The ecological and evolutionary consequences of sperm chemoattraction

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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 experimental evidence that sperm chemoattraction directly affects the magnitude of fertilization success. The recent discovery of L-tryptophan as a potent attractant chemotaxis significantly promote contacts. Our results show further that attractant release by means of 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 (Haliotis fulgens) abalone are only minor contributors to maintaining reproductive isolation. Because abalone typically live in dense, multispecies 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 evolve to locate the right target within mixed gamete suspensions of closely related species.

Soluble sperm attractants are potentially critical agents mediating fertilization success and driving speciation, operating upstream of cell-surface proteins and before gamete contact (2, 5, 12). 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 (13). In contrast, a series of peptides described from sea urchin egg jellies increases sperm respiration and/or motility at nanomolar levels (14, 15). Peptides with similar functions were isolated from eggs of other echinoderms (16). These peptides are broadly active for species within a given family, but it is unclear whether they are effective at physiological pH (2). Furthermore, only one peptide triggers chemotaxis (14, 15).

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. By using bioassay-guided fractionation and NMR spectroscopy, we recently established that the free-amino acid L-tryptophan is the natural sperm attractant for red abalone (Haliotis rufescens) (12). This discovery has permitted experiments on 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

Sperm Behavior in Response to a Natural Tryptophan Gradient. Procedures for abalone collection, maintenance, and spawning, and for measuring tryptophan concentrations in adult abalone tissues and release rates from individual eggs, are described in Supporting Methods, which is published as supporting information on the PNAS web site. 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 μl of filtered seawater (FSW) alone, or as one of the following five treatments. (i) A single egg was placed in a solution of tryptophanase (2 μg/ml). This enzyme digests tryptophan in the medium around the egg, thus eliminating the signal. Before use, tryptophanase was activated by incubation with 100 μM pyridoxal-5’-phosphate in FSW (pH 7.9) at 37°C for 1 h to ensure maximum formation of the holoenzyme (17). (ii) 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. (iii and iv) An egg was placed in a solution of either denatured (boiled) tryptophanase or 10^-7

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M tyrosine. The addition of tyrosine controlled for nonspecific effects of elevated aromatic amino acid concentration on sperm swimming. (v) Assays were run in FSW, by using both sperm and eggs bathed in a tryptophanase solution prepared as above, but were rinsed free of enzyme before tests.

To begin each trial, red abalone sperm (2.5 × 10^5 cells per ml) was gently pipetted into a chamber. Integrating with respect to time, fluid dynamic theory predicts that a concentration gradient created by continuous tryptophan release should reach steady state in ~10 min, within 300 μm of an egg surface (18). Swimming speeds and directions were therefore videotaped of individual cells over 30 s, beginning 10 min after sperm introduction. The camera (NEC model TI 23A) was mounted on an Olympus IX70 compound light microscope at ×90 magnification and had a 100-μm depth of field. Fluid dynamic theory further predicts that drag forces have especially strong effects on flagellar motion within ~10 sperm body lengths of a wall, or microscope slide surface (19, 20). To minimize potential artifacts, we assayed sperm motility in response to live eggs at 0.4–0.5 mm, or ~15 sperm body lengths from the nearest chamber wall.

Images were digitized at 30 frames per s by using a computer-assisted video motion analysis system (Motion Analysis model VP 320 and custom software) interfaced with a Sun SPARC2 computer workstation. To avoid problems of parallax, we discarded short paths (~10 frames) in which sperm changed >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 was averaged over each path. By using nonmotile sperm as tracers for flow visualization, fluid motion due to convection was insignificant (<5 μm/s) compared with swimming speeds of live cells. The angle of sperm orientation was measured with respect to an origin (0°), defined as the shortest tangent between each cell and the egg surface. Circular statistics; in particular, the mean vector (r), were used to describe the average direction of sperm movement at 50-μm intervals from an egg surface to 250 μm away. A Rayleigh’s test was used to compare each mean direction against a uniform circular distribution to determine the significance of cell movement toward the egg surface. Gamete encounter rate was measured as the elapsed time (0.053 s accuracy) for a sperm to contact and attach to an egg.

The Consequences of Sperm Chemoattraction for Fertilization Success. Before chemoattractant effects could be determined, an initial experiment established conditions for fertilization assays. By using a factorial design, crosses were run for a wide range of red abalone egg (10^6 to 10^8 gametes per ml) and sperm (10^4 to 10^8 gametes per ml) density combinations, over interaction intervals of 5–2,400 s. Results showed, first, that percent fertilization more strongly depended on the ratio of sperm to eggs than on the density of either gamete type (21). As the ratio of sperm to eggs was increased 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. 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 (10^5 eggs per ml). As before, we used six chemical treatments (FSW, tryptophanase, denatured tryptophanase, 10^-7 M tryptophan, 10^-7 M tyrosine, and sperm and egg exposed to tryptophanase, then rinsed free of enzyme with FSW before bioassay] were employed to describe chemoattractant effects. 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 Individual Eggs. As male gametes of red abalone approached within 100 μm of a conspecific egg in FSW, they accelerated significantly and navigated directly toward the egg surface (Figs. 1A and 2). Control solutions [denatured (boiled) tryptophanase, 10^-7 M tyrosine, and sperm and egg exposed to tryptophanase, then rinsed with FSW before bioassay] were not effective without effect on sperm behavior (Figs. 1 B–D and 2). After addition of tryptophanase (1-tryptophan indole lyase, EC 4.1.39.1) to solution, however, sperm ceased to orient toward an egg or to swim faster (Figs. 1 F and 2). In contrast, cells swam significantly faster, but failed to navigate toward egg surfaces when presented with an elevated, uniform, tryptophan concentration (10^-7 M, Figs. 1E and 2). 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 hemolymph, 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 × 10^-4 pmol per egg per min as long as they remained viable (Fig. 3A). This empirically derived rate constant was used in a three-dimensional Fickian diffusion model to estimate signal strength (18). 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 μm (see above). As calculated here (Fig. 3B), the minimum effective dose (~4 × 10^-9 M) closely matched a threshold value (~10^-8 M) previously determined for abalone sperm chemotaxis (using an exogenous source of tryptophan without eggs present) (12).
from experiments described above, video images were processed for rates of sperm attachment to conspecific eggs. Rates were indistinguishable for gametes in FSW, tyrosine and denatured enzyme, and for gametes transiently exposed to enzyme before assays (Fig. 7, which is published as supporting information on the PNAS web site). 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 Chemotaxis for Fertilization Success. To quantify the extent to which red abalone sperm chemotaxis 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 (23–25). For each of the six chemical treatments, a logistic regression described the relationship between percentage of fertilized eggs and sperm-to-egg ratio (Fig. 4, F test: $F \geq 8.61, df = 1/99$, $P < 0.0001$, all comparisons). When the ratio was too low (1.0 sperm:1.0 egg) or too high (10,000 sperm:1.0 egg), sperm were either limiting or saturating, respectively. Under these conditions, chemotaxis did not affect fertilization. In contrast, at intermediate ratios (10–1,000 sperm per egg), fertilization success increased significantly as a function of chemotaxis. To compare across treatments, logistic regression equations were used to calculate effective sperm-to-egg ratios ($ER_{50}$) fertilizing half of all eggs (26). As calculated, $ER_{50}$s were almost identical (range: 88.0–95.4 sperm:1.0 egg) for gametes held in FSW, denatured tryptophanase, and $10^{-7}$ M tyrosine, or transiently exposed to enzyme before bioassays. In contrast, $ER_{50}$s for $10^{-7}$ M tryptophan and tryptophanase were elevated, significantly, by 2.87 times (149.5 sperm:1.0 egg) and 5.60 times (450.6 sperm:1.0 egg) relative to FSW.

The Consequences of Sperm Chemotaxis for Reproductive Isolation. To investigate how chemoattraction contributes to reproductive isolation, we dissected experimentally the effects due to sperm navigation from processes occurring after gamete contact. Sperm of red ($H. rufescens$) and green ($H. fulgens$) abalone responded to egg factors in a species-specific manner, navigating toward conspecific but not heterospecific eggs (Fig. 5, and Table 1, which is published as supporting information on the PNAS web site). Sperm of both species swam significantly faster within 100 μm of a conspecific egg, and movement was directed toward the egg surface; in contrast, sperm did not change speed or direction around heterospecific eggs (Table 1). Moreover, soluble sperm attractants significantly promoted gamete encounter rates when sperm and eggs were drawn from the same species (Fig. 8, which is published as supporting information on the PNAS web site).

Logistic regression lines describing relationships between mean percentages of fertilized eggs and sperm-to-egg ratio were determined for each of four conspecific or heterospecific crosses (Fig. 6, F test: $F \geq 38.53, df = 1/69$, $P < 0.0001$, all comparisons). Due to low hybridization rates, we extrapolated regressions for heterospecific crosses to predict effective sperm-to-egg ratios ($ER_{50}$) fertilizing half of all eggs (26). These $ER_{50}$s were 112,201 (green sperm × red egg); 30,199 (red sperm × green egg); 91.2 (red sperm × red egg); and 76.3 (green sperm × green egg). As calculated, $ER_{50}$s were significantly higher for heterospecific than for conspecific crosses (one-way ANOVA with post hoc Scheffé test, $P < 0.0001$, all comparisons). Moreover, conspecific sperm achieved 330–1,470 times the fertilization success of heterospecific sperm.

Gamete Encounter Rates. Straighter and faster swimming paths need not indicate that chemically mediated behavior increases encounter rates, or ultimately enhances fertilization success.
The block against hybridization could lie before or after gamete contact. To evaluate these possibilities, we compared the fertilization success of nonnavigating red sperm (Fig. 4) with that of green sperm (Fig. 6). Although neither could respond to egg-derived tryptophan (Figs. 1 and 5), nonnavigating red sperm still held an fertilization advantage over green sperm, when each was mixed with red abalone eggs. For green and red abalone, reproductive isolation must therefore 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 (5). Chemical communication between sperm and eggs is purportedly critical in sexual reproduction, but the contribution of soluble egg factors has been elusive. Nearly all previous investigations introduced an attractant, by means of 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 (1, 3, 13–15). 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 almost always limited to the first minute after 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.

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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 (18). For these conditions, egg-derived tryptophan induced both chemotaxis and chemokinesis in red abalone (H. 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 contributed equally to promoting fertilization.

Selective pressures must drive the evolution of sperm chemotaxis. 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 (28). 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. Because the probability of encounter between male and female gametes is directly proportional to egg radius (29, 30), remote chemical communication is an effective means of promoting sperm-egg contacts. Our results indicate that attractant release by means of diffusion creates a chemical concentration gradient that doubles the effective target size of red abalone eggs which, in turn, substantially increases fertilization success.

**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 (5). There are ~60 species of abalone worldwide, many with overlapping breeding seasons and habitats, yet hybrids are rare (27, 28). It is unresolved whether remote 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 (H. fulgens) sperm are necessary to achieve fertilization of red abalone (H. rufescens) eggs. Is this result because green abalone sperm do not navigate toward red abalone eggs, or because their membrane proteins do not bind to cognate receptors on the egg? Either mechanism could potentially block hybridization, however, before 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 nonnavigating red versus green abalone sperm. This experiment

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**Fig. 4.** Logistic regression lines describing relationships between mean (± SEM) percentages of red abalone-fertilized eggs and sperm-to-egg ratio for experimental controls (A) and experimental treatments (B) relative to FSW control (Egg alone). Twenty replicate trials were performed for each data point. Some SE bars are smaller than the size of the symbols. 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 Scheffe tests, $P < 0.001$).

**Fig. 5.** Swimming behavior of red and green abalone sperm, respectively, near an isolated conspecific or heterospecific egg, as determined by using computer-assisted video motion analysis. (See the Fig. 1 legend for explanation of symbols.)

**Fig. 6.** 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. Some SE bars are smaller than the size of the symbols.
thus quantified how much of the impeded fertilization was due to interference with sperm chemotraction. In terms of their ability to fertilize red abalone eggs, nonnavigating red sperm were impaired by a factor of 5, whereas nonnavigating green sperm were impaired by a factor of \( \approx 1,250 \). Thus, the 250× difference in fertilization success between nonnavigating red and green abalone sperm can be due only 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 (ref. 28 and D. L. Leighton, unpublished data). Red, pink (Haliotis corrugata), and green abalone are more distant relatives of each other, having diverged in the Pacific northeast over a period of 4–20 million years ago. In contrast, red and white (Haliotis sorenseni) abalone are closely related and diverged only within the past 1–2 million years (31). For the heterospecific crosses of pink sperm × red egg and white sperm × red egg, calculated ER\(_{50}\)s were 159 and 95 times higher, respectively, than for the conspecific cross of nonnavigating red sperm × 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.

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, multispecies 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.

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