

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

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Summary

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 sea water by conspecific eggs. Sperm in proximity to individual 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 high-performance liquid chromatography. Chemical characterization by nuclear magnetic resonance spectroscopy indicated that the free amino acid L-tryptophan was the natural sperm attractant in *H. rufescens*.

Eggs released L-tryptophan at concentrations that

triggered both activation and chemotaxis in sperm, exhibiting significant activity at levels as low as $10^{-8} \text{ mol l}^{-1}$. 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 on orientation. In experimental treatments involving an elevated, uniform concentration of tryptophan ($10^{-7} \text{ mol l}^{-1}$) 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.

Key words: sperm, egg, fertilization, attractant, chemotaxis, tryptophan, abalone, *Haliotis rufescens*.

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} mol l⁻¹).

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 is therefore a logical starting point to investigate the importance of soluble egg factors and chemosensory-mediated sperm behavior in fertilization and evolution. Furthermore, natural populations of abalone have been heavily exploited and adult densities are at historic lows (Tegner et al., 1989, 1996; Davis et al., 1996; Alstatt et al., 1996). If free-spawned sperm and eggs are diluted before gametes can make contact, fertilization success may be 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, USA) were held in aquaria of running sea water (15 °C). The animals were fed fresh kelp *Macrocystis pyrifera*, collected twice weekly. Ripe adults were identified by gonadal growth beyond the shell (Hahn, 1989), and the sexes were separated and fed *ad libitum* for 2 weeks prior to spawning induction (Morse et al., 1977). Individuals were placed singly in chambers and the sea water pH was raised to 9 by adding 6.5 ml of 2 mol l⁻¹ tris-hydroxymethyl-aminomethane (Tris-base) per liter, followed by 4 ml of 6% H₂O₂ per liter. After 2.5 h exposure, the chamber was emptied, rinsed and refilled with 0.45-µm filtered sea water (FSW). Spawning occurred within 2–4 h of H₂O₂ exposure, with males releasing gametes 10–30 min before females. A typical male (12 cm length) spawned 20–60 ml of sperm solution (10⁹ sperm ml⁻¹), whereas a female of similar size spawned 10–20 ml of egg suspension (10⁵ eggs ml⁻¹). 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. Fertilization assays were run

in combination with each experiment to confirm the viability of harvested gametes (see methods in Gee and Zimmer-Faust, 1997). Material from each of the spawns produced 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 one-way analysis of variance (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 approximately 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 × 10 mm × 5 mm (length × width × depth) Plexiglas chamber held at 15 °C. Images of sperm (at 2.5 × 10³ cells ml⁻¹) 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 approximately 2 mm, or about 70 sperm body lengths, away from the nearest chamber wall.

Video images of sperm were digitized at 30 frames s⁻¹ and processed over 10 s intervals using a CAVMA system (Motion Analysis Corp. model VP 320, ExpertVision, and custom-designed 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 (≤ 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 6 μl , 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^{-1}) 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 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 \times 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^{-1}) 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 s. Alternatively, as a control, a brine shrimp *Artemia salina* 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 using CAVMA to quantify sperm speed and orientation. Swim speeds of sperm near abalone *versus* brine shrimp eggs were compared using an unpaired two-tailed *t*-test. Circular statistics were applied to describe sperm navigation around individual eggs. Both the vector mean length (\mathbf{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 three 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 eliminate particulates. A portion 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^{-1} . Each conditioned seawater solution was assayed by gently injecting a 5 μl sample through a microsyringe into the chamber, and recording activity of sperm over 10 s within 300 μm of the syringe tip. Sperm swimming behavior was analyzed using 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 sea water (ESW). This solution was prepared by bathing 10^3 eggs ml^{-1} in FSW for 18 h 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 resuspended 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) high-performance liquid chromatography (HPLC) on a Phenomenex C_{18} column (250 mm \times 10 mm, 5 μm particle size). Elution was isocratic at 1 ml min^{-1} 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% trifluoroacetic acid (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). Samples (40 μl) were taken from fractions corresponding to one major and three minor peaks observed at 220 nm, dried to remove all solvents and then resuspended in 20 ml FSW for bioassays.

Final purification of the bioactive peak employed size-exclusion HPLC, using a Bio-Sep SEC-S2000 column (300 mm \times 7.8 mm) eluting at 1 ml min^{-1} in water. The column was calibrated with an HPLC peptide standard mixture (Sigma), and the effect of \log_{10} -transformed molecular mass 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 resuspended 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 ^1H detection (Glycobiology Core Facility, UC San Diego, USA). 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 ^1H NMR spectra and two-dimensional (2D) COSY spectra were acquired using a ^1H 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 using analytical HPLC. Prior to chromatography, attractant or standard solutions were treated with *o*-phthalaldehyde (OPA), which reacts with primary amines to generate fluorescent derivatives. A solution of OPA (50 mg) dissolved in absolute methanol (1.25 ml) was added to 0.4 mol l^{-1} sodium borate buffer (11.2 ml, pH 9.5) with 2-mercaptoethanol (50 μl). The attractant or a synthetic standard dissolved 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. Fluorescent derivatives were separated by RP HPLC on an Ultrasphere ODS column (4.6 mm \times 25 cm, 5 μm particle size). 1 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 mol l^{-1} sodium acetate; Solvent B, 80:20 methanol:0.05 mol l^{-1} sodium acetate). Solvents were pH 6.8 and elution was at 1 ml min^{-1} . 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). 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 dose of half-maximal response (ED_{50} ; 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} mol l^{-1} with FSW and red abalone ESW as negative and positive controls, respectively. 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 analysis of covariance (ANCOVA) (Sokal and Rohlf, 1981). To provide information on structure-activity relationships and the specificity of the 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^{-1}) was incubated with 100 $\mu\text{mol l}^{-1}$ 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 tryptophanase, activated as described above and used at the same final concentration (2 $\mu\text{g ml}^{-1}$). This enzyme digests tryptophan in the medium around the egg, preventing the establishment of a gradient. In the second treatment, an egg was placed in a solution of 10^{-7} mol l^{-1} 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. In the final treatment, an egg was placed in a solution of 10^{-7} mol l^{-1} 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^{-1}) were gently pipetted into the chamber 10 min after addition of an egg to a solution, the shortest delay that 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 s once fluid came to rest. Video images were digitized and analyzed using 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.

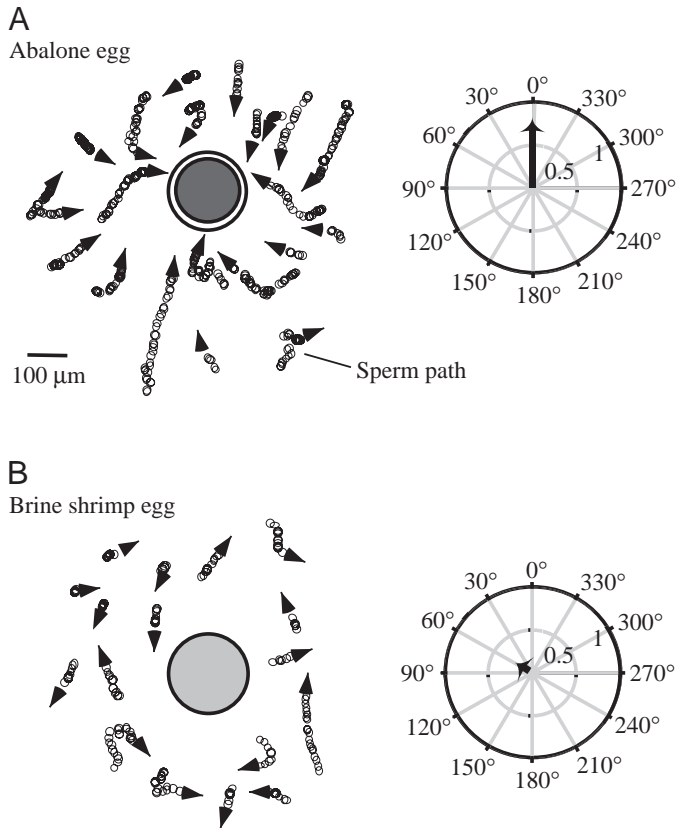


Fig. 1. Orientation of abalone sperm to individual eggs. Sperm were videotaped for 30 s in the presence of a red abalone egg (A) or brine shrimp egg (B) (negative control). Polar plots show the vector mean angle of sperm swimming, relative to the egg surface (0°); the bar represents the vector mean length (r). On the left, representative swimming paths of individual sperm are plotted around an abalone or control egg. Circles correspond to video images captured at 0.033 s intervals, and arrowheads correspond to the directions of travel for individual cells.

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 (Fig. 1A; 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; 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 two-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

Freshly spawned eggs were incubated in sea water for brief

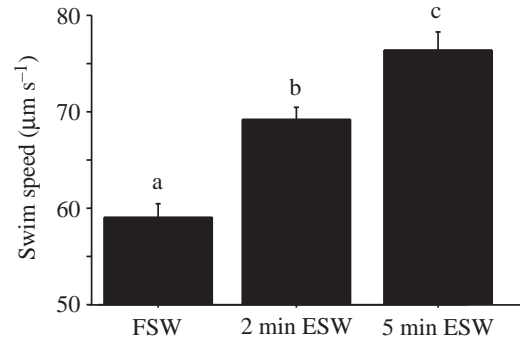


Fig. 2. Accumulation of the sperm attractant in sea water around live eggs. Freshly spawned eggs were suspended in filtered sea water (FSW) and incubated for 2 min or 5 min. The resulting egg-conditioned sea water (ESW) solutions were filtered to $0.22\ \mu\text{m}$ and immediately bioassayed using fresh sperm. Swimming speed was quantified using computer-assisted video motion analysis. Values are means + S.E.M.; means labeled with different letters differed significantly (one-way ANOVA with *post-hoc* Scheffé test, $P<0.005$).

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 (Fig. 2; one-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 (Fig. 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 sea water 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 C₁₈ Sep-Pak cartridge, desalted with water, and eluted with a gradient of 0%–100% methanol. When column fractions were diluted back to the original volume of sea water and assayed, only material eluting in 25% methanol significantly increased sperm swimming speeds compared with FSW controls (one-way ANOVA: $F_{6,312}=13.2$, $P<0.0001$; Scheffé test, $P<0.005$). The 25% methanol fraction induced the same swimming speed as unfractionated ESW (Scheffé test, $P>0.99$).

The active fraction was concentrated and then partially purified by RP-HPLC on a C₁₈ column, eluting with a gradient of 5% to 50% methanol in water/0.1% TFA. One major and three minor peaks were observed at 220 nm (Fig. 3A). 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 (Fig. 3B; one-way ANOVA: $F_{5,93}=3.8$, $P<0.005$). Mean swim speeds for FSW controls and inactive fractions

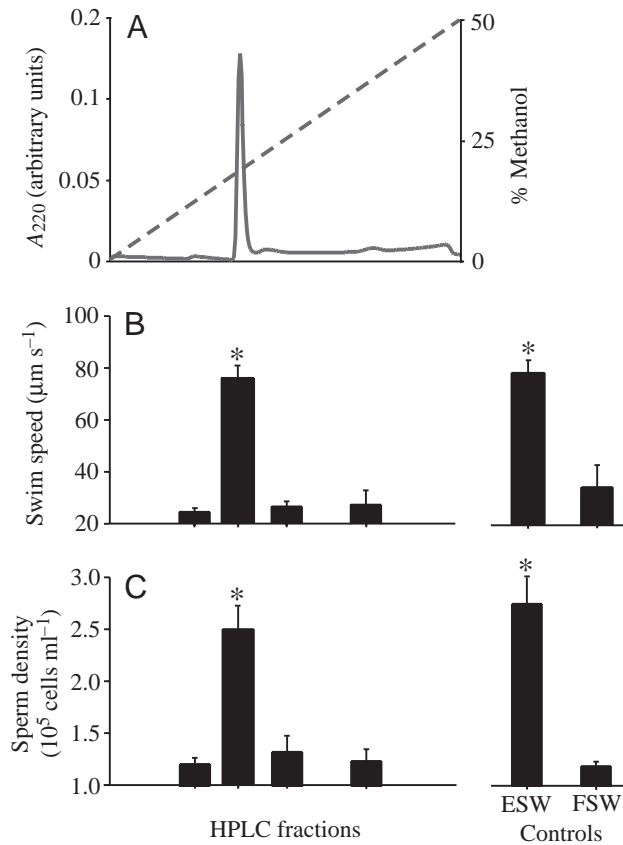


Fig. 3. Partial purification of the sperm chemoattractant by RP-HPLC. The active fraction from egg-conditioned sea water (ESW) was eluted from a Sep-Pak cartridge in 25% methanol, concentrated, and injected onto a C₁₈ column. Compounds were eluted over a 30 min run using a gradient from 5% to 50% methanol in 0.1% TFA. Fractions (0.5 ml) were pooled as indicated, lyophilized, and bioassayed for sperm motility and orientation. (A) Absorbance spectrum at 220 nm. (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 using computer-assisted video motion analysis. Values are means + S.E.M. Fresh ESW and filtered sea water (FSW) were used as positive and negative controls, respectively. *Means 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. Values are means + S.E.M.

ranged from 25–37 $\mu\text{m s}^{-1}$, while sperm exposed to ESW or the active fraction swam at 75–79 $\mu\text{m s}^{-1}$. The bioactive peak also induced significant attraction of sperm in the flat capillary assay (Fig. 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 (Fig. 4A). Only the major peak significantly elevated sperm swim speeds, compared with FSW controls (Fig. 4B; one-way ANOVA: $F_{5,138} = 15.8$, $P < 0.0001$; Scheffé test, $P < 0.0001$). The three minor peaks that eluted at earlier

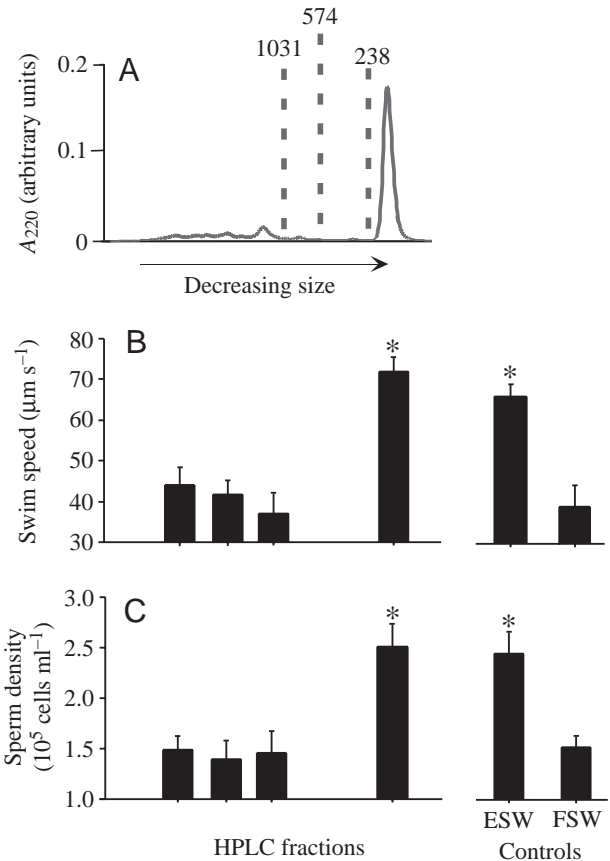


Fig. 4. Final purification of the sperm chemoattractant by size-exclusion chromatography. (A) The bioactive fraction from RP-HPLC was chromatographed on a size-calibrated Bio-Sep S-2000 column, eluted at 1 ml min⁻¹ in water and absorbance monitored at 220 nm. Fractions corresponding to detected peaks were pooled, lyophilized, resuspended 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. Values are means + S.E.M. Fresh egg-conditioned sea water (ESW) and filtered sea water (FSW) were used as positive and negative controls, respectively. *Means significantly greater than negative controls ($P < 0.0001$). (C) Chemotactic response of abalone sperm to isolated size fractions. Values are means + S.E.M.

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 (Fig. 4C; one-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 masses, obtaining a standard curve of log₁₀-transformed molecular mass versus retention time. The approximate molecular mass of the attractant was 200 Da, based on its time of elution from the sizing column.

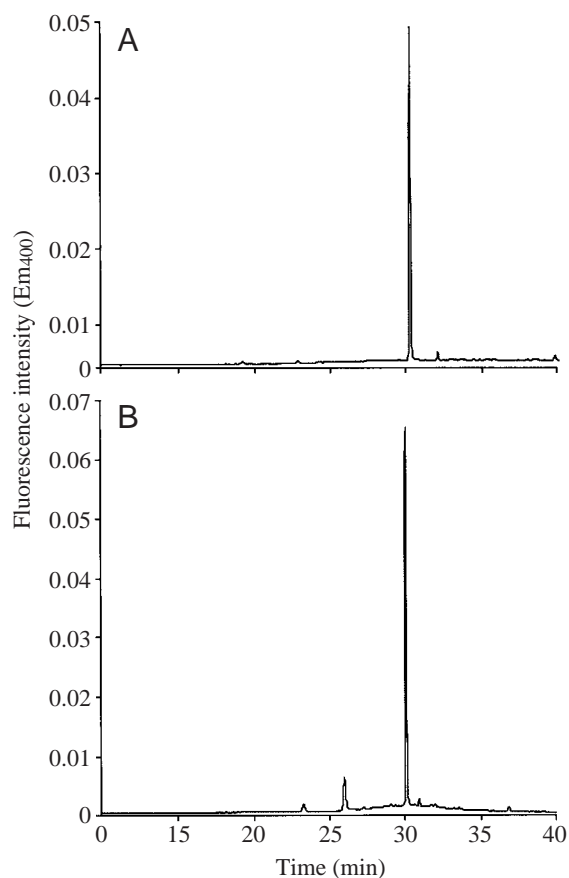


Fig. 5. Analytical HPLC comparison of the natural attractant and a synthetic tryptophan standard. Samples were reacted with *o*-phthalaldehyde (OPA) prior to injection, producing derivatives that were identified by RP-HPLC with fluorescence detection at 400 nm (Em_{400}). (A) A sample of the purified natural sperm attractant corresponding to 0.25 nmol l^{-1} , showing the diagnostic retention time and purity of the sample. (B) Co-injection of the previous sample with 0.25 nmol l^{-1} of a synthetic standard of L-tryptophan, showing co-elution as a single peak.

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 ^1H 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 tenfold 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. A sample of the attractant was derivatized and compared with a

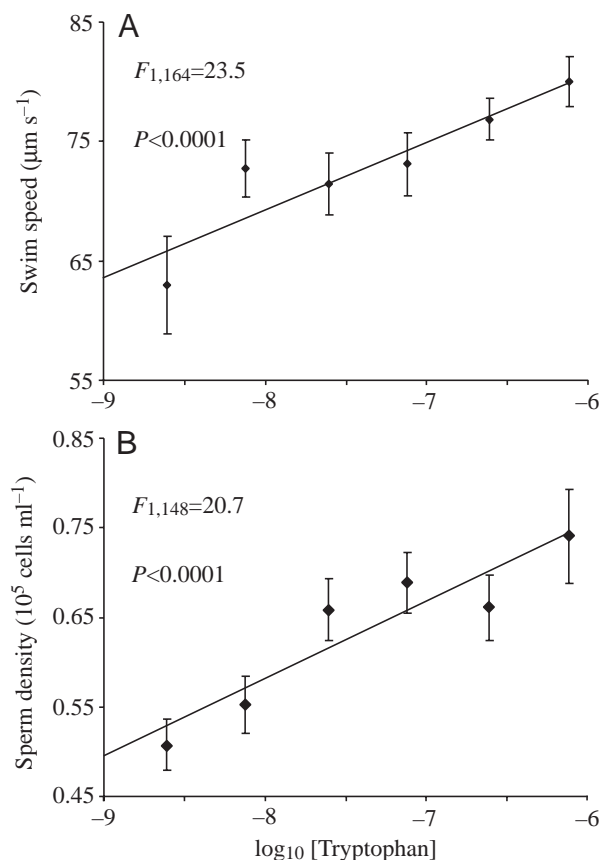


Fig. 6. Dose-response relationship of red abalone sperm to L-tryptophan. Values are means \pm s.e.m. with the regression line calculated using the raw data. (A) Results of a linear regression of \log_{10} -transformed dose on sperm swim speed. (B) Results of a linear regression of \log_{10} -transformed dose on sperm density. The dose producing the half-maximal response (ED_{50}) was calculated from the regressions to be $3 \times 10^{-8} \text{ mol l}^{-1}$ for both swim speed and cell density.

synthetic tryptophan standard by RP-HPLC. The derivatized attractant yielded a single peak with the same retention time as the co-injected synthetic standard (Fig. 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-dependent manner, exhibiting elevated swim speeds and demonstrating chemotaxis in capillary assays. At 10^{-6} – $10^{-7} \text{ mol l}^{-1}$, L-tryptophan increased sperm swim speed and density by the same amount as unfractionated ESW, relative to FSW controls (one-way ANOVA: $F_{5,195}=7.6$, $P<0.0001$; Scheffé test, $P<0.05$). Levels of tryptophan in ESW solutions were approximately 1 – $2 \times 10^{-7} \text{ mol l}^{-1}$. When plotted as \log_{10} -transformed data, the dose of tryptophan had a significant effect on sperm behavior (Fig. 6; Model 1 regression of dose x on swim speed y_1 : $y_1=6x+115$; $P<0.0001$, and Model 1 regression of dose x on cell density y_2 : $y_2=8.5x+126$; $P<0.0001$). Doses above $10^{-6} \text{ mol l}^{-1}$ did not produce further increases in speed or density (results not

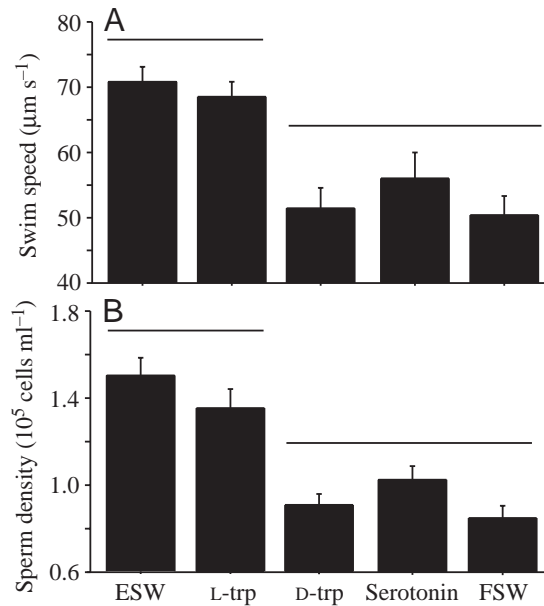


Fig. 7. Structural specificity of the sperm response to L-tryptophan. Red abalone sperm were assayed for behavioral responses to natural egg-conditioned sea water (ESW), and to synthetic standards tested at $10^{-7} \text{ mol l}^{-1}$ concentration. Both the natural L-enantiomer (L-trp) and the unnatural D-enantiomer (D-trp) 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. Values are means \pm S.E.M.; means not linked by a horizontal bar differed significantly (one-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.

shown). The ED_{50} was $3 \times 10^{-8} \text{ mol l}^{-1}$ 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 $10^{-7} \text{ mol l}^{-1}$ solution of L-tryptophan was identical in activity to unfractionated ESW (Fig. 7), both in swimming speed (Scheffé test, $P > 0.97$) and chemotaxis assays ($P > 0.69$). However, a $10^{-7} \text{ mol l}^{-1}$ solution of the unnatural isomer D-tryptophan was inactive (Fig. 7), inducing no more activity than FSW controls in swimming speed (one-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

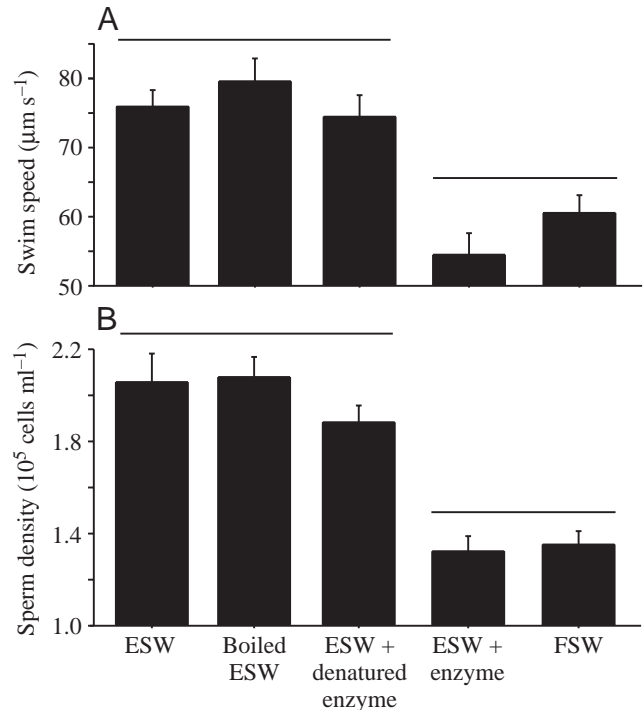


Fig. 8. Effect of enzymatic treatment with tryptophanase on bioactivity of egg-conditioned sea water (ESW). Active ESW solutions were incubated with the enzyme tryptophanase, which cleaves the indole ring from L-tryptophan; solutions were boiled to quench the reaction after 10 min. In one positive control (boiled ESW), ESW solutions were boiled without enzyme, to determine effects of the deactivating step in enzyme treatments. In a second positive control (ESW+denatured enzyme), 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 filtered sea water (FSW). Values are means \pm S.E.M.; means linked by a horizontal bar did not differ significantly (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.

biosynthetic derivative of tryptophan, had no significant effect on abalone sperm when assayed at the same concentration (Fig. 7; swimming speed, $P > 0.83$; chemotaxis, $P > 0.51$).

To establish that no component 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 (Fig. 8). Enzymatic treatment of ESW reduced sperm swimming speeds (one-way ANOVA: $F_{4,141}=12.9$, $P < 0.0001$) and cell densities (one-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 $10^{-7} \text{ mol l}^{-1}$ tryptophan solution with the enzyme produced

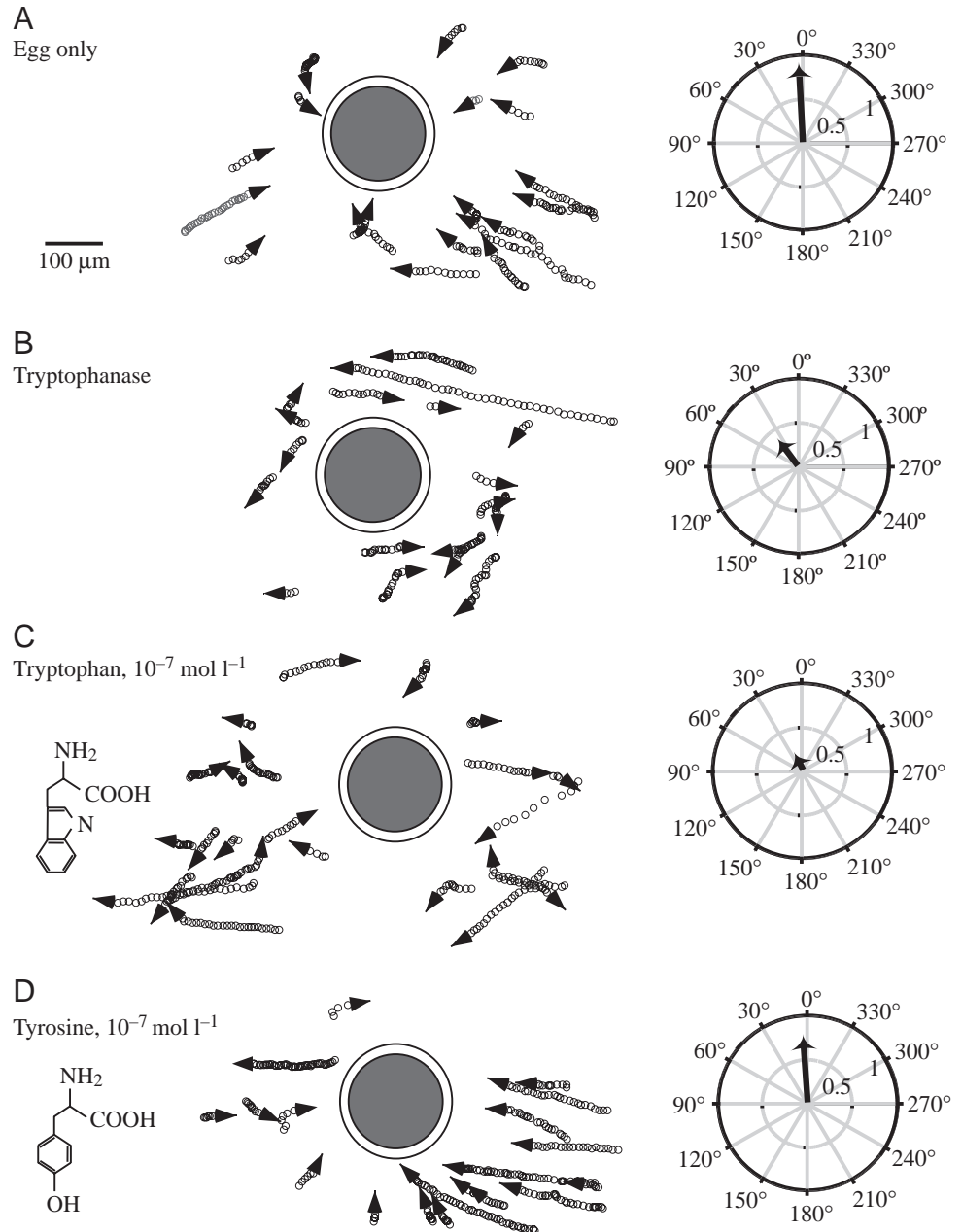


Fig. 9. Disruption of the tryptophan gradient around live eggs prevents navigation by sperm. Individual red abalone eggs were placed in 400 μl of filtered sea water (FSW) or the indicated test solution, after which sperm were added and video-recorded for 30 s. Representative swimming paths are shown as in Fig. 1; the scale bar applies to all panels. Polar plots show the vector mean direction of sperm swimming, with angles measured relative to the egg surface (0°); the bar indicates the vector mean length (r). (A) Sperm in FSW near the surface of a live egg. (B) Sperm in the presence of the enzyme tryptophanase, which selectively digests L-tryptophan as it diffuses from the egg. Enzyme treatment had no effect on sperm viability. (C) Sperm in a uniform solution of $10^{-7} \text{ mol l}^{-1}$ L-tryptophan. (D) Sperm in a uniform solution of $10^{-7} \text{ mol l}^{-1}$ L-tyrosine. The tyrosine solution controlled for any effects of elevating the concentration of an aromatic amino acid in the sea water medium.

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 (Fig. 9). In positive controls using a live egg in FSW, a significant proportion of sperm oriented and swam towards the egg (Fig. 9A; 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 (Fig. 9B; 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 test further if sperm require a chemical gradient for navigation, eggs were placed in a $10^{-7} \text{ mol l}^{-1}$ 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 $10^{-7} \text{ mol l}^{-1}$ L-tryptophan (Fig. 9C; 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 $10^{-7} \text{ mol l}^{-1}$ solution of L-tyrosine (Fig. 9D). We tested L-tyrosine because it is 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 $10^{-7} \text{ mol l}^{-1}$ L-tyrosine, as in FSW controls (Fig. 9D; 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 $10^{-8} \text{ mol l}^{-1}$, 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, 5-HT). 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-HT₁ receptors that result in adenylate cyclase activation, and 5-HT₃ receptors that cause the opening of ion channels (Kadam and Koide, 1990; Bandivdekar 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. Sperm activation may result from cAMP-dependent 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 substitutions (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 seven 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 sea water, indeed contain several tryptophan derivatives (J. A. Riffell, P. J. Krug and R. K. Zimmer, manuscript in preparation). 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 (J. A. Riffell, P. J. Krug and R. K. Zimmer, 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.

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