et al., 1998). For the heterospecific crosses of pink sperm X red egg and white sperm X red egg, calculated ER_{50s} were 159-times and 95-times higher, respectively, than for the conspecific cross of non-navigating red sperm X red egg. Because differences in fertilization success varied by only a factor of 2.6 between distantly (green and red) and closely (white and red) related species, generalizations are permissible to other abalone species. Membrane recognition proteins, not sperm attractants, evidently are the principal barriers to hybridization in haliotids.

Although playing only a minor role in blocking reproduction between red and green abalone, sperm activation and chemotaxis were nonetheless species-specific. Only red abalone sperm responded to the natural tryptophan gradient around red eggs, and only green abalone sperm responded to dissolved factors from green eggs. Given the high sperm-to-egg ratios necessary to achieve fertilization in red-green crosses, sperm that contact heterospecific eggs were wasted reproductive effort. Because abalone typically live in dense, multi-species aggregations, chemically mediated navigation would prevent sperm from pointlessly tracking heterospecific eggs. Thus, even though reproductive isolation fundamentally resides at the level of membrane recognition proteins, species-specific sperm attractants may have evolved to locate the right target within mixed gamete suspensions of closely related species.

Chemical analyses show that abalone eggs contain a suite of tryptophan metabolites. The species-specific response of red and green abalone sperm suggests that

each species is co-adapted to recognize the chemical signature of conspecific eggs. Future efforts are likely to reveal the chemical identities of sperm attractants for additional abalone species. Species-specificity may result from recognition of distinct tryptophan metabolites, unique natural products, or mixtures of egg-derived attractants and inhibitors, as are commonly found in multi-component sex pheromone blends (Linn and Roelofs, 1989; Hildebrand and Shepherd, 1997).

Table 2-1. Mean (± SEM) speeds, mean vector lengths (**r**) and angles (θ) describing swimming behavior of sperm near conspecific or heterospecific eggs (see text and Fig.2-2 caption for details).

Abalone species		Variable	Distance from egg (μm)				
Egg	Sperm		50	100	150	200	250
Red	Red	Speed	70.2 (± 2.1)**	69.0 (± 3.0)**	60.2 (± 4.1)	55.3 (± 2.3)	51.8 (± 5.2)
		r	0.94**	0.79**	0.49	0.06	0.08
		θ	2°	3°	25°	137°	140°
Green	Green	Speed	86.0 (± 4.3)**	72.1 (± 6.5)**	61.8 (± 4.1)	55.1 (± 4.7)	56.3 (± 4.0)
		r	0.94**	0.79*	0.49	0.06	0.08
		θ	7°	17°	110°	92°	121°
Green	Red	Speed	61.8 (± 4.7)	62.5 (± 2.6)	63.2 (± 3.9)	57.4 (± 3.7)	60.8 (± 5.0)
		r	0.19	0.11	0.08	0.13	0.10
		θ	118°	161°	45°	171°	63°
Red	Green	Speed	52.9 (± 3.8)	52.3 (± 3.3)	54.7 (± 3.3)	57.2 (± 4.1)	55.6 (± 4.6)
		r	0.16	0.10	0.17	0.13	0.18
		θ	18°	55°	292°	152°	354°

*$P < 0.01$, ** $P < 0.001$; an asterisk denotes a significant acceleration as sperm move towards an egg (one-way ANOVA with *post-hoc* Scheffé test), or a significant deviation in swim direction from a uniform circular distribution (Rayleigh's test).

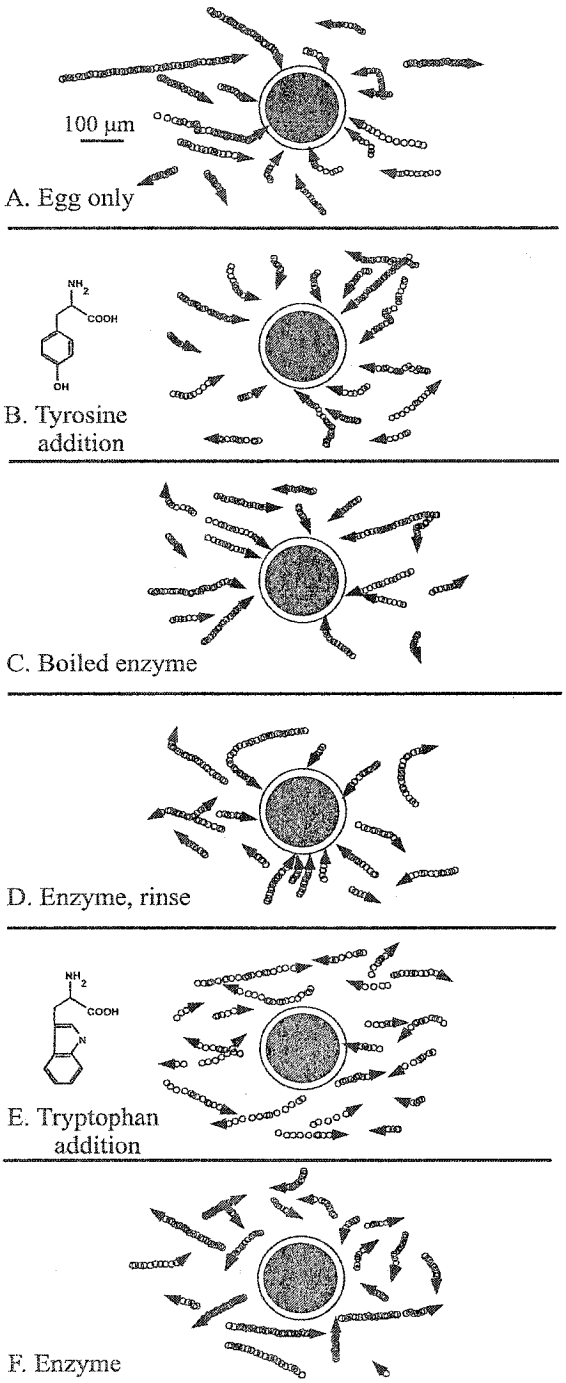

Figure 2-1. Swimming behavior of red abalone sperm near an isolated conspecific egg using computer-assisted video motion analysis. Open circles correspond to video images captured at intervals of 0.033 s; arrowheads indicate directions of travel of individual cells. To eliminate selection bias, we used a random numbers generator to choose representative paths for each of six chemical treatments.

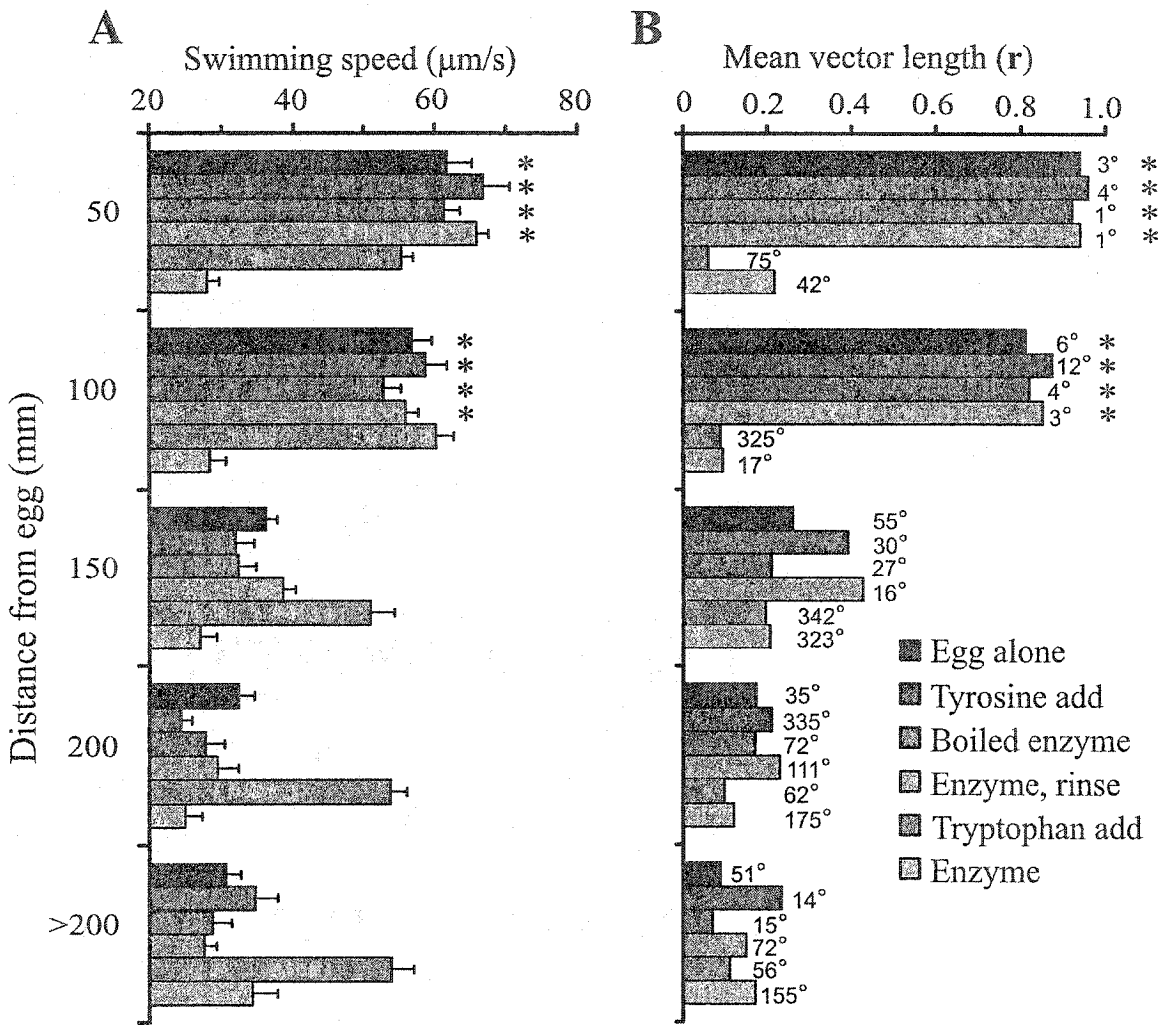

Figure 2-2. (A) Mean (± SEM) speeds and (B) mean vector lengths (r) describing the swimming behavior of red abalone sperm. A mean vector length of 1 indicates that all cells swim in a single, common direction; a vector length of zero indicates random motion. The mean angle of sperm orientation appears above each histogram bar; angle measurements were made with respect to an origin (0^o), defined as the shortest tangent between each individual cell and the surface of an egg. Complete data sets – not representative paths – were used to calculate these mean values. A minimum of 30 paths was analyzed for each of the six treatments. Asterisks (* $P < 0.001$) denote a significant acceleration as cells move towards an egg (one-way ANOVAs: $F \geq 36.9$, df = 4/126, $P < 0.001$, all comparisons), or a significant deviation in swim direction from a uniform circular distribution (Rayleigh's test: $r \geq 0.82$, $z \geq 8.06$, $P < 0.001$, all comparisons). Sperm are shown to swim faster in a uniform gradient of 10^{-7} M tryptophan, but they do not accelerate as they approach an egg.

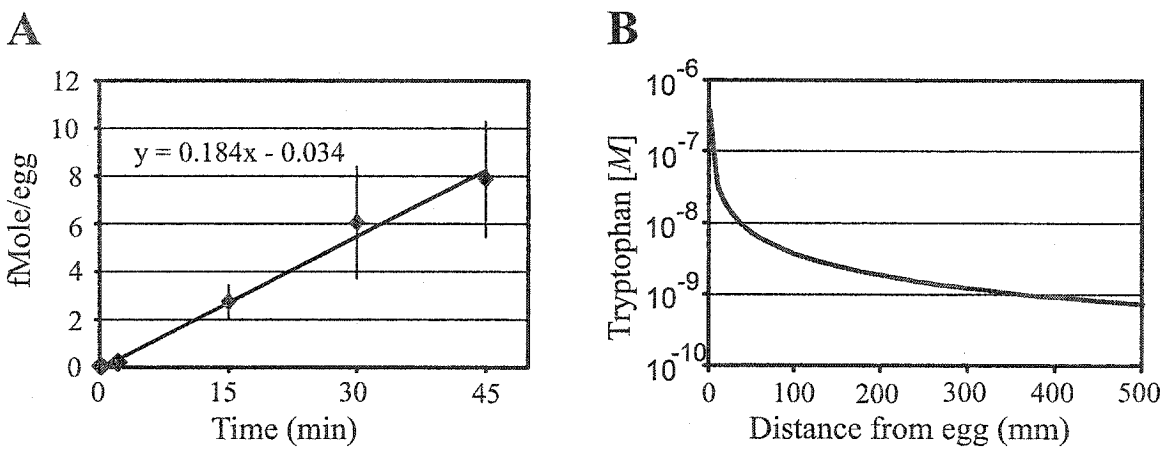

Figure 2-3. The accumulation of tryptophan in solution over time, after release from individual eggs; (A) values are means (± SEM). There is a constant rate of tryptophan release during a 45-min experimental period (Least squares regression: $F = 24.6$, df = 1/23, $P < 0.001$). (B) Tryptophan concentrations surrounding an egg, as predicted using the empirically derived rate constant and applying a 3-dimensional Fickian diffusion model (see text for details).

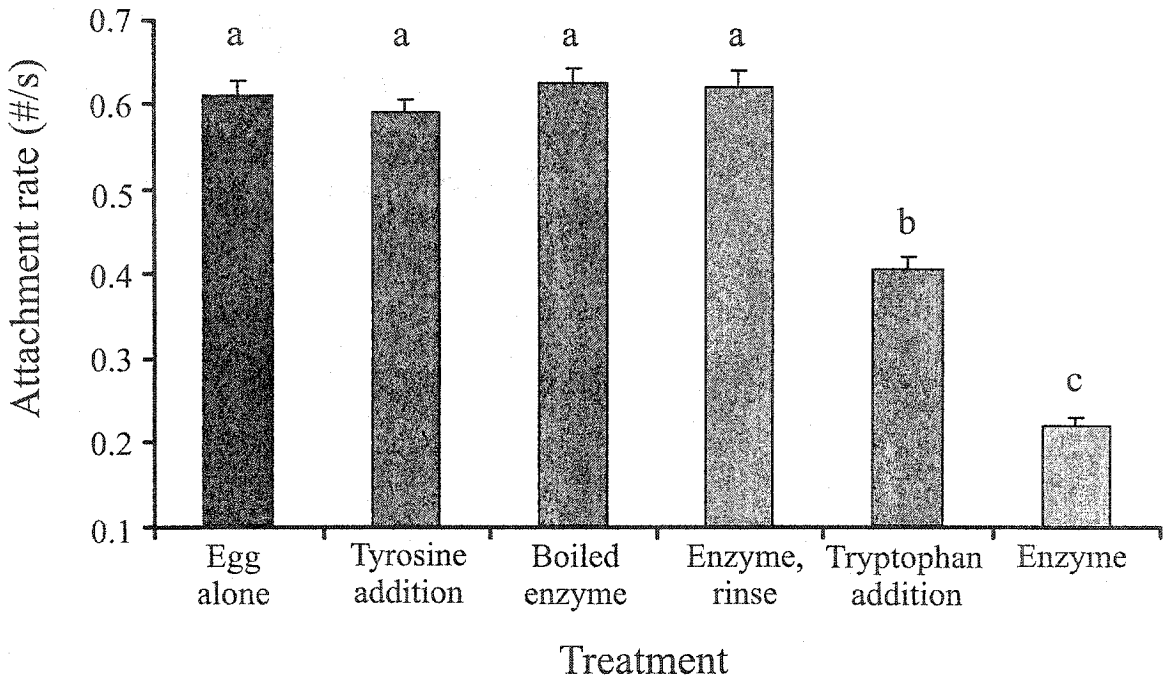

Figure 2-4. Mean (± SEM) rates at which red abalone sperm attach to the vitelline envelope of conspecific eggs, as measured using computer-assisted video motion analysis. The relative effect of each chemical treatment on attachment could be predicted from behavioral results (see Figs. 1 and 2). Means labeled with different letters are significantly different (one-way ANOVA with *post-hoc* Scheffé tests, $P < 0.005$). A total of five replicate trials was performed for each of the six chemical treatments.

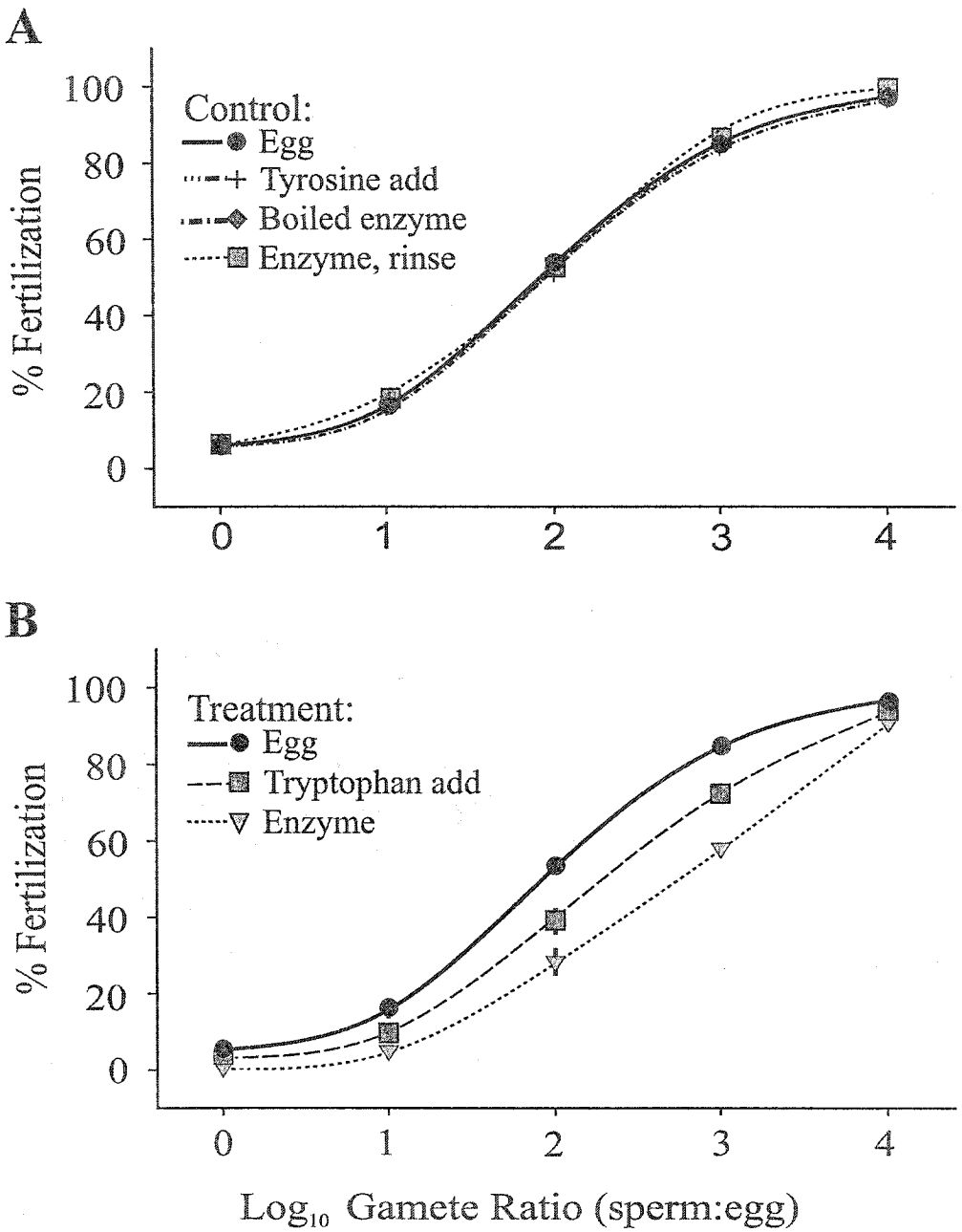

Figure 2-5. Logistic regression lines describing the relationships between mean (± SEM) percentages of red abalone fertilized eggs and sperm-to-egg ratio for (A) control treatments, and (B) test treatments, relative to filtered seawater control ('Egg alone'). When a standard error bar fails to appear, it is smaller than the symbol size describing the mean. Twenty replicate trials were performed for each data point. There is no significant difference between control treatments (one-way-ANOVA: $F = 0.038$, df = 3/348, $P > 0.99$). In contrast, each test and seawater control treatment differ significantly from one another (one-way ANOVA with *post-hoc* Scheffé tests, $P < 0.001$).

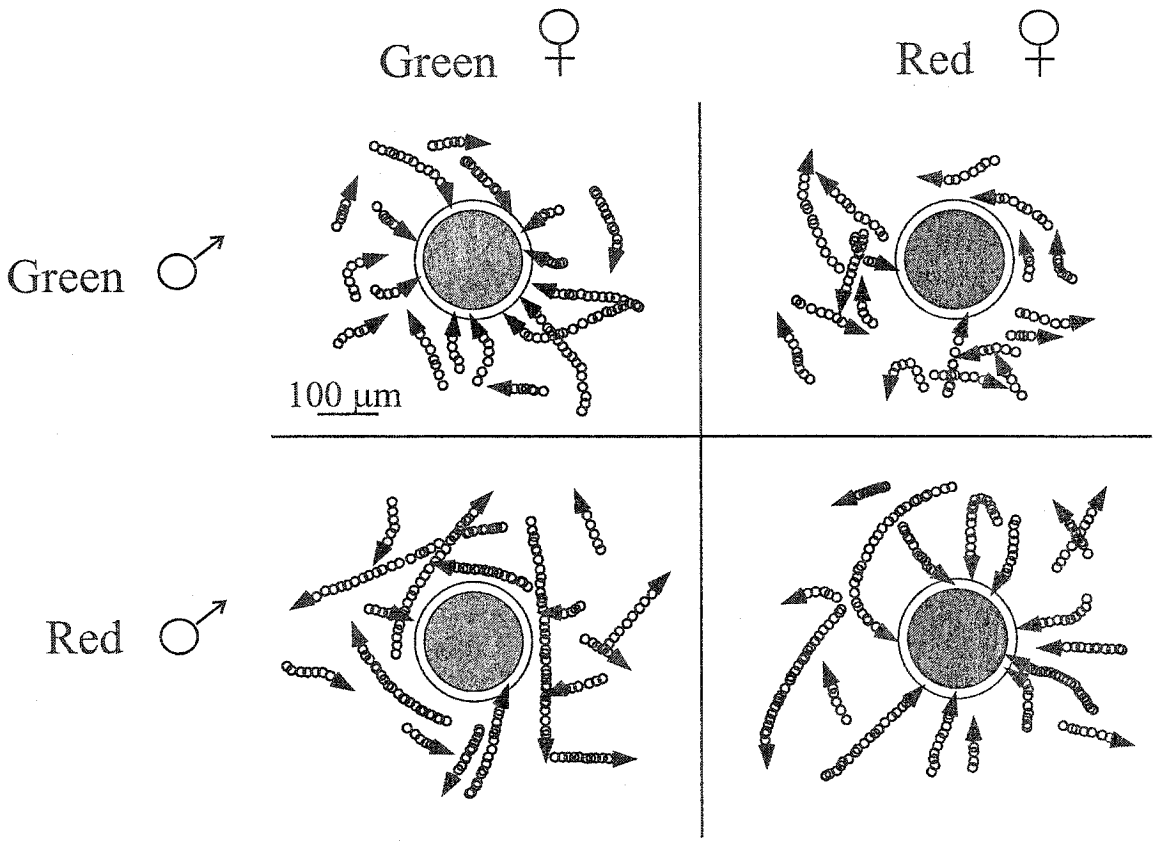

Figure 2-6. Swimming behavior of red and green abalone sperm, respectively, near an isolated conspecific or heterospecific egg. Determinations were made using computer-assisted video motion analysis. Open circles correspond to video images captured at intervals of 0.033 s; arrowheads indicate directions of travel of individual cells. To eliminate selection bias, we used a random numbers generator to choose representative paths for each of the four crosses.

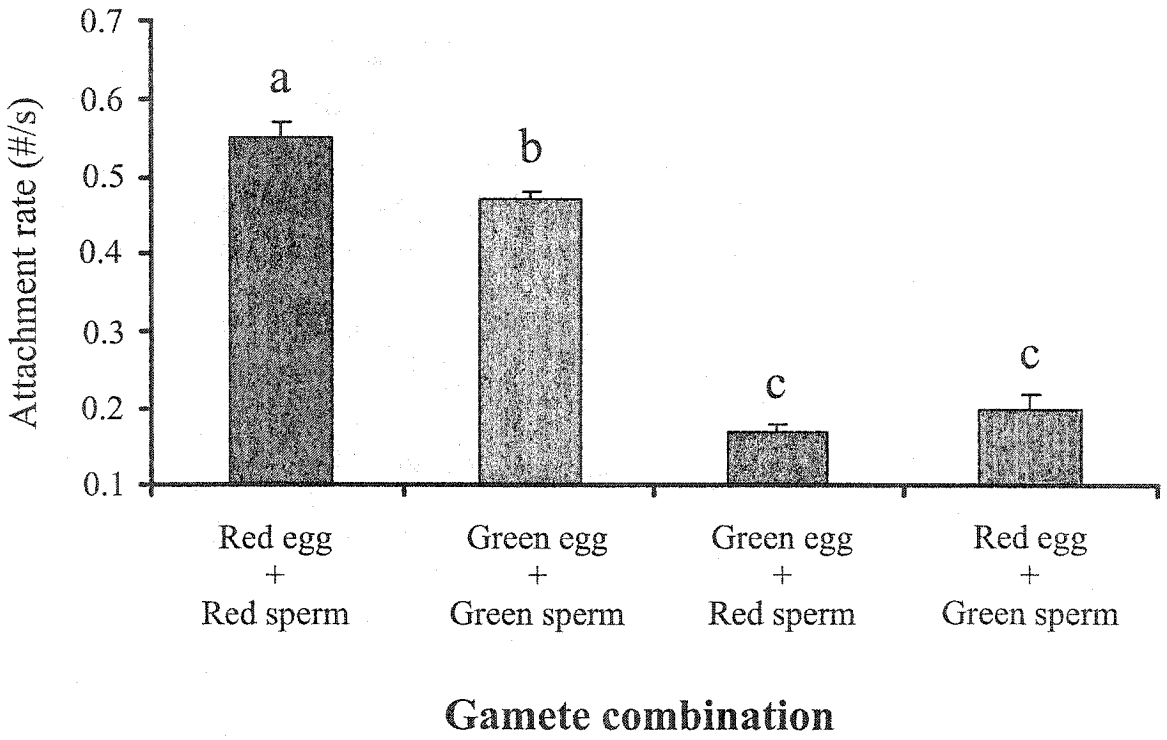

Figure 2-7. Mean (± SEM) rates at which red and green abalone sperm attach, respectively, to the vitelline envelope of conspecific or heterospecific eggs. Determinations were made using computer-assisted video motion analysis. Means labeled with different letters are significantly different (one-way ANOVA with *post-hoc* Scheffé test, $P < 0.0001$). A total of seven replicate trials was performed for each of the four crosses.

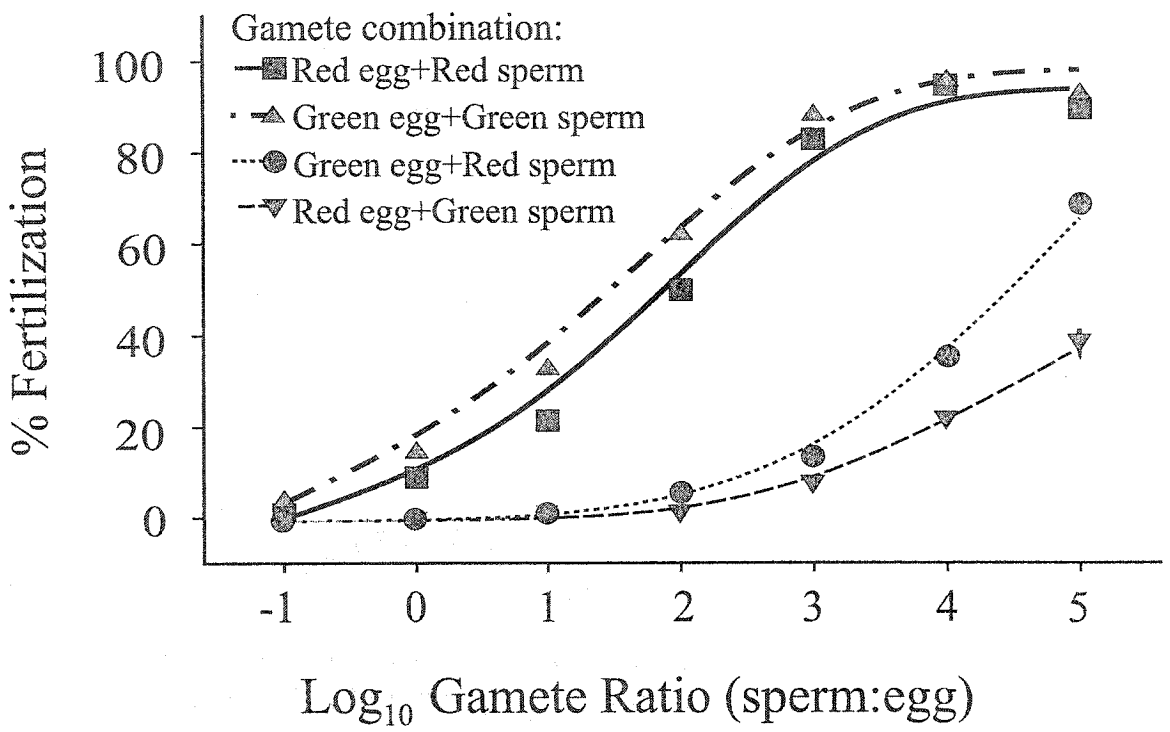

Figure 2-8. Logistic regression lines describing the relationships between mean (± SEM) percentages of fertilized eggs and sperm-to-egg ratio for each of four conspecific or heterospecific crosses. When a standard error bar fails to appear, it is smaller than the symbol size describing the mean. Ten replicate trials were performed for each data point. Due to low hybridization rates, we extrapolated the regression lines for heterospecific crosses to predict effective sperm-to-egg ratios (ER_{50s}) fertilizing half of all eggs (28). These ER_{50s} were 112,201 (green sperm X red egg); 30,199 (red sperm X green egg); 91.2 (red sperm X red sperm); and, 76.3 (green sperm X green egg). As calculated, ER_{50s} were significantly higher for heterospecific than for conspecific crosses (one-way ANOVA with *post-hoc* Scheffé test, $P < 0.0001$, all comparisons).

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Chapter III:

Broadcast Spawning: A Test of the Sperm Limitation Hypothesis

Abstract

Sperm limitation is widely purported as the major determinant of reproductive success for organisms that spawn their gametes into the sea. This paradigm is based largely on studies of relationships between fertilization success and either sperm density or gamete longevity. Yet, other critical variables include egg density, sperm-to-egg ratio, contact interaction time, and physiochemical properties of the gamete suspensions. Using a factorial design, egg (10^1 to 10^5/ml) and sperm (10^2 to 10^8/ml) density combinations, and interaction intervals (5 – 1,200 s), were tested for effects on red abalone (*Haliotis rufescens*) fertilization. These experiments showed, first, that sperm-to-egg ratio explained $\geq$ 60% of the variation in fertilization success. Thus, sperm concentration, per se, played only a minor role in determining reproductive rate. Second, contact time and gamete longevity mattered little. Whereas eggs and sperm are viable for at least 30 min, percent fertilization asymptoted within 15 s of initial gamete contact. Additional experiments established that egg suspensions were negatively buoyant (1.09 g/ml) and sank rapidly (3.7 mm/s) compared to sperm solutions (density: 1.0275 g/ml; fall velocity: 0.01 mm/s). Egg suspensions remained highly viscous, even at relatively

low concentrations (10^2 eggs/ml) and high shears ($\geq$ 10/s). Yet, viscosity of sperm solutions decayed quickly, as a function of increasing shear and decreasing gamete concentration. Given these biomechanical properties of sperm and egg, behavior of reproductive fluids in turbulent flow cannot be predicted by classic Gaussian or Fickian advection-diffusion models. Moreover, egg, not sperm, limitation is the probable agent controlling gamete contact rate, and hence, fertilization success in red abalone.

Introduction

Many marine organisms reproduce through the release, or broadcast, of gametes into the ocean environment, where fertilization takes place external to the body cavity. Consideration of the events controlling fertilization, termed "fertilization ecology", has led to valuable insight into factors influencing population dynamics and life history evolution of marine organisms. Because sperm concentrations can quickly drop to such low levels that fertilization is no longer possible, studies of fertilization ecology have traditionally focused on the events where low sperm abundance regulates reproduction (Lillie, 1915; Pennington, 1985; Levitan, 2002). Today, sperm limitation is argued as the primary determinant of reproductive success for many marine organisms, and a major force in the evolution of reproductive behavior, gender, anisogamy, and egg size (Goodwillie, 2001; Levitan, 1993, 2002). Recent evidence of high fertilization rates in natural spawning events, however, coupled with the existence of intense polyspermy blocks in eggs, has suggested that sperm limitation may occur only infrequently, and may not be as prevalent as previously believed (Brawley, 1987; Babcock and Mundy, 1992;

Coma and Lasker, 1997; Kiflawi et al., 1998; reviewed in Yund, 2000). Thus, it remains unclear whether sperm limitation may be the dominant component influencing fertilization, or other factors may be involved.

There are several motivating factors for why sperm limitation is believed to regulate reproduction and has been the focus of intense research for the past 30 years. First, few investigations have sought to resolve the relative influence of sperm and egg concentration on the fecundity of broadcast spawners. Indeed, most laboratory studies have concentrated solely on the influence of sperm concentration and longevity on fertilization rates. Given that most of the study organisms inhabit turbulent environments where sperm and eggs have only seconds for gamete contacts to take place, gamete longevity - often on the scale of hours - probably is not a bottleneck for successful reproduction (Table 3-1). Moreover, by holding egg concentration constant while only varying sperm concentration, fertilization success has always covaried with sperm concentration. Another reason why sperm limitation dominates fertilization ecology is that, together with the laboratory experiments, manipulative field studies may have artificially induced sperm limitating conditions. For example, releasing already dilute sperm in IV bags or hypodermic syringes (male mimics), and using fixed mesh bags or baskets to hold eggs (female mimics), disregards the behaviors and properties of natural spawns (Table 3-1). Therefore, the experimental designs used in past laboratory and field studies may have inadvertently produced unrealistic predictions on the severity of sperm limitation in natural populations.

Important features of the gametes that may explain the dramatic differences in fertilization between natural spawns and experimental studies are the biomechanical properties of the released sperm and eggs (Thomas, 1994a; Thomas and Bolton 1999). Naturally spawned gametes exist as dense aggregations and clumps, and exhibit complex fluid dynamic behavior due to their physical properties (e.g., viscosity, fall velocity; Thomas, 1994a, b). For example, sperm-egg viscosity, a measure of the stickiness of the fluid, will dampen turbulent water flow and decrease the dilution rate of the gametes. Gametes that are negatively buoyant will fall out of the water column, remaining in close proximity to the spawning adults. By not taking into account these biophysical properties of the gametes, previous investigations may have circumvented some of the basic strategies used to counteract gamete dilution.

Given the above uncertainties to which sperm limitation may influence fertilization, we conducted this initial laboratory study to establish whether sperm limitation is the primary determinant of reproductive success. First, we examined the gamete longevities to establish whether age may be a limiting factor. Next, using a factorial design, egg and sperm density, and contact intervals were tested for effects on red abalone (*Haliotis rufescens*) fertilization. Using traditional analyses of past fertilization ecology studies, we examined the percentage of fertilized eggs as a function of sperm-to-egg ratio. Rather then relying on the fraction of fertilized eggs as our sole metric of reproductive success, we also examined the influence of sperm and egg density on the total number of fertilized eggs. Finally, as gamete concentration may be one of several important, though related, factors controlling fertilization success, we additionally

examine the biophysical properties of the gametes to establish how they may influence gamete interactions in the field. By using these analyses in a simplified laboratory setting, we were able to identify the critical factors that regulate reproductive success, and demonstrate that sperm limitation is not the primary factor controlling reproductive success.

Materials and Methods

Study organism and experimental procedures

Experiments were performed at Scripps Institution of Oceanography, University of California at San Diego. Mature *Haliotis rufescens* (red abalone) from Cultured Abalone, Inc. (Goleta, CA) were held in aquaria of running seawater (15 ^{o}C) and spawned using the methods of Riffell et al. (2002). Briefly, animals were fed fresh kelp, *Macrocystis pyrifera*, and induced to spawn through exposure to hydrogen peroxide (6%) for 2 hours followed by rinse and exposure to 0.22-μm filtered seawater (Morse et al., 1977). Once individuals initiated spawning, gametes were collected above the excurrent tremata and placed in centrifuge tubes. Sperm concentration for each male was determined by four replicate haemocytometer counts, and egg concentration for each female was determined by four replicate counts of 50-μl aliquots.

Approach for testing the sperm limitation hypothesis

The sperm limitation hypothesis predicts 2 primary factors influencing fertilization success of broadcast spawners; the first is that sperm longevity is extremely

short relative to egg. The second prediction is that reproductive success is more dependent upon sperm concentration then egg concentration or sperm-to-egg ratio. The following experiments and analyses will test each of these predictions on the fecundity of the red abalone.

Effect of gamete age on fertilization success

Gamete longevity has long thought to be an important factor controlling successful fertilization in free-spawning marine invertebrates (Pennington, 1985). However, in the natural kelp-forest habitat in which gametes are spawned, eggs and sperm have only seconds to a few minutes in which to encounter one another before gametes become too dilute. To establish whether egg age may be the dominant factor regulating fertilization at these time scales, approximately 100 freshly spawned eggs from each of 5 females were placed into 32 mm diameter petri dishes containing filtered seawater, 4 replicates per female for each time period. At every ten minute interval, for a total duration of 120 min, 1-ml aliquots of 10^5 sperm/ml stock solution from 5 males were added to each petri dish. Fertilization success was assessed by counting the number of eggs having gone through division 3 h post spawn. At time of the final treatment period, freshly spawned eggs from two females were fertilized with the same sperm in order to assess any changes in sperm viability over the duration of the experiment.

In parallel with the experiment on egg longevity, an experiment was performed to determine the influence of age on sperm viability. Six different time periods (0, 3, 6, 9,

and 12 h) were used at a sperm concentration of 10^5 sperm/ml. A 1-ml aliquot of diluted sperm was placed in a 32 mm diameter petri dish containing 100-200 freshly spawned eggs, with 8 replicates for each time period. Each time period used freshly spawned eggs from 4 females (a total of 20 females). Percent fertilization values were arcsine transformed prior to analysis.

Influence of sperm-egg encounter duration and concentration on fertilization

Once gametes are spawned in the field, the concentration and total duration at which sperm-egg interactions occur vary widely due to gamete dilution. To assess the influence of varying gamete concentration and contact duration in the laboratory, we varied three factors simultaneously in a factorial design, namely egg concentration, sperm concentration, and sperm-egg contact time. Natural concentrations of sperm and eggs in freshly spawned gamete plumes typically contain 10^9 and 10^5 gametes/ml, respectively. Thus, five egg concentrations (10^5, 10^4, 10^3, 10^2, and 10^1 eggs/ml), seven sperm concentrations (10^8, 10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 sperm/ml), and eight sperm-egg contact time periods (5, 15, 30, 60, 120, 240, 600, and 1200 s) were used, with 6 replicates for each treatment combination. Plastic tubes with one end covered with 55-μm mesh screen were placed in containers that held the sperm solutions. Aliquots (1-ml) of egg suspensions were pipetted into the partially immersed tubes. After the prescribed encounter time, immersed eggs were removed from the sperm solution, rinsed several times, and placed in containers of filtered sea water. Microscopic examinations indicated that the rinse eliminated all sperm near the eggs surfaces, except those cells attached to

the vitelline envelope. After 4 h, the time at which fertilized eggs will have undergone cleavage and reached the 16 – 32 cell stage, eggs were placed in eppendorf tubes, fixed with 5% formalin, and scored for the number of fertilized eggs.

Fertilization success was scored in two different ways. First, we established the percentage of eggs fertilized. This dimensionless ratio was useful in addressing independently of egg number the effects of contact time, gamete densities, and sperm-egg ratio, on fertilization success across widely varying treatments. However, the link between percent fertilization and reproductive fitness is unclear. Fitness is defined ultimately as the number of progeny (or genes) passed by an adult into the next generation. As a second, alternative means of scoring fertilization success, we therefore determined the total number of fertilized eggs (hereafter termed "embryos") resulting from each trial cross. Prior to statistical analysis, fertilization data was log transformed to achieve homogeneity of variances.

Sperm versus egg limitation

To establish whether sperm or egg limitation was occurring, three-dimensional surface plots were then created using the number of embryos as a dependent variable (z-axis) and sperm (x-axis) and egg (y-axis) densities as independent variables. A separate plot was generated for each contact time. The rate of change in fertilization success (∂F, number of embryos) was determined as a function of the rate of change in sperm (∂S) or egg (∂E) density. Sperm limitation was thus defined by the inequality

$$\partial F/\partial S > \partial F/\partial E. \quad (1)$$

In contrast, a reversal in the sign of inequality indicated that eggs, not sperm, were limiting the rate of change in fertilization success

$$\partial F/\partial S < \partial F/\partial E. \quad (2)$$

For each contact time, rates ($\partial F/\partial S$ and $\partial F/\partial E$) were derived empirically by integrating across each of 35 interaction "windows – 7 sperm concentrations by 5 egg concentrations. The overall contribution of sperm, or egg, limitation was defined by the relative proportion of interaction windows with a ">" or "<" inequality sign, respectively.

Gamete physical properties

For free-spawning marine invertebrates, gamete physical characteristics have the potential to strongly influence both gamete concentration and contact duration within the water column. To examine the physical properties of the gametes, we specifically chose to study the viscosity, density, and gravitational fall velocities of eggs and sperm. Viscosity was measured using a cone and plate viscometer (Wells-Brookfield model DV-II) maintained at a temperature of 15°C using a constant temperature bath. Each viscosity measurement was made on 0.5 ml of gametes, the volume required by this model of viscometer. Measurements were begun at a low shear rate of 0.6/s and increased in a step-wise fashion to 1.2, 3.0, 6.0, and 12.0/s, and then reversed from the highest to lowest shear. Shear rates tested lie in the natural range within crevices and in exposed kelp forest where abalones are found (Riffell, in prep.).

To determine whether the viscosity of the gametes exhibits shear-thinning behavior (decrease in viscosity with increasing shear), and to determine the field conditions most likely to produce the greatest change in viscosity, we assumed that fluid viscosity is proportional to a power of the applied force,

$$\eta = k(du/dr)^{n-1}, \quad (3)$$

where η is the viscosity, u is the velocity of the fluid, r is the distance from the wall, du/dr is the shear rate (rate at which force is applied), n is the power coefficient, and k is the proportionality constant. A value of n less then 1 indicates that the fluid is shear-thinning, while k describes the viscosity of the fluid, with higher values of k indicating greater viscosity over all shear rates.

Gravitational fall velocities of the gametes were conducted at 15°C within a water-jacketed fall chamber in the dark, following procedures of Hannan (1984) and Butman et al. (1988). Gametes within the chamber were illuminated with a narrow, focused light sheet created using an IR (830 nm, 25 mWatt) laser diode (Coherent model NT54-029), equipped with a concave line-generating lens. Images were captured using an IR-sensitive video camera (COHU Model 6415-2000, with active heads) oriented 90 degrees to the axis of the laser sheet. Video images were digitized at 30 frames/s and processed over 10 s intervals using a Computer-Assisted Video Motion Analysis (CAVMA, Motion Analysis Corp. model VP 320, ExpertVision, and custom software) interfaced with a Sun SPARC 2 computer workstation (Gee and Zimmer-Faust, 1997). Gamete densities were determined by pipetting eggs and sperm into a series of vials

containing an upper layer of seawater and a lower layer of diluted Percoll (Pharmacia Inc.). Gametes were denser than the lower layer if they continued to sink through it.

To ensure that all gametes used in these trials were viable, fertilization assays in each experiment were run in parallel. Material from each of the spawns produced 86-99 % fertilization when sperm and eggs were mixed.

Results

Gamete age

Freshly spawned eggs were incubated in filtered seawater before being exposed to sperm. Time had a significant affect on egg viability (Fig. 3-1), with fertilization success quickly decreasing to 0% 90 min after egg release (one-way ANOVA: $F = 330.5$, $df = 13/98$, $P < 0.001$). To establish the relationship between percentage of fertilization and egg age, a logistic regression was fit to the data (Fig. 3-1A, F-test: $F = 8.92$, $df = 1/103$, $P < 0.001$). Based on the logistic regression equation, the F_{50} of egg viability - the time at which half of all eggs were fertilized - was 51min post-spawn. In contrast to the limited viability of eggs, sperm longevity extended on the order of hours (Fig. 3-1B). The F_{50} value from the logistic regression was 394 min post spawn (Fig. 3-1B, F-test: $F = 3.52$, $df = 1/19$, $P < 0.05$). Thus, egg age, not sperm, may be the limiting factor at time periods exceeding 30 min after spawning. However, given that the turbulent kelp forest environment in which abalones spawn will preclude gamete contacts to only the first few minutes, gamete age may not be a significant factor controlling fertilization success in this organism.

Sperm-egg encounter time

Next, to establish the degree to which sperm determines reproductive success, we used a factorial design that allowed us to separately establish the relative influence of egg, sperm, and contact time on fertilization success.

To establish the influence of sperm-egg encounter duration on fertilization rates, the percentage of fertilized eggs were analyzed for each time period (5-1200 s). Results indicated gamete contact time mattered little, with difference in fertilization rates becoming non-significant after only 5 s (Fig. 3-2; one-way ANOVA for time: $F_{7,1703} = 4.99$, $P < 0.001$; Scheffé test: $P > 0.53$ for encounter times after 5 s). Thus, fertilization was extremely rapid, even in still water, with time having no effect on reproductive success beyond the first 5 s.

Gamete concentration

The percentage of fertilized eggs was subsequently analyzed to establish the influence of gamete concentration, egg and sperm, on fertilization success. Both egg and sperm density had a highly significant effect on fertilization (one-way ANOVA for egg: $F_{4,1706} = 64.83$, $P < 0.0001$; one-way ANOVA for sperm: $F_{6,1706} = 371.92$, $P < 0.0001$). Moreover, a step decrease in sperm density had nearly the same effect as an equivalent step increase in egg density, and *vice versa* (Table 3-2). Thus, suggesting that both gamete densities may be important factors controlling the percentage of fertilized eggs, with egg density having an equal effect to that of sperm.

Gamete ratio

To integrate the data into a more manageable form, and to understand the relative contribution between egg and sperm density on fertilization success, the ratio of sperm-to-eggs was used to non-dimensionalize the gamete concentrations. Results show that percent fertilization was highly dependent upon the gamete ratio, with percent fertilization increasing monotonically from 0% to 100% as the ratio of sperm-to-eggs was raised from 1 to 10,000 (Fig. 3-2). For each contact time, a forward stepwise multiple regression was applied to determine the percent variation in reproductive success explained by sperm concentration, egg concentration, and sperm-to-egg ratio. A significantly larger effect of male gamete concentration is predicted by the sperm limitation hypothesis. The stepwise regression analyses demonstrate that while both gamete ratio and sperm concentration had significant effects, sperm-egg ratio was the best predictor of fertilization success. Regression values indicated that the gamete ratio cumulatively explained 60 to 82%, and sperm concentration explained 0.1 to 6% of the variation, respectively (Table 3-3). Thus, percentage fertilization is not solely dictated by sperm density, but instead is controlled by the ratio of the sperm-to-egg concentration.

Embryo production

The above analyses strongly demonstrate that egg and sperm, together, are critical for the percentage of fertilized eggs. Yet, using the percentage of fertilized eggs as the only measure of fecundity may underestimate the total reproductive output of broadcast

spawning individuals (Podolsky and Strathmann, 1996). Therefore, to better quantify the relative role of egg and sperm density on fertilization success, and to establish whether sperm, or egg, is the dominant factor influencing reproduction, we examined the total number of embryos produced (16 – 32 cell stage), rather then the fraction of fertilized eggs, for each time period and sperm-egg density interaction.

Results demonstrate that both egg and sperm concentration had a statistically significant affect on the total number of embryos (one-way ANOVA for egg: $F_{4,1492} = 1871.69$, $P < 0.0001$; one-way ANOVA for sperm: $F_{6,1492} = 73.8$, $P < 0.0001$). However, when graphically displayed, the relative contribution of the gametes on embryo production is dramatically clear (Fig. 3-3, *Top*). For each time period, a matrix of sperm concentration by egg concentration was constructed with the total number of embryos for each sperm-egg density interaction. Egg density had a striking affect on embryo production; a single egg density can produce nearly the same number of embryos over 3 orders of magnitude sperm density, while a single-order lower egg density produces an equivalent 1-order lower number of embryos over 3 orders of magnitude sperm density. For example, at a contact time of 15 s, a 3-order decrease in egg concentration caused a 440-fold drop in the total number of embryos (34,998 to 79 embryos). In contrast, a 3-order magnitude decrease in sperm caused only a 5-fold drop in the number of embryos (34,998 to 6310 embryos). Thus, both egg and sperm density may critically affect embryo production, but egg density had an 80-fold greater influence then that of sperm.

Gamete limitation

The influence of gamete density on zygote production does not necessarily establish the extent to which sperm, or eggs, control fertilization. Therefore we next measured the rate at which embryos were produced relative to each sperm-egg density, and used this change in embryo production as the measure of egg or sperm limitation. From the embryo production surface plots (Fig. 3-3, *Top*), the degree to which egg-, or sperm-limitation occurred was integrated over the entire surface map. There was a significant time effect for the proportion of egg limitation to sperm limitation (Fig. 3-3, Table 3-4, one-way ANOVA: $F_{7,24} = 9.21$, $P < 0.0001$). However, after 5 s there was not a significant difference between time periods in the degree at which either egg or sperm limitation occurred (Table 3-4, *post-hoc* Scheffé test: $P > 0.23$). Moreover, egg limitation, not sperm limitation, significantly dominated the surface plots, with eggs being limiting ~55% of the fertilization landscape (one-way ANOVA: $F_{1,62} = 20.2$, $P < 0.0001$) . Thus, while eggs and sperm may both be important agents controlling the percentage of fertilized eggs, egg concentration may regulate the total number of developing embryos, and ultimately drive reproductive success.

Gamete physical properties

Gamete density has a critical effect on reproductive success, yet could be one of several important, though related, characteristics of the gametes that influence sperm-egg interactions. Once spawned into the water column, gametes have the potential to influence their density and interaction time through the inherent physical properties of the spawned material. Thus, controlled laboratory experiments were performed to examine

how physical properties of the spawned material may influence the potential for gamete interactions in the field.

Gravitational gamete fall velocities and densities - To ascertain the sinking rates of the gametes, a gravitational fall velocity chamber was used. Because eggs are typically spawned at high concentrations, the influence of egg number on the sinking rates of eggs was determined. Results indicate that concentration had a significant affect, with egg aggregates composed of 100-200 eggs having ~10-fold higher fall velocity then that of single eggs, 3.72 ± 0.15 *versus* 0.41 ± 0.21 mm/s, respectively (one-way ANOVA: $F_{2,57}$ = 882.99, $P < 0.0001$; Scheffé test: $P < 0.0001$). Moreover, sperm had a significantly lower sinking rate then eggs, approximately 0.012 ± 0.01 mm/s (Scheffé test: $P < 0.0001$ both comparisons). The density of eggs (Table 3-5; 1.092 to 1.095 g/ml) was higher then seawater, while sperm (1.0275 to 1.03 g/ml) was close to the seawater density (1.023 g/ml at 15°C). Hence, once spawned, eggs will rapidly sink out of the water column, while sperm, being close to neutrally buoyant, will remain in the water column longer.

Gamete viscosity - Viscosity of eggs and sperm, and the effect of gamete concentration on viscosity, was measured using a cone-and-plate viscometer. Both eggs and sperm displayed shear-thinning behavior; that is, viscosity decayed as a function of the shear imposed upon the gametes (Table 3-5, Fig. 3-4). This shear-thinning behavior is apparent by the power coefficients (n) used to calculate the regression equations, with both female and male gametes exhibiting n values much less then 1 (Table 3-5). The difference

between male and female viscosities can also be ascertained by their relative difference in k, a description of the consistency of the fluid, with female gametes having a much larger k coefficient then males. Moreover, when abalone gametes are compared with sea urchin gametes, blood, and other fluids, we find that the gametes and blood are similar to one another, with small power-coefficients and a rapid drop in viscosity with increasing shear (e.g., low ES_{50s}). Previous studies examining the physical characteristics of blood have found that the cell-cell interactions and protein content in the fluid are the critical determinant of blood's non-Newtonian behavior. Thus, gametes from different organisms may have similar physical properties derived from the cellular components (e.g., sperm, eggs) and material making up the spawned gamete plumes.

To understand the relative viscosity of seawater to that of the spawned gametes, and to establish how viscosity of the gametes will change as they become more dilute, the ratio of gamete viscosity (V_{gam}) to seawater (V_{sea}) was used (Fig. 3-4). This analysis allows a quantitative understanding the effects of dilution on gamete viscosity, and insight into the physical conditions (shear), or dilution, which will cause the gametes to behave like seawater (e.g., as V_{gam}/V_{sea} approaches unity). Moreover, eggs and sperm have completely different physical characteristics, thus, this comparison gives insight into how eggs or sperm may influence gamete interactions in the field.

For both gametes, increasing shear had a significant reciprocal decrease in viscosity (one-way ANOVA: $F_{4,175} > 6.85$, $P < 0.0001$ for both eggs and sperm). Additionally, there was a dramatic difference between egg and sperm viscosity at all concentrations, with eggs being more viscous then sperm by 3-orders of magnitude (one-

way ANOVA: $F_{1,357} = 31.69$, $P < 0.0001$). Logistic regressions were used to describe the relationship between gamete viscosity and shear (F-test: $F_{1,30} > 4.05$, $P < 0.05$, both comparisons). Calculated ES_{50s} from the regression equations – the shear which produced the half-maximal response – were dramatically different between sperm and eggs. The ES_{50} for the egg viscosity was at a shear of 2.1/s, while sperm ES_{50} was at 1.0/s. Moreover, at this shear, eggs were still 5000-times more viscous then seawater, while sperm was only 3-times that of seawater. Concentration also had a strong influence on gamete viscosity. With one-order dilution, sperm had nearly the same viscosity as that of seawater ($V_{gam}/V_{sea} = 1.7$). In contrast, egg viscosity after a single dilution was ~3500-times more viscous then seawater, and even after a five-fold dilution remained highly viscous ($V_{gam}/V_{sea} = 3.7$).

Taken together, these results show that eggs behave in quite a different manner than sperm. Once spawned, eggs will remain at high concentrations by clinging together, resisting the shear caused by turbulent eddies, and will rapidly fall out of the water column. On the other hand, sperm, being close to neutrally buoyant, will remain in the water column and behave much like a dye, dispersing as it is spawned.

Discussion

For nearly a century, studies on the reproductive biology of aquatic organisms have implicated sperm limitation as a major factor controlling successful fertilization (Lillie, 1915; Levitan, 1995). In this study, sperm-to-egg ratio, not sperm concentration, was found to be the critical determinant for the percentage of fertilized eggs, with a

decrease in sperm concentration having the same effect on fertilization as an equivalent increase in egg concentration. Moreover, using the total number of embryos produced rather then the percentage fertilized eggs as a measure of reproductive success, a 3-order magnitude decrease in sperm density caused a 5-fold drop in the number of embryos, while a 3-order magnitude decrease in egg concentration caused a 440-fold decrease in the number of embryos. Thus, egg density had an 80-times greater influence then sperm on embryo production. Finally, when the gamete physical properties were examined to establish how they may potentially influence sperm-egg interactions, eggs behaved quite differently then sperm, with much greater viscosity, density, and sinking rates. Sperm on the other hand, was close to neutrally buoyant and once diluted had nearly the same characteristics as that of seawater. Thus, spawned eggs will remain clumped and rapidly sink to the bottom at high concentrations, while sperm will remain in the water column and be transported for much longer distances. Because of the biomechanical characteristics of the gametes, studies using Gaussian advection-diffusion models to predict transport and mixing rates of the gametes will under- or overestimate fertilization rates. When these results are placed in context of our fertilization results, eggs may have an equal, or greater, influence then sperm on gamete contact rates and fertilization success.

Egg versus sperm limitation

Previous fertilization ecology studies have already examined the effects of sperm-egg ratio, and egg and sperm concentration on fertilization. However, in the case of

sperm-to-egg ratios, few studies have held egg densities per unit volume (e.g., #/ml) constant, while manipulating the gamete ratios (Lillie, 1915; Vogel et al., 1982; Levitan et al., 1991). Instead, most studies have manipulated gamete ratios by changing the sperm concentration (#/volume), and manipulated the number of eggs exposed to the sperm, ignoring the egg concentration (#/volume; Benzie and Dixon, 1994; Baker and Tyler, 2001). In reality, only the sperm concentration is changing in these experiments, rather then the sperm-to-egg ratio (# sperm per volume: # egg per volume), so that the percentage of fertilized eggs in these experiments will always scale with sperm concentration, rather then with the gamete ratio.

Lillie's (1915) study using the sea urchin *Arbacia* was the first deliberate laboratory test on the effects of gamete kinetics and concentration on fertilization. He found that sperm-egg ratio was critical, but only when sperm-egg concentrations were near equal, and noted that fertilization was less sensitive to egg density then to sperm density; a 16-fold decrease in sperm concentration caused approximately the same decrease in percent fertilization as a 64-fold increase in egg concentration. More recently, studies using sea urchins (Vogel et al., 1982; Levitan et al., 1991) found similar results, where percentage fertilization was more sensitive to sperm than egg concentration. However, these studies examined only limited egg densities (10-fold change in density) in comparison to sperm densities (1000 to 10,000-fold change), and both measured reproductive success as the percentage of fertilized eggs, rather then the total number of embryos. Our own results using the red abalone directly contrast those studies, indicating that a decrease in sperm concentration having the same effect as that

of an equivalent increase in egg concentration (Table 3-2), and that egg limitation may strongly influence embryo production. The disparity in results obtained in this study compared to previous studies can be resolved if we examine Lillie's (1915), Vogel et al.'s (1982), and Levitan's (1991) results as a function of the number embryos produced and egg and sperm concentration (Fig. 3-5). These were the first studies to quantitatively examine the relative influence of egg and sperm density on fertilization rates, and have provided the foundation for studies on the fertilization ecology of marine organisms. However, in those studies we find that the range of gamete concentrations tested small (Lillie, 1915; Vogel et al., 1982), and the concentration between sperm and eggs unequal (Levitan, 1991). Thus, in those studies, sperm limitation may have inadvertently appeared to dominate reproductive success.

Model systems in fertilization ecology

Studies of fertilization in marine organisms have traditionally used echinoderms, in particular, sea urchins. Because of the ease at which sea urchins are cultured, the often easy field collection for common species, and the rapid spawning method using HCl, has made echinoderms premier organisms in which to study the mechanisms influencing fertilization success, particularly for manipulative field studies. Today, pioneering work with bryozoans, ascidians, and algae, among others, has increased our understanding of the factors influencing reproduction in marine organisms. However, many of these studies use organisms that are brooders, where fertilization occurs internally after sperm are spawned into the water column and actively filtered out of the water. Gaps yet

remain in our understanding of the mechanisms controlling fertilization success for broadcast spawning organisms – organisms that release both eggs and sperm into the water column – besides those of echinoderms.

The red abalone provides an additional system in which to study the fertilization ecology of broadcast spawners. Molecular studies on the factors controlling sperm-egg interactions have been better characterized for abalone (genus *Haliotis*) than for any other organism (Vacquier, 1998). That, in combination with the ability to maintain the red abalone reproductively active year round, provides an excellent model system in which to study the factors controlling fertilization. Moreover, the red abalone provides an interesting contrast to fertilization ecology studies conducted with other marine invertebrates. With the limited viability of the eggs and extended longevity of the sperm, both contrasts to many echinoderm, ascidian, and horse shoe crab species with their extended egg viability and limited sperm longevity (Pennington, 1985;; Havenhand, 1991; Benzie and Dixon, 1994), combined with the fact that abalones spawn the gametes out in jets from their excurrent holes without any external morphology, like spines in sea urchins, to catch and concentrate the gametes (e.g., Thomas, 1994a,b), makes the abalone an interesting system to study fertilization. Furthermore, the complete biochemical signaling system leading to natural spawning behavior has been fully elucidated in the red abalone (Morse, 1977). This is in stark contrast to the chemical inducer, KCl, commonly used to initiate spawning in many echinoderms. Whereas in abalone, addition of H_2O_2 to seawater causes the same innate biochemical-signaling cascade that precipitates spawning behavior to those abalones that naturally spawn in the field. In sea

urchins, however, the chemical KCl is a potent muscular neurotoxin, and when injected into the gonad of sea urchins, initiates intense muscular contractions causing the sea urchins to eject their gametes into the sea water. Thus, induced gamete release rates, production, and behaviors by urchins may dramatically differ from natural spawning events, hence causing laboratory flume experiments and manipulative field studies to potentially be subject to artifact.

Evolutionary implications for egg limitation

Evolutionarily, populations of abalone occurred in highly dense, clumped aggregations, located deep within crevices and ledges (Cox, 1962). Through intense predation by sea otters, abalone populations were restricted to these refuges, and on the central coast of California where sea otters exist, abalones are typically found within this complex relief (Tegner, 2000). In these microhabitats, turbulent water flow is dampened by several orders of magnitude in comparison to the outer reef system (Riffell, in prep.). Typical shear rates within crevices and ledges range from 0.1 to 1.4/s. Thus, spawned gametes, due to their physical properties and ability to withstand shears of 1.0 to 2.0/s, will be retained at high concentrations and for extended periods of time. Field observations of spawning abalones have supported this view, with spawning events occurring during extremely calm conditions, and with low dilution rates of gametes (Breen and Adkins, 1980; Stekoll and Shirley, 1993). Under such conditions, low sperm abundance will not occur, and sperm competition may be dominant. Molecular evidence in abalone has demonstrated that evolution of egg receptors drives rapid selection for

sperm with the appropriate egg-binding protein (Swanson and Vacquier, 1998). If sperm were limiting, selective pressure imposed by the eggs would be impossible (Metz and Palumbi, 1996; Palumbi, 1999). Moreover, evidence of the restricted longevity of the eggs in contrast to that of the sperm, and the existence of intense polyspermy blocks, suggests that eggs may have evolved under sperm saturating conditions, implicating egg limitation as an important factor influencing reproduction in this organism (Jaffe and Gould, 1985; Vacquier et al., 1997; Swanson et al., 2001; Riffell et al., 2004).

Within the context of the natural habitat, our results suggest that successful fertilization may be regulated by both egg limitation and sperm limitation. In contrast to previous fertilization ecology studies that have primarily used echinoids, our study with a marine archaegastropod suggests that the current paradigm of sperm limitation controlling fertilization may be unfounded. Moreover, fertilization ecology studies should adequately scale laboratory conditions to that of the natural habitat (Mead and Denny, 1995; Denny et al., 2002). In doing so, it may be possible to elucidate the underlying mechanisms dictating fertilization. Future work should address how egg limitation may influence fertilization in the field, and the conditions, both chemical and physical, that may control successful fertilization. As abalones are severely impacted and are at historic low densities, such determinations could have critically important implications for population recovery and sustainability.

Table 3-1. Previous fertilization ecology studies.

Taxon	Setting	Sperm concentration (cells/ml)	Egg concentration (cells/ml)	Contact time (s)	Reference
Echinodermata					
Echinoidea					
Arbacia	Laboratory	10^6 - 10^7	10^4 - 5.0 x 10^5	NA	*Lillie*, 1915
Paracentrotus lividus	Laboratory	10^7 - 10^4	2.0 x 10^3 - 6.0 x 10^3	100	*Vogel et al.*, 1982
Strongylocentrotus franciscanus	Laboratory	10^7 - 10^3	0.5 x 10^3 - 5.0 x 10^3	180	*Levitan et al.*, 1991
Strongylocentrotus droebachiensis	Laboratory	10^0 - 10^{10}	~10^1	10	*Pennington*, 1985
Dendraster excentricus	Laboratory	10^7 - 10^1	10^2	15	*Podolsky*, 2002
Clypeaster rosaceus	Laboratory	10^6 - 10^2	~10^1	60	*Levitan and Young*, 1995†
Asteroidea					
Acantaster planci	Laboratory	10^5 - 10^1	10^2 - 0.5	387	*Benzie and Dixon*, 1994
Mollusca					
Bivalvia					
Laternula elliptica	Laboratory	10^9 - 10^1	NA	1440	*Powell et al.*, 2001
Chlamys bifrons	Laboratory	10^7 - 10^2	103	210	*Styan and Butler*, 2000
Prosobranchia					
Haliotis tuberculata	Laboratory	10^7 - 10^2	102	0.08 - 40	*Baker and Tyler*, 2001
Haliotis laevigata	Laboratory	10^7 - 10^2	~10^3	0.11 - 40	*Babcock and Keesing*,1999
Urochordata					
Pyura stolonifera	Laboratory	10^8 - 10^1	103	180	*Marshall et al.*, 2000
Echinodermata					
Echinoidea					
Evechinus chloroticus	Field	induced (10^3 - 10^7)	induced (NA)	3	*Franke* et al., 2002
Strongylocentrotus franciscanus	Field	syringe (10^8)	syringe (10^4)	2	*Levitan*, 1998
Strongylocentrotus franciscanus	Field	syringe (10^{10})	basket (10^2)	30	*Levitan et al.*, 1991
Clypeaster rosaceus	Field	syringe (NA)	mesh bag (10^1)	20	*Levitan and Young*, 1995†
Strongylocentrotus droebachiensis	Field	induced (NA)	syringe (NA)	10	*Pennington*, 1985
Asteroidea					
Oreaster reticulatus	Field	induced (NA)	induced (NA)	1	*Metaxas et al.*, 2002
Mollusca					
Haliotis laevigata	Field	IV bag (10^8)	pump (10^2)	NA	*Babcock and Keesing*,1999

Table 3-2. Percent of eggs fertilized at different sperm and egg concentrations during a 15 s contact time. Egg concentration had a reciprocal effect to that of sperm on fertilization success, with a drop in sperm concentration exerting the same influence as an increase by the same factor in egg concentration, and vice versa. The percentage fertilization for each sperm-egg density interaction is the mean (± SEM) from 6 trials (see Fig. 3-2). The results shown are typical of all the time periods after the first 5 s contact time (*post hoc* Scheffé test: $P > 0.29$). Sperm concentrations of 10^8 and 10^2 sperm/ml were omitted because they lie in the tails of the fertilization curve.

Sperm concentration (#/ml)	Egg concentration (#/ml)				
	10^5	10^4	10^3	10^2	10^1
10^7	46.5 (5.0)	62.2 (4.3)	85.0 (3.0)	84.4 (3.5)	92.5 (4.7)
10^6	27.3 (3.2)	43.1 (7.5)	63.0 (4.5)	83.5 (2.7)	75.7 (4.8)
10^5	4.0 (1.9)	45.0 (12.8)	43.8 (5.1)	61.6 (2.8)	82.5 (4.7)
10^4	2.0 (0.9)	11.5 (5.3)	24.7 (3.7)	38.3 (5.2)	72.5 (8.5)
10^3	2.5 (1.8)	1.0 (0.7)	5.7 (1.8)	6.1 (2.0)	29.2 (9.6)

Table 3-3. The results of a forward stepwise multiple regression model for each time period examining the relative influences of sperm concentration, egg concentration, and gamete ratio on the percentage fertilized eggs. This analysis revealed that fertilization is highly contingent upon gamete ratio, rather then sperm concentration alone.

Time (s)	Source	df	F-value	% of variance explained
5	Gamete ratio	1/248	391.9*	61.1
	Sperm	2/247	26.9*	4.2
	Egg	3/246	2.4	<0.1
15	Gamete ratio	1/250	845.5*	77.2
	Sperm	2/249	3.4	<0.1
	Egg	3/248	1.3	<0.1
30	Gamete ratio	1/238	953.5*	80.0
	Sperm	2/237	18.6*	1.5
	Egg	3/236	0.1	<0.1
60	Gamete ratio	1/250	1012.4*	80.0
	Sperm	2/249	2.1	<0.1
	Egg	1/248	3.6	<0.1
120	Gamete ratio	1/250	1183.7*	82.6
	Sperm	2/249	9.7*	0.6
	Egg	3/248	2.2	<0.1
240	Gamete ratio	1/240	653.2*	73.1
	Sperm	2/239	15.6*	1.7
	Egg	3/238	0.3	<0.1
600	Gamete ratio	1/243	578.3*	70.4
	Sperm	2/242	4.2*	0.5
	Egg	3/241	2.3	<0.1
1200	Gamete ratio	1/246	382.7*	60.9
	Sperm	2/245	51.2*	6.7
	Egg	3/244	2.1	<0.1

* $P < 0.0001$.

Table 3-4. The proportion occurrence of egg or sperm limitation. Each time period is the mean (± SEM) from 4 complete trials, with each individual trial containing 35 sperm-egg concentration interactions, or "windows" (7 sperm concentrations by 5 egg concentrations) that made up the limitation and zygote production plots (see Fig. 3-3).

Time (s)	% Egg limitation (n = 4)	% Sperm limitation (n = 4)
5	38.6 (± 0.8)*	61.4 (± 0.8)*
15	48.6 (± 2.0)	51.4 (± 2.0)
30	54.3 (± 1.6)	45.7 (± 1.6)
60	54.3 (± 1.6)	45.7 (± 1.6)
120	57.9 (± 3.8)	42.1 (± 3.8)
240	57.1 (± 2.6)	42.9 (± 2.6)
600	52.9 (± 2.7)	47.1 (± 2.7)
1200	54.3 (± 1.2)	45.7 (± 1.2)

* Asterick denotes significant difference between time periods, as determined by a *post-hoc* Scheffé test (* $P < 0.05$). There was no difference between time periods beyond the first 5 s.

Table 3-5. The density and viscosity properties of the red abalone, *Haliotis rufescens*, male and female gametes. The gamete viscosity critical shear rate (ES_{50}), power coefficient (n), and proportionality constant (k) were derived from equation (3).

		Density (10^{-3} kg/m^3)	Viscosity		
			ES_{50} (1/s)	n	k
Haliotis rufescens	Female	1.09 – 1.095	2.1	0.1	10.1
	Male	1.027 – 1.03	1.0	0.2	0.02
*Colobocentrotus atratus**	Female	1.03	1.3	0.24	6.8
	Male	1.07	1.0	0.38	1.5
Human blood[†,‡]		1.053	0.85	0.38	0.09
Human semen[§,α]		1.037	2.3	0.81	0.12

Values are taken from: * Thomas, 1994b; [†] Technical Manual of the American Association of Blood Banks, 1993; [‡] Chien, 1970; [§] Mendeluk et. al., 2000; [α] Al-Mogazy et. al., 1993.

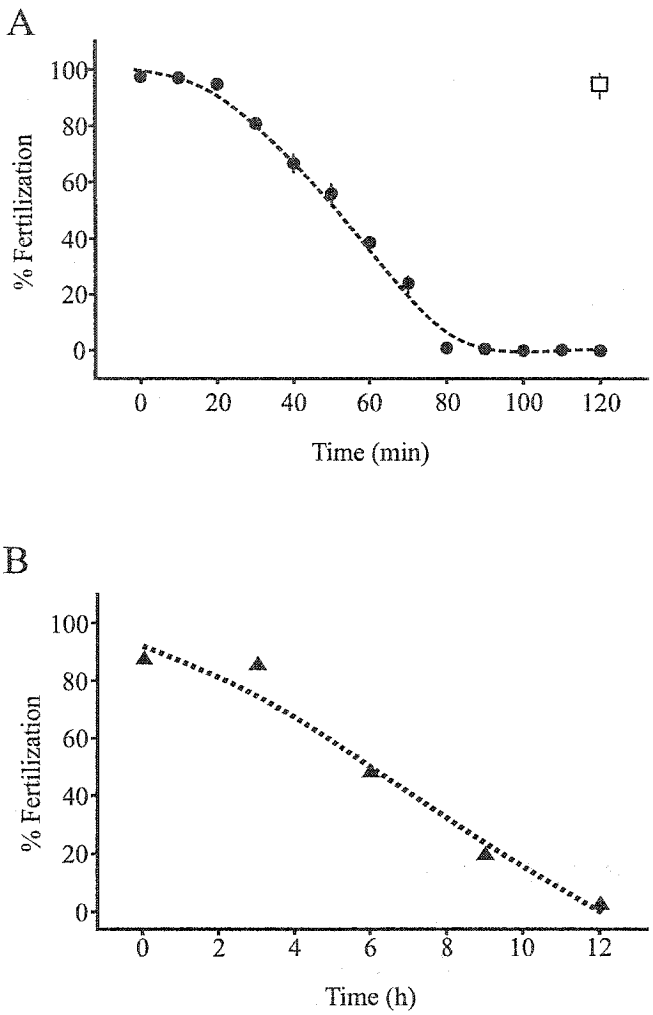

Fig. 3-1. The influence of gamete age on fertilization success. (A) Logistic regression line describing the relationship between time after spawn and fertilization success for eggs. Each filled circle represents the mean of eight replicate trials. When the standard error bar fails to appear, it is smaller then the symbol size describing the mean. After the first twenty minutes fertilization dropped precipitously until, at 90 min, fertilization reached 0%. There was no significant difference between eggs fertilized within the first 20 min and eggs freshly spawned at the end of the experiment and fertilized with the same sperm (white square symbol; one-way ANOVA with *post-hoc* Scheffe test: $P > 0.99$). Thus, sperm viability did not change over the course of the experiment. (B) Logistic regression lines describing the relationship of time after spawn and sperm concentration on fertilization success. Six replicate trials were performed for each data point (filled triangles). When the standard error bar fails to appear, it is smaller then the symbol describing the mean. After 3.5 h, there was a significant decline in fertilization (one-way ANOVA with posthoc Scheffe test: $P < 0.001$). Note the difference in the x-axes between (A) and (B).

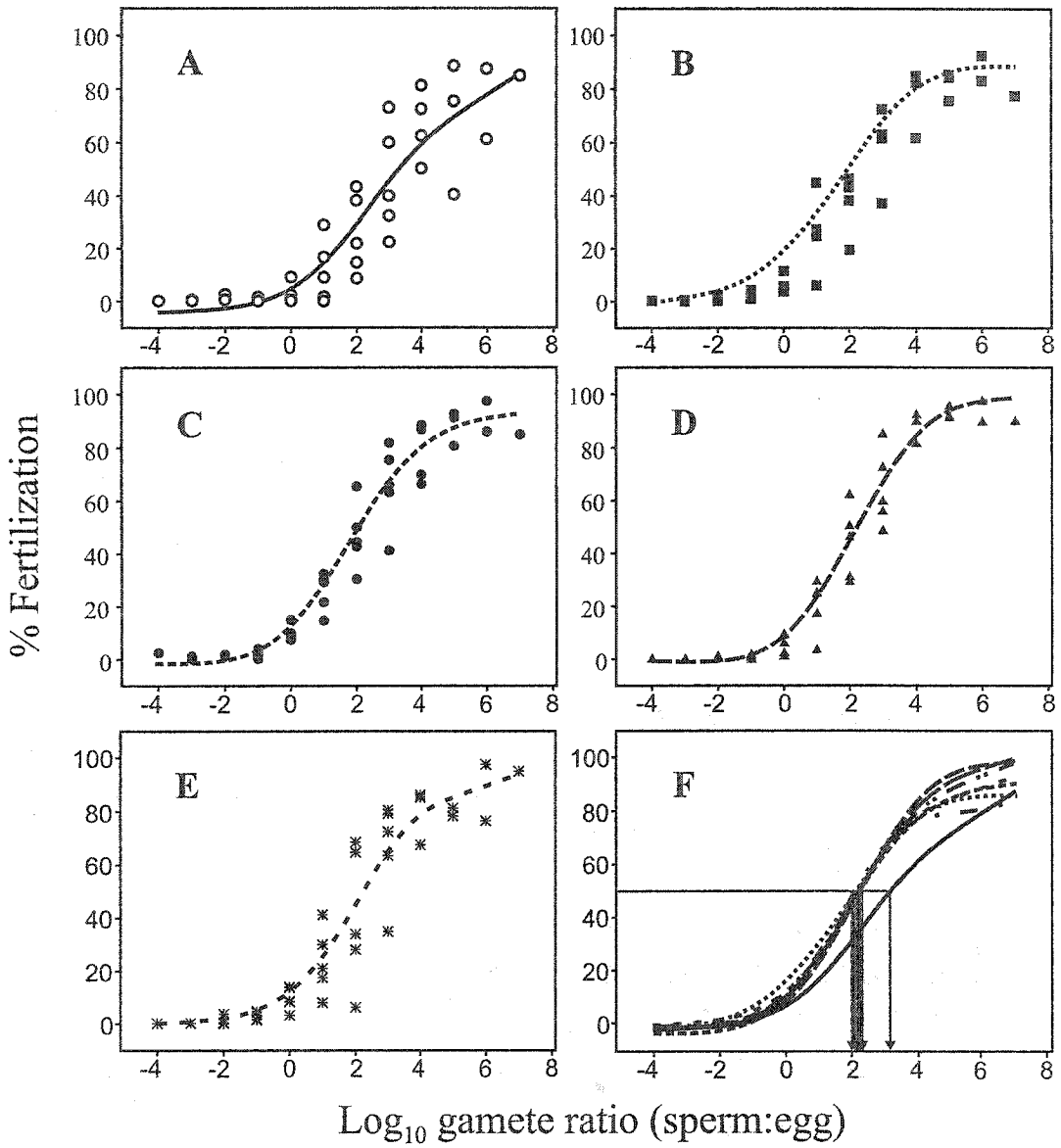

Fig. 3-2. Proportion of fertilized eggs as a function of sperm: egg ratio through time. Plots A – E correspond to time periods 5, 15, 30, 60, and 600 s. Logistic regression lines were fitted to the data, thus describing the relationship between gamete ratio and fertilization success for each time period. Each data point represents the mean of 4 – 6 replicate trials. When the standard error bar fails to appear, it is smaller then the symbol size. (F) After 5 s, fertilization rates and location of ED_{50s} are not significantly different from one another (one-way ANOVA with *posthoc* Scheffé test: $P > 0.89$ for both comparisons). To show differences in fertilization rates between all time periods (5 – 1200 s), only the logistic regressions were plotted.

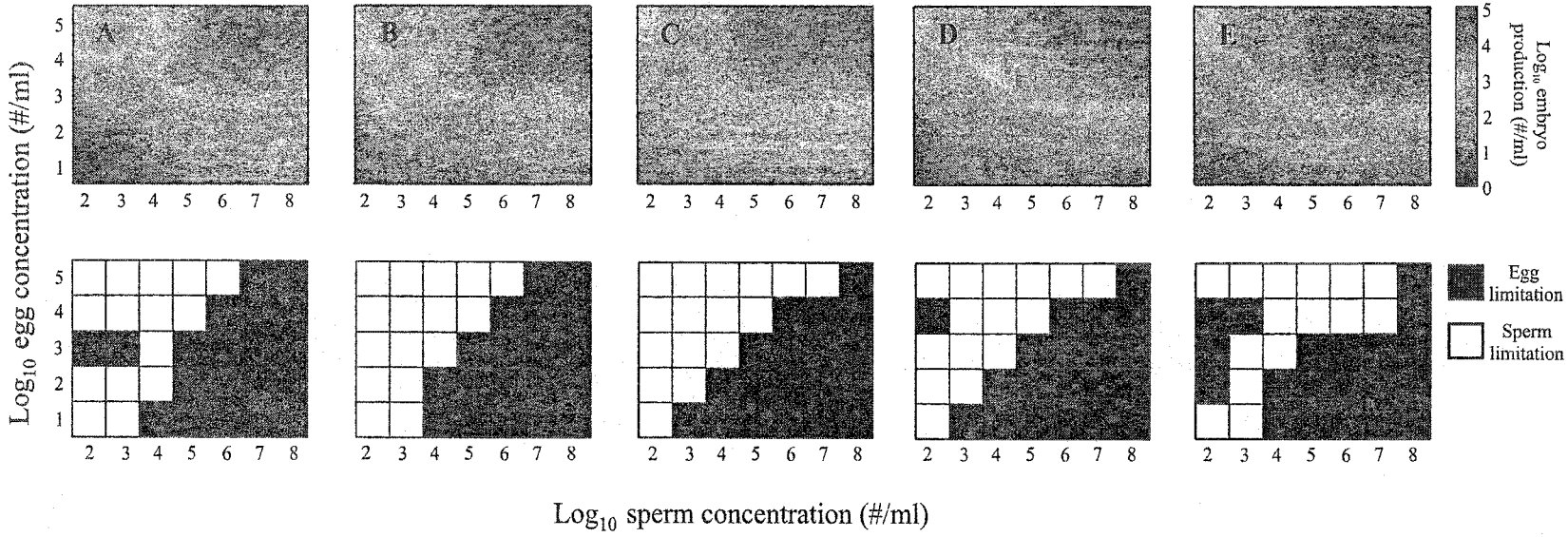

Fig. 3-3. Surface plots representing embryo production and gamete limitation. A – E *Top*: The change in number of zygotes (shown by the color bar) as a function of both sperm and egg density (mean # embryos, n = 4, for each sperm-by-egg concentration interaction), corresponding to a contact time of 5, 15, 30, 60, and 600 s. Note that the number of embryos produced is highly dependent upon egg density, and remains constant over several orders of magnitude sperm concentration. However, both sperm and egg density had a significant affect on embryo production (one-way ANOVA: $F > 64.8$, $P < 0.0001$ for both comparisons), thus suggesting that both egg and sperm limitation can co-occur. *Bottom*: Gamete limitation plots for 5, 15, 30, 60, and 600 s, demonstrating the influence of either egg or sperm limitation on embryo production. The change in the number of produced embryos from the surface plot (*top*) were used to establish whether egg limitation ($\partial F/\partial S < \partial F/\partial E$), or sperm limitation ($\partial F/\partial S > \partial F/\partial E$) occurred for each sperm-egg density combination.

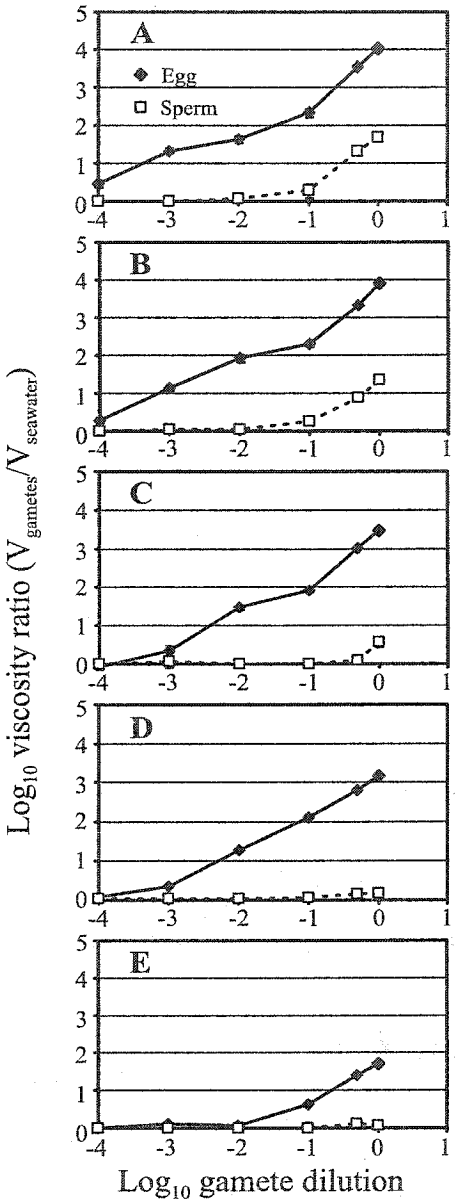

Fig. 3-4. Non-dimensional gamete viscosity graphs showing the change in viscosity (V_{gam}/V_{sea}) as a function of egg (◆) and sperm (□) concentration. Five replicate trials were performed for each data point, and error bars (often obscured by the symbols) represent one standard error. Gametes did not exhibit thixotropic characteristics (change in viscosity with duration of shear), and viscosities were the same in either ascending or descending shear intensity (data not shown). Graphs A – E represent shear rates of 0.6, 1.2, 3.0, 6.0, and 12.0/s. As shear increases, there is a correspondingly significant decrease in viscosity ($P < 0.0001$ for both gametes). Moreover, both gametes exhibited shear-thinning behavior, producing n values < 1.0 ($n = 0.1$, $k = 10.1$ for egg; $n = 0.2$, $k = 0.02$ for sperm). For all shear rates and concentrations, egg viscosity was significantly greater then sperm viscosity (one-way ANOVA: $F_{1,357} = 31.69$, $P < 0.0001$).

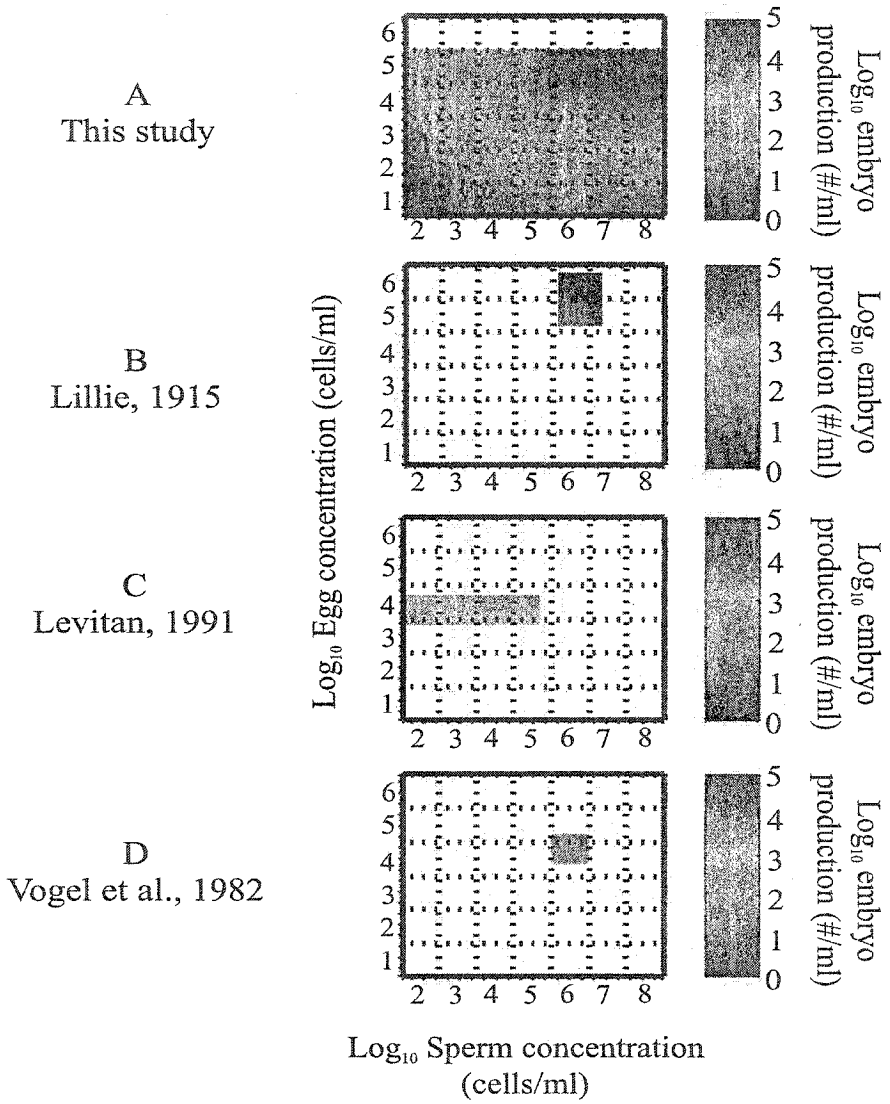

Fig. 3-5. Embryo production as a function of egg and sperm concentration determined in this study (A; 15 s contact time), Lillie (1915; B), Levitan (1991; C), and Vogel et al. (1982; D), using gametes from the red abalone, *Haliotis rufescens*, and the sea urchins *Arbacia*, *Strongylocentrotus franciscanus*, and *Paracentrotus lividus*, respectively. The number of embryos produced was obtained by multiplying the percentage of fertilized eggs by the egg concentration. Typical egg and sperm concentrations spawned by the urchins are ~10^6 and 10^{11} cells/ml, respectively; while corresponding egg and sperm concentrations spawned by abalone are ~10^5 and 10^9 cells/ml. Spawned gamete concentrations were either stated in the study (Vogel et al., 1982; Levitan, 1991; this study) or, in the case of Lillie (1915), were calculated based on the volume of a sphere (egg), and cylinder (sperm), and multiplied to obtain the number of cells per cm^3 (Harvey, 1956).

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