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Sperm Chemotaxis as Revealed With Live and Synthetic Eggs

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Fertilization is one of the least understood fundamental biological processes. How sperm search for and find an egg remains enigmatic. Sperm attraction to egg-derived chemical cues may be significant evolutionarily for maintaining species barriers and important ecologically for increasing gamete encounters. New tools are needed, however, to resolve the functional consequences of these dissolved signal molecules. Freshly spawned eggs from red abalone (Haliotis rufescens) *naturally release* L*-tryptophan, which stimulates chemotactic responses by conspecific sperm. Here, microspheres were manufactured to the approximate size and the same shape as female gametes and formulated to emit controlled doses of chemoattractant, imitating natural* L*-tryptophan release rates. When experimentally tested for effectiveness, male gametes did not distinguish between chemically impregnated mimics and live eggs, demonstrating that* L*-tryptophan alone is both necessary and sufficient to promote chemotaxis, and confirming the identity of a native sperm attractant. The techniques that we describe can be used to create synthetic eggs for most animal and plant species, including humans. Egg mimics increase the capacity for experimental manipulation and enable realistic studies of sperm behavior even in the absence of female gametes.*

Gamete communication is fundamental to sexual reproduction. Successful gamete fusion for internally and externally fertilizing organisms relies on chemical signals transmitted through dynamic-fluid environments (1–7). To date, investigations of sperm chemosensory-mediated behavior have usually involved microscope slides or custom-built micro-chambers. Most commonly, a chemical source well created an attractant concentration gradient *via* diffusion into an observation arena (8–11). Additionally, caged attractants were used to control response timing precisely (6, 12–13). Caging inactivated an attractant compound to establish a gradient prior to activation by flashes of UV light (14–15), allowing for nearly instantaneous physiological and behavioral measurements.

Despite providing valuable insights into sperm behavior, these methods have potential artifacts. Sperm were placed within shallow arenas where viscous wall effects can interfere with male gamete behavior. The beating waveform of a sperm flagellum contains three-dimensional components that cause trapping along solid interfaces (16). Moreover, drag forces imposed by walls, such as microscope slide surfaces and equivalent platforms, modify sperm swimming speeds and directionalities (17–19). Whereas existing methods may advance understanding of sperm physiology and behavior (2, 5–6, 10, 20–21), they sacrifice the physical realism of actual encounters between male and female gametes.

New methods, therefore, are needed to simulate *in vitro* both physical and chemical aspects of egg signaling. Here, we used drug delivery technology to produce microspheres that mimic these essential properties of eggs, and tested their effectiveness relative to live eggs and placebos (Fig. 1). Freely spawned eggs from red abalone (*Haliotis rufescens* Swainson) naturally release L-tryptophan, which evokes sperm behavioral responses (4, 22–23). Egg mimics were constructed with a microcrystalline cellulose core, surrounded by a layer of L-tryptophan, using fluidized-bed

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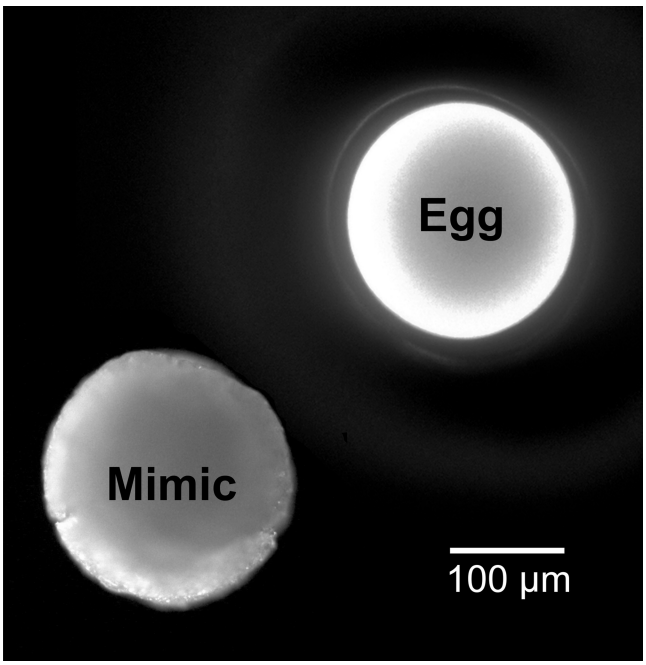

Figure 1. Images of live and synthetic red abalone eggs.

technology and a bottom spray-coating method (Mini-Glatt system coupled with a Micro-Kit [Glatt Air Technologies, Inc., Ramsey, New Jersey]). Each sphere was then covered with a thin, ethyl cellulose film, incorporating a channelizer and a plasticizer whose formula precisely regulated L-tryptophan release rate (24–27). As revealed by HPLC analysis, similar release profiles (0.2–0.3 fmole tryptophan egg^{-1} min^{-1}) characterized freshly spawned eggs and their mimics (see methods in 23). Moreover, in experiments, mean diameters were largely equivalent among red abalone eggs (221 μm $\pm$ 2.5 μm SEM, $n = 3$), attractant-impregnated microspheres (259 μm $\pm$ 7.6 μm SEM, $n = 3$), and microsphere placebos (251 μm $\pm$ 6.3 μm SEM, $n = 3$; identically prepared, without tryptophan addition). Drug delivery technology thus produced reliable physical and chemical facsimiles of female gametes for studies on sperm behavior.

Experiments tested for effects of eggs, mimics, or placebos on behavioral responses of red abalone sperm (mean length: 32 μm $\pm$ 0.8 μm SEM, $n = 100$). Each trial was conducted using fresh gametes (defined as 10–30 min postspawn), according to methods detailed in several previous studies (4, 22–23, 28). A single egg, mimic, or placebo was placed in the center and on the bottom of a clear acrylic plastic observation chamber containing 400 μl of filtered seawater, held at 15 °C ($n = 3$ trials for each treatment). Before placement, mimics and placebos were bathed in a stirred, filtered seawater solution (15 °C) to equilibrate for 30 min. This interval was required for channelization of the bead, and hence for optimization of L-tryptophan release. After equilibration and egg or microsphere addition, a 10-μl aliquot of freshly spawned red abalone sperm (1×10^5 cells ml^{-1}) was gently pipetted into each experimental chamber. Integrating with respect to time, fluid-dynamic theory predicts that a concentration gradient created by continuous tryptophan release would reach steady state in $\approx$10 min within 300 μm of an egg surface (22, 29). Swimming speeds and directions of individual cells, therefore, were videotaped over 30 s in a 300-μm (length) $\times$ 300-μm (width) viewing field, beginning 10 min after sperm introduction. A camera (NEC model TI 23A) was mounted on an Olympus IX70 compound light microscope at 90$\times$ magnification (100-μm viewing depth). Video tracks were recorded as sperm swam 100 to 200 μm above the bottom, thus minimizing interactions between flagella and the solid boundary.

Images were digitized at 30 frames s^{-1} using a computer-assisted video motion-analysis system (Motion Analysis model VP 320, ExpertVision, and custom software) interfaced with a Sun SPARC2 computer workstation. To avoid potential problems of parallax, short paths ($\leq$4 frames) were discarded, as well as those in which sperm changed $>$20% in apparent size. In a previous study, sperm responded behaviorally to egg-derived attractant release in still water at a distance of $\leq$100 μm from an oocyte surface (22). Consequently, sperm tracks were analyzed in two categories as defined by cells swimming either $\leq$100 μm or $>$100 μm away from the surface of a freshly spawned egg or a microsphere. Swimming speed of each individual sperm was determined on a frame-by-frame basis and averaged over each path.

The angle of sperm orientation was measured with respect to a circular coordinate system. Here, θ is the angle relative to an operationally defined origin (0°), and $\boldsymbol{r}$ is the unit vector for n cells in a population (30). A unit vector length of 1 indicates that all sperm swam on a single trajectory for a given treatment. In contrast, a length of zero denotes random movements without a shared bearing. Sperm swimming trajectory was evaluated with respect to the position of an egg, mimic, or placebo. In this case, the origin was defined as the tangent between each cell head and the nearest location of the egg or sphere surface. Sperm moving directly toward an egg or sphere thus would follow a 0° heading. For each treatment, a Rayleigh's test initially compared the mean direction swum against a uniform circular distribution. If significantly different, a subsequent *V*-test determined the fit with respect to the origin. In addition, a Watson-Williams test compared the mean sperm headings with respect to an egg, mimic, or placebo.

Male gametes exhibited chemotactic behavior (Fig. 2). Within 100 μm of the egg or mimic surface, sperm swimming direction was non-uniform, and sperm oriented toward the target (Rayleigh's test: live egg: mean angle =345.2°, $\boldsymbol{r} = 0.516$, $z = 4.526$, $P = 0.009$; mimic: mean angle = 0.1°,

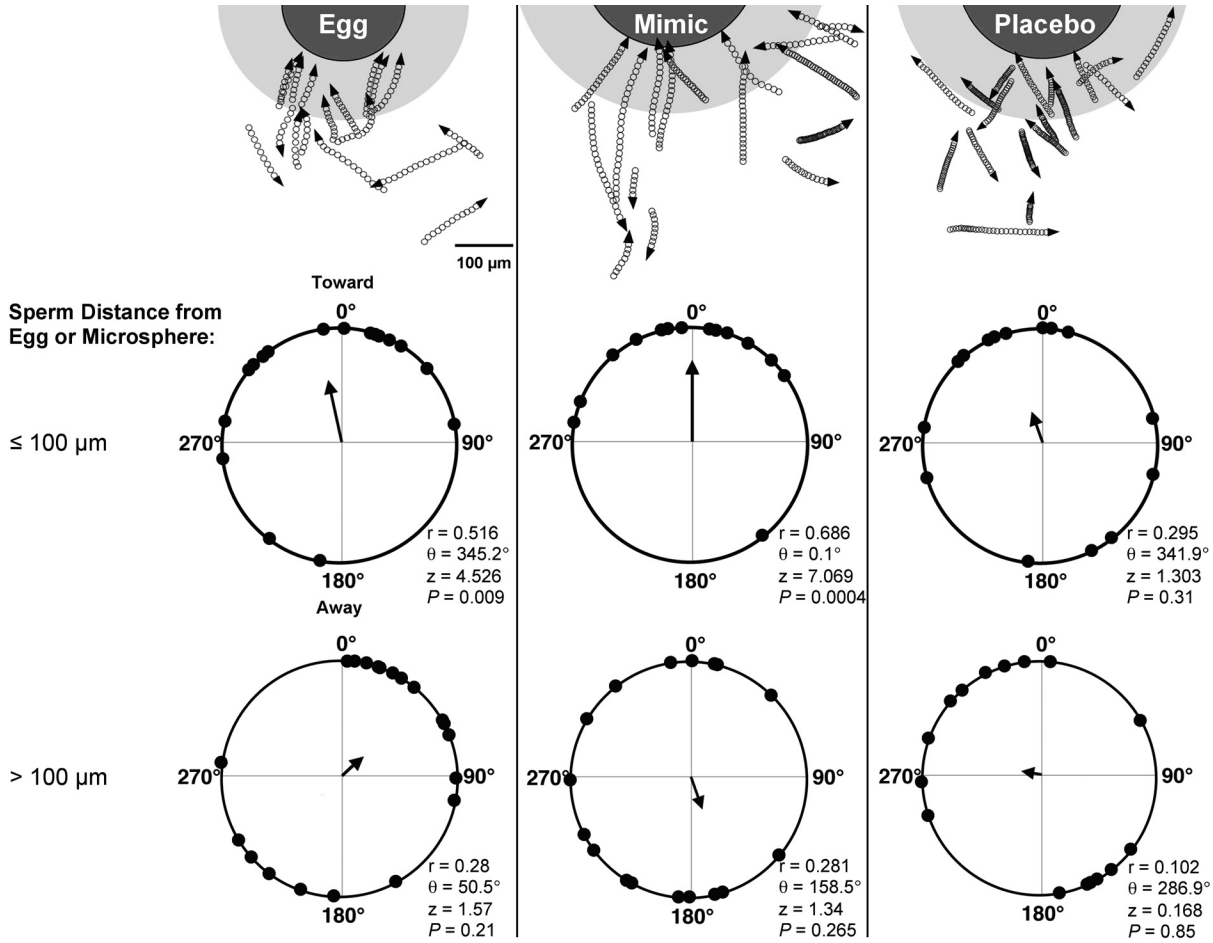

Figure 2. *Top panel:* Representative swimming paths of individual red abalone sperm surrounding a live conspecific egg, egg-mimic, or placebo. The shaded area highlights a region within 100 μm from the egg or microsphere surface. Small (open) circles are successive digital images of sperm heads captured at 0.033-s intervals, and each arrowhead denotes the direction of travel for an individual cell. To eliminate selection bias, a random numbers generator was used in choosing paths for each treatment. *Middle and lower panels*: Sperm swimming orientation for cells positioned $\leq$ 100 μm or $>$ 100 μm from the surface of a live conspecific egg, egg-mimic, or placebo, respectively. Complete data sets (*i.e.,* not representative paths) were used to establish circular distributions and in calculating mean unit vector lengths ($\boldsymbol{r}$) and angular headings (θ) relative to an origin. In these cases, the origin was defined as a tangent between each sperm head and nearest location of the egg or sphere surface. For example, individual sperm (small closed circles) moving directly toward an egg would follow a 0° heading. A Rayleigh's test (z-value) was used to compare each mean direction against a uniform circular distribution and to calculate the P value.

$\boldsymbol{r} = 0.686$, $z = 7.069$, $P = 0.0004$). In contrast, orientation was at random within 100 μm of the placebo (Rayleigh's test: placebo: mean angle = 341.9°, $\boldsymbol{r} = 0.295$, $z = 1.303$, $P = 0.31$). A V-test demonstrated that sperm significantly oriented toward the origin, directly aimed at both the egg and mimic (V-test: egg: $V = 8.481$, $u = 2.909$, $P < 0.0025$; mimic: $V = 10.297$, $u = 3.76$, $P < 0.0005$). The average angle did not differ significantly between mimics and eggs (Watson-Williams test: $P > 0.50$). At distances greater than 100 μm from the egg and mimic surface, sperm swimming direction was random (Rayleigh's test: live egg: mean angle = 50.5°, $\boldsymbol{r} = 0.280$, $z = 1.573$, $P = 0.212$; mimic: mean angle = 158.5°, $\boldsymbol{r} = 0.281$, $z = 1.341$, $P = 0.265$). These results indicate that close to the surface, the L-tryptophan gradient associated with a mimic stimulated chemotaxis equivalent to that observed near live eggs, whereas a directional response did not occur near tryptophan-free placebos. Furthermore, sperm navigated directly toward the gradient source when in proximity to the surface, but not at greater distances.

An L-tryptophan gradient also induced marked chemokinesis near eggs and mimics alike. Within 100 μm of both the eggs and mimics, speeds of red abalone sperm approximately doubled relative to those near placebo microspheres

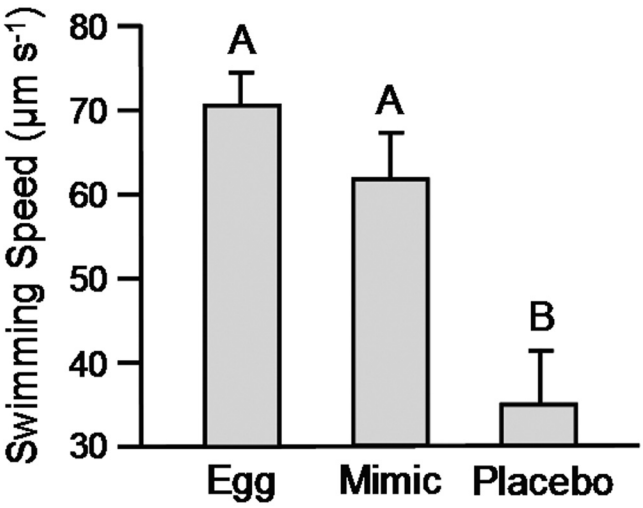

Figure 3. Swimming speed of red abalone sperm within 100 μm of a live conspecific egg, egg-mimic, or placebo. Values are means ± standard errors ($n \geq 15$ cells). Means marked with the same letter did not differ significantly (one-way ANOVA, followed by a *post hoc* Scheffé test [$P > 0.05$] for unplanned multiple comparisons).

(Fig. 3) (one-way ANOVA: $F_{2,40} = 21.1$, $P << 0.001$; Scheffé tests: $P < 0.05$). Sperm swimming speeds near mimics and live eggs were indistinguishable (Scheffé test: $P > 0.25$).

Knowledge of L-tryptophan release dynamics over the lifetime of a viable abalone egg afforded the opportunity to reproduce these properties in synthetic microspheres (4, 22–23, 31). Historically, heterologous systems have provided valuable experimental tools for testing relationships between gene structure and function in protein expression (7, 32). Similarly, investigations employing synthetic eggs can be used to establish cause-and-effect between a specific chemical signal and sperm response. Here, results show unequivocally that L-tryptophan alone is both necessary and sufficient for red abalone sperm attraction. Synthetic eggs, therefore, are critical tools for testing theories on gamete communication and sperm behavior.

Limitations in acquiring live eggs have restricted sperm behavioral work to date, particularly for internal fertilizers. Most understanding of sperm-egg interactions comes from broadcast-spawning species for which eggs are attainable in large numbers. As a result, detailed knowledge of sperm attractants arises largely from investigations on marine invertebrates (2, 5, 13, 22, 33–35; for a review, also see 36). Constraints to working with live human eggs have hindered, for example, basic research into behavioral interactions that may have important implications for fertility and contraception. Synthetic eggs are adaptable tools that can be designed to imitate, both physically and chemically, eggs from different species and would be especially useful for studying the consequences of sperm behavior. Egg mimics increase the capacity for experimental manipulation in pursuit of understanding the fundamental principles of egg-derived attractant signals and chemical communication among gametes.

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