Fertilization in the sea: Establishing the minimum population sizes and environmental conditions for successful fertilization in abalone

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A. PROJECT SUMMARY

The influence of sperm behavior and fluid motion on mediating gamete encounters is thought to be a critical component in fertilization. Fertilization has been implicated as an important factor regulating population dynamics in many animals, particularly in marine organisms that broadcast their sperm and eggs into the sea. However, little is known about the mechanisms controlling sperm-egg interactions in flowing water. Using the red abalone (*Haliotis rufescens*) as the model organism for exploring fertilization dynamics, this study established the influence of gamete behavior, concentration, and fluid motion on sperm-egg interactions. This was accomplished through three phases of this project: (i) Subtidal surveys of adult abalone populations and flow measurements to characterize aspects of the hydrodynamic environment within typical abalone habitat (subtidal kelp beds), allowing us to scale our laboratory flow-tank experiments, (ii) laboratory flow tank experiments that examined the influence of turbulence (shear) and gamete concentration on fertilization, and (iii) sperm chemosensory-mediated behavior was examined under simulated natural flow conditions. In parallel to our studies with abalone, we additionally began experiments examining chemosensory response of human sperm. Using the human sperm, we were able to use the human genome to target specific olfactory receptors that may function in sperm chemotaxis. Therefore, using these two systems, we were able to examine the physiological and genetic mechanisms controlling sperm behavior (human), and examine how chemosensory-mediated behavior by sperm influences reproduction (abalone). Abalones are commercially valuable but ecologically endangered resource. By providing new information on physical and behavioral mechanisms mediating fertilization success, this study should have significant implications for managing and restoring threatened abalone populations.

B. PROJECT DESCRIPTION

Introduction

For adult populations, the influence of juvenile supply on population dynamics has been a controversial topic for decades (Young, 1990; Connell and Green, 2000; Underwood and Keough, 2001). While supply of progeny to adult populations is thought to be critical, little attention has been focused on the events that occur upstream of juvenile delivery, and until recently, fertilization was rarely considered as an important rate-limiting step in juvenile production. Because many organisms either produce vast quantities of gametes, or have behavioral mechanisms to maximize sexual encounters, high fertilization rates seemed inevitable, even for marine organisms that broadcast, or release, their gametes into the sea (Ginsberg and Milner-Gulland, 1994; Leviatan, 1995; Underwood and Keough, 2001). Such conjecture is now only of historical significance for both terrestrial and oceanic environments (Levitan, 1998; Moody-Weis and Heywood, 2001; but see Yund, 2000). In the marine environment, the current view for external spawners favors sperm concentration as a limiting factor in fertilization, particularly for animals living in habitats with strong water flow (Levitan and Peterson, 1995). For some

species, individuals separated by only a meter or less are unable to cross-fertilize (Denny and Shibata, 1989; Grosberg, 1991). The study of fertilization ecology is thus of critical importance in population ecology and conservation biology.

For marine animals that broadcast spawn, the turbulent nature of the ocean environment into which the gametes are released can drastically influence fertilization rates. Fluid motion can transport and mix the gametes together, facilitating commingling between sperm and eggs, or it can have a detrimental effect by causing rapid dilution and prevention of sperm-egg encounters (Pennington, 1985; Denny and Shibata, 1989; Babcock et al., 1994). Indeed, flow-dependent fertilization has been demonstrated for many marine free-spawners, but the mechanisms responsible for those trends have only been inferred and not directly tested (Levitan, 1998). Although flow has long been recognized as a critical factor influencing fertilization, at the scale of the gametes, little is known about the influence of fluid motion on mediating gamete encounters (but see Mead and Denny, 1994).

At the scale of the sperm and egg, turbulent fluid motion is thought to influence the ability for a sperm to encounter, and then fertilize an egg. Turbulence will also influence the dilution rate of the gametes, thus influencing the time available for the gametes to contact one another. Therefore, active behavior by the sperm may be critical for successful fertilization. Sperm chemoattractants, released from conspecific eggs, are thought to increase the probability of contact between sperm and egg, consequently enhancing fertilization in turbulent aquatic environments (Ward and Kopf, 1993; Miller and Vogt, 1996). Indeed, sperm chemoattractants have even been found in mammalian reproductive tracts, suggesting that such chemical signaling between gametes may play an important role in fertilization even in terrestrial systems (Eisenbach, 1999). However, little is known about how sperm are able to navigate to the egg plume under natural flow conditions.

To understand the importance of gamete behavior and fluid motion on fertilization, controlled laboratory studies under simulated natural environmental conditions are necessary and vital. Our proposed research will elucidate the mechanisms by which fertilization occurs, and determine the relative influences of fluid motion and gamete behavior and density on fertilization. This study will also seek to advance understanding in the role of chemical signaling in fertilization success. Waterborne sperm attractants may compensate for the dilution of gametes that occurs when sperm and eggs are shed into the ocean, but how such signals operate in flowing water remains unexplored. Sperm behavior has never been imaged under hydrodynamic conditions simulating turbulent fluid motion. In this proposed study, we will determine how sperm and eggs encounter one another at different densities, and how sperm navigate towards eggs while experiencing flow regimes similar to those occurring in the field. Together, these results will improve understanding of how the physical environment affects fertilization, and how chemical communication between single-celled gametes occur.

RESEARCH GOALS

The goals of this research was to establish the influence of fluid shear on sperm-egg interactions, and to provide insight into how dissolved signal molecules are utilized by sperm to locate eggs. The following objectives were addressed:

(1) Characterize the flow regime of the abalone microhabitat in the field
(2) Determine the influence of shear and gamete density on fertilization
(3) Establish the hydrodynamic effects on sperm chemoattraction and gamete encounters
(4) Understand the molecular mechanism controlling chemosensory-mediated behavior by sperm.

The premise to the combined field/laboratory approach for this study is that flow characteristics of abalone microhabitats, at the scale of the sperm and egg, can be simulated in laboratory flow tanks. A field measurement program to characterize the hydrodynamic regime in abalone microhabitat within subtidal kelp beds (Objective 1, Years 1 and 2) provided measurements allowing us to specify the range of conditions to be tested in hypothesis driven, flow-tank experiments in the laboratory. Broadcast spawning organisms inhabit turbulent aquatic environments, but there is no information on the mechanics on how sperm encounter eggs in flowing water. Investigations on sperm-egg encounters and gamete density (#/ml) under simulated natural hydrodynamic conditions (Objective 2, Year 1 and 2) placed laboratory results within a field context. The role of chemosensory-mediated behavior was next determined by examining sperm swimming and orientation towards eggs (Objective 3, Year 2) under flow, in parallel with numerical modeling of the influence of shear on attractant distribution around eggs. Studies of sperm-egg interactions in flow will mechanistically complete the picture of how turbulence influences fertilization, and how chemical communication, at the level of single cells, can mediate fertilization.

A mechanistic understanding of sperm-egg interactions under natural environmental conditions, and the relative roles of hydrodynamics and chemosensory behavior, can both lead to greater insights into the factors that mediate fertilization. The California red abalone provides an excellent opportunity for such studies. Abalones are an important ecological and commercial resource in California, but populations are severely threatened; human fishing and disease have resulted in decimated populations with adults scattered over large areas (Tegner at al., 1989; Davis et al., 1996; Friedman et al., 1997). These unnaturally low densities of adults implicate fertilization success as the limiting factor preventing recovery of abalone populations. Understanding the mechanisms at which turbulence and chemosensory behavior mediates fertilization could significantly aid conservation efforts.

D. RESEARCH ACTIVITIES

***Isolation and purification of abalone sperm attractant*:** Surface proteins that mediate sperm-egg interactions have been better characterized in abalone then in any other organism, thus providing a well characterized system to investigate the importance of soluble egg-factors in fertilization ecology. We tested whether live red abalone eggs released an attractant to which sperm respond, through quantifying the sperm behavior in proximity to live abalone eggs (Riffell et al., 2002). Sperm swam significantly faster in proximity to a live abalone egg also oriented towards the egg (Fig. 1). Swim speed did not change for sperm near a brine shrimp egg, and orientation to brine shrimp eggs was random.

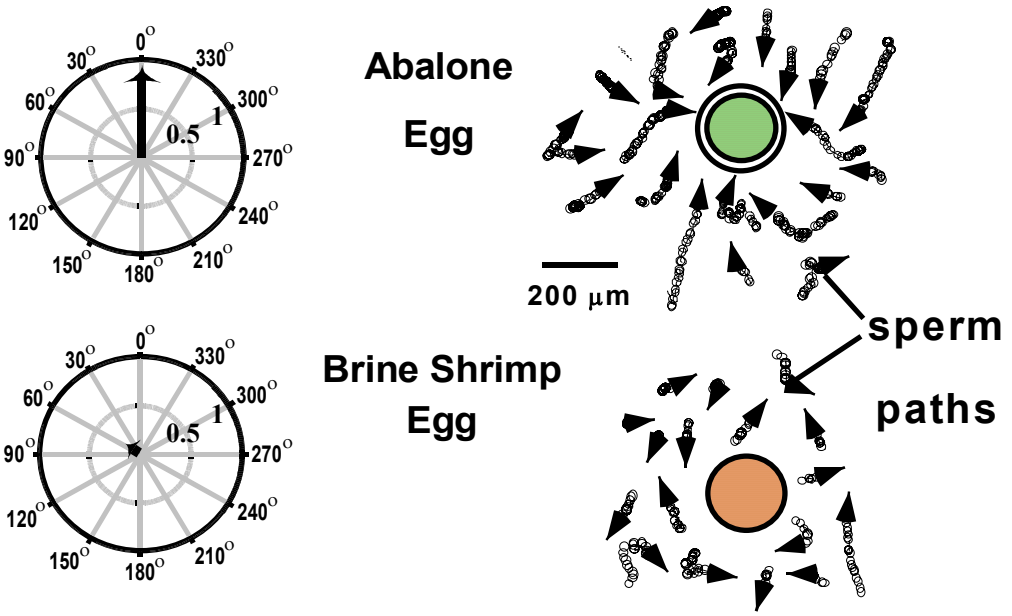

Figure 1. Sperm responses to red abalone versus brine shrimp eggs. **Left:** Polar plots of mean sperm swimming directions videotaped for 30 s, with angles relative to the egg surface (0°). **Right**: Sperm paths over 30 s in the presence of a single live abalone or brine shrimp egg. Dots are images captured at 0.017 s intervals; arrows are direction of travel for individual cells.

The sperm attractant was isolated by bioassay-guided fractionation of egg-conditioned seawater (ESW). The ESW was desalted using a C_{18} Sep-Pak cartridge, then concentrated and partially purified with reversed-phase HPLC on a C_{18} column, eluting with a gradient of 5 to 50% CH_3OH in water/0.1% TFA. All bioactivity was contained in one peak absorbing at 220 nm. Final purification by size-exclusion HPLC yielded a single compound roughly 200 Da in molecular weight. This molecule was fully active in both bioassays, thus precluding the need to work with complex mixtures as in other systems (i.e., Coll et al., 1994). Final elucidation of the isolated chemoattractant chemical structure by 2D heteronuclear NMR indicates that the sperm attractant is the amino acid, tryptophan.

***Fertilization kinetics and properties of gametes*:** As a critical prelude to the proposed investigations on fertilization dynamics in flow, we performed laboratory

experiments in still water to identify basic gamete properties (Riffell and Zimmer, in prep.). These experiments were run on red abalone, because gravid adults were readily available through commercial suppliers and because both males and females could be easily spawned using existing methods (Morse et al., 1977). An initial set of trials was conducted to determine the percentage of eggs fertilized while varying gamete age. These tests showed that sperm remained without significant changes in swimming speed or fertilization rate for 3 h following initial spawn. In contrast, for eggs, percent fertilization decayed rapidly beginning 30 min after release. Thus, fertilization success is limited by egg, rather than by sperm, longevity.

It remains unclear, however, whether gamete longevity is an important constraint in natural habitats. Field studies in other free-spawning invertebrates have shown, for example, that fertilization can be limited by gamete dilution within one or two minutes of gamete release, or shorter (Pennington, 1985; Yund, 1990; Levitan, 1998). We tested effects of gamete density and contact time on percent fertilization using a factorial design. Natural concentrations of eggs and sperm in freshly spawned gamete plumes contained 10^5 and 10^9 gametes/ml suspensions, respectively. Crosses were performed for a wide range of egg ($10^1 – 10^5$ gametes/ml) and sperm ($10^1 – 10^8$ gametes/ml) concentrations, over contact times of 5 to 2,400 s. Results indicated that, first, percent fertilization was dependent upon the ratio of sperm-to-eggs but not on the concentration of either gamete type. Second, contact time mattered little. Asymptotic percent fertilization was achieved within 15 s of initial gamete contacts. Hence, fertilization is extremely rapid, even in still water.

Environmental context of sperm-egg interactions: At the scale of fertilization (0.1-1 mm), sperm encounter eggs while being mixed within a sheared (velocity gradient) viscous flow. This fluid-dynamic regime occurs inside the smallest eddies (of order 1 mm) in the flow, where fluid is dominated by viscosity (Fig. 2). Thus, water particles tend to move as a sheared unit. Much like a pot of honey stirred by a spoon, the fluid moves in a lazy path along the track of motion and quickly stops when the action. However, because of the turbulent nature of the eddies, these laminar velocity gradients shift over time and space, thus causing eggs and sperm to tumble and contact one another (Mead and Denny, 1995; Karp-Boss et al., 1996). Such flow-dependence has been demonstrated for fertilization in other free-spawning animals, but the mechanisms responsible for these trends have only been inferred and not tested (Pennington, 1985; Levitan, 1998).

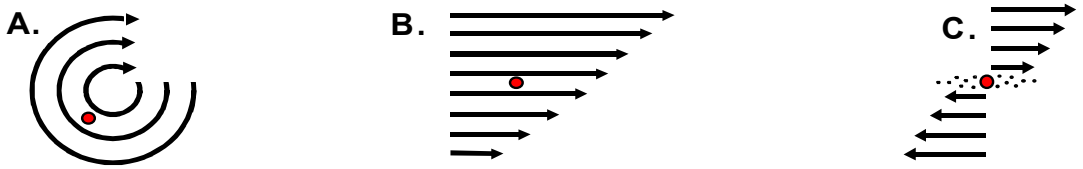

Figure 2. Fluid-dynamic environment experienced by sperm and eggs. **(A)** Egg inside turbulent eddy. **(B)** Laminar-shear flow within eddy, straightening flow velocity vectors in A. **(C)** Flow from perspective of egg and through which sperm must navigate. An odor plume of sperm attractant (dots) is generated by the shear.

Establishing the principles that mediate signal production and transport is critical for interpreting biological responses to chemical cues released into the environment. Prior studies of chemosensory systems have tended to ignore essential properties of physical transport on interactions between single cells (Zimmer and Butman, 2000). During the past year, we measured the flow regime in microhabitats where abalone spawning and fertilization take place. Large aggregations of abalone (3-7 adults/m^2) were found only at localized "hot spots" within crevices (0.5-0.8 m deep, openings $\leq$ 0.3 m wide) and under ledges (27-40 cm high) (Table 1). Flow speeds were measured at these hot spots using an acoustic Doppler velocimeter (ADV) mounted on the articulating arm of a stable tripod. The small size and sample volume (0.3 ml) of our custom-built probe allowed high-speed (30 Hz) measurements ~1 cm above abalone living in crevices and under ledges. Flow measurements were also made in open areas away from hot spots, regions of strong oscillatory, cross-shelf currents (Table 1). Water flow in hot spots was 5-10 times slower than in exposed areas, with smaller Reynolds stresses and eddy dissipation rates, indicating low turbulent mixing; abalone thus aggregated at sites with limited water motion. Moderate flow and turbulent mixing can enhance gamete contact rates, increasing fertilization rates over both still water and energetic flow conditions (Mead and Denny, 1995; Karp-Boss et al., 1996).

Table 1. Flow properties of microhabitats that are typical hot spots for red abalone (*Haliotis rufescens*) and nearby open areas from Point Loma, San Diego, CA and Harris Point, San Miguel Island, CA (n = 10 sites per habitat type). Data were collected for >10 min intervals at 30 Hz

Depth (m)	Habitat type	Long-shelf current (cm/s)	Cross-shelf flow Speed (cm/s)	Cross-shelf flow Wave Period (s)	Turbulent energy dissipation rate (cm^2/s^3)	Reynolds stress (N/m^2)	Shear rate, G (1/s)
10-20	crevice	N/A	2-5	N/A	0.002-0.01	3-6	0.2-0.4
"	ledge	N/A	4-7	N/A	0.02-0.08	5-9	0.5-1.0
"	open	0.4-1.2	9-13	10-15	0.09-0.5	12-16	1.1-2.5

N/A = not applicable

Effects of steady shear on sperm-egg interactions: In this study, laboratory experiments were performed that simulate the fluid-dynamic regimes both of abalone hot spots and nearby open areas. To our knowledge, these experiments are the first to determine the effects of fluid shear associated with turbulent mixing on sperm chemosensory-mediated behavior and fertilization success. Two Taylor-Couette apparatuses (Fig. 3) allowed us to investigate the encounters between sperm and egg under conditions of laminar-shear flow similar to that found in the natural environment. Each unit consists of two coaxial cylinders (28.0 cm tall, 6.2 cm and 7.0 cm radii) that rotate in opposite directions by means of computer-controlled stepper motors. The apparatuses are padded and placed on air-cushioned, vibration-free tables to eliminate any effects of the stepper motors, in a 15° temperature-controlled room. Seawater (0.22

μm filtered) fills the 8-mm gap between the inner and outer cylinders. The sheared fluid associated with each cylinder moves in opposite directions, producing a predictable cross-over point of no translational velocity about midway through the fluid-filled space. Gametes can be observed through our long-range video microscopes (Titan Tool Supply Co.) attached to custom-built infrared (IR)-sensitive video cameras.

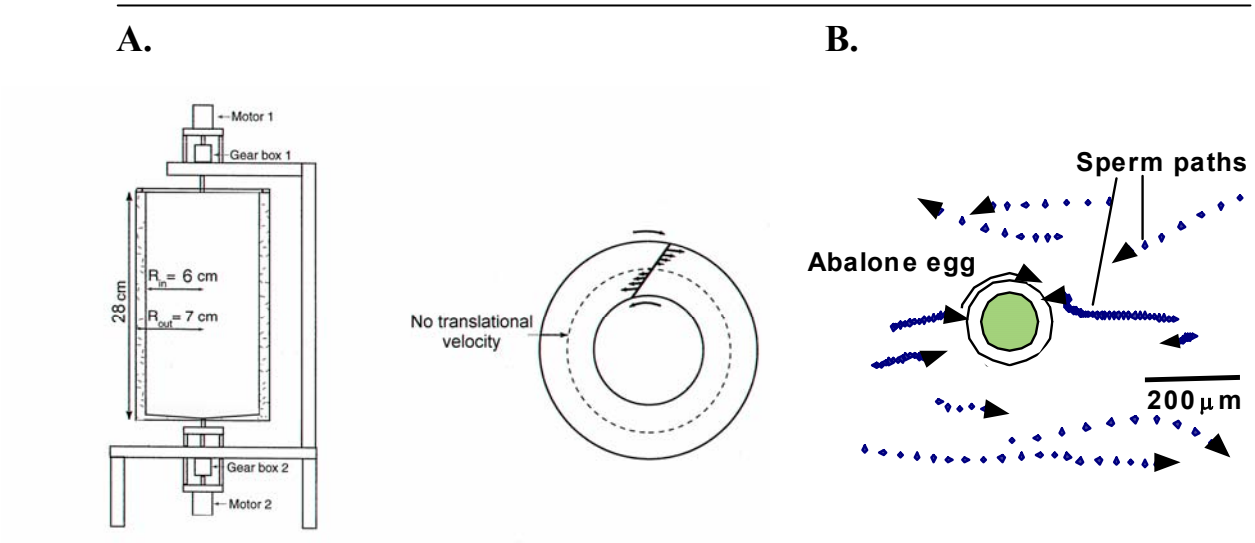

Figure 3. (A) *Left:* Taylor-Couette apparatus (not to scale) used in simulating steady shear flows in the laboratory. *Right:* View from above of flow within the annular gap between the cylinders (not to scale). The counter-rotating cylinders drag adjacent fluid 'particles' in opposite directions. The cross-over point (dashed line) between the two velocity fields is the point of no translational motion. **(B)** Sperm paths relative to an abalone egg within the zone of no translation (shear of 0.5/s). The rotation of the egg induced by the shear may influence sperm attachment.

Gametes placed in the apparatus were video imaged by an IR laser sheet and a long-range zoom microscope, interfaced with a custom computer-assisted video analysis system to quantify the gamete interactions. Using results from a theoretical prediction on the bulk transport of tryptophan from a simulated egg, we examined sperm behavior within and without the predicted 10^{-8} *M* area (the sensory threshold of sperm) around live eggs (Fig. 4). At shear flows of 0.1 – 1.0/s, sperm within the modeled tryptophan plume accelerated significantly and navigated directly towards the egg. Outside of the plume, however, sperm cells swam slower and had random orientation. Together, these results demonstrate that shear increases the natural tryptophan broadcast distances from live eggs and that sperm can respond as far as 1 mm away. Active sperm behavior, however, is shear-dependent. Chemosensory-mediated behavior by sperm decreased with increasing shear until, at > 2.0/s, shear completely dominated sperm swimming, and cells were passively transported with flow. Thus, while shear may increase the transport distances of tryptophan from eggs in comparison to still water, sperm behavior is only important at low shears.

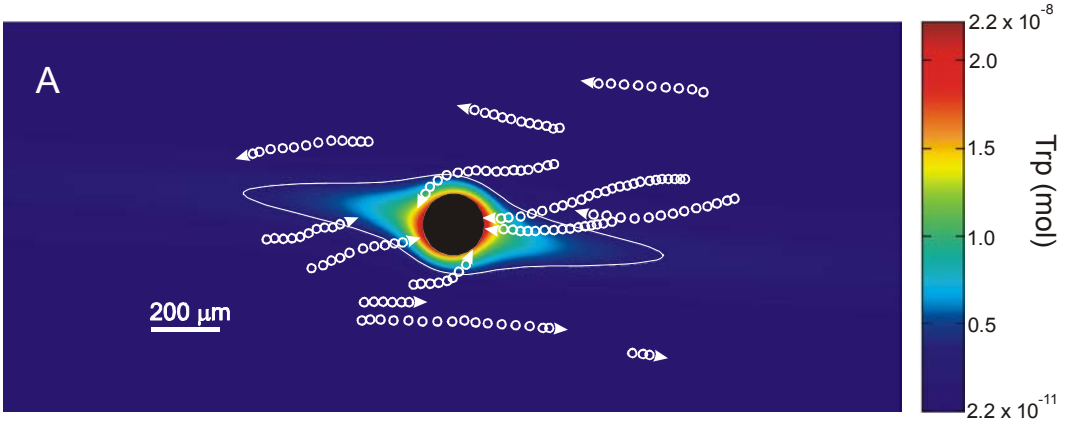

Figure 4. (A) Swimming behavior of abalone sperm near an egg within the Taylor-Couette apparatus by using computer-assisted video motion analysis. Open circles correspond to video images captured at intervals of 0.016 s, and arrowheads indicate directions of travel of individual cells. To eliminate selection bias, a random numbers generator was used to choose representative paths. The surface map displays the modeled tryptophan plume. Sperm chemosensory responses correlate well with the model prediction of the ED_{50} tryptophan concentration (white contour).

Human sperm chemosensory responses: Human sperm chemotaxis is a critical component of the fertilization process, but the molecular basis for this behavior remains unclear. Recent evidence shows that chemotactic responses depend on activation of the sperm olfactory receptor, hOR17-4. Certain floral scents, including bourgeonal, activate hOR17-4, trigger pronounced Ca^{2+} fluxes, and evoke chemotaxis (Fig. 5). Here, we provide evidence that hOR17-4 activation is coupled to a cAMP-mediated signaling cascade. Multidimensional protein identification technology was used to identify potential components of a G-protein-coupled cAMP transduction pathway in human sperm. These products included various membrane-associated adenylate cyclase (mAC) isoforms and the G_{olf}-subunit. Using immunocytochemistry, specific mAC isoforms were localized to particular cell regions. Whereas mAC III occurred in the sperm head and midpiece, mAC VIII was distributed predominantly in the flagellum. In contrast, G_{olf} was found mostly in the flagellum and midpiece. The observed spatial distribution patterns largely correspond to the spatiotemporal character of hOR17-4-induced Ca^{2+} changes. Behavioral and Ca^{2+} signaling responses of human sperm to bourgeonal were bioassayed in the presence, or absence, of the adenylate cyclase antagonist SQ22536. This specific agent inhibits particulate AC, but not soluble AC, activation. Upon incubation with SQ22536, cells ceased to exhibit Ca^{2+} signaling, chemotaxis, and hyperactivation (faster swim speed and flagellar beat rate) in response to bourgeonal. Particulate AC is therefore required for induction of hOR17-4-mediated human sperm behavior and represents a promising target for future design of contraceptive drugs.

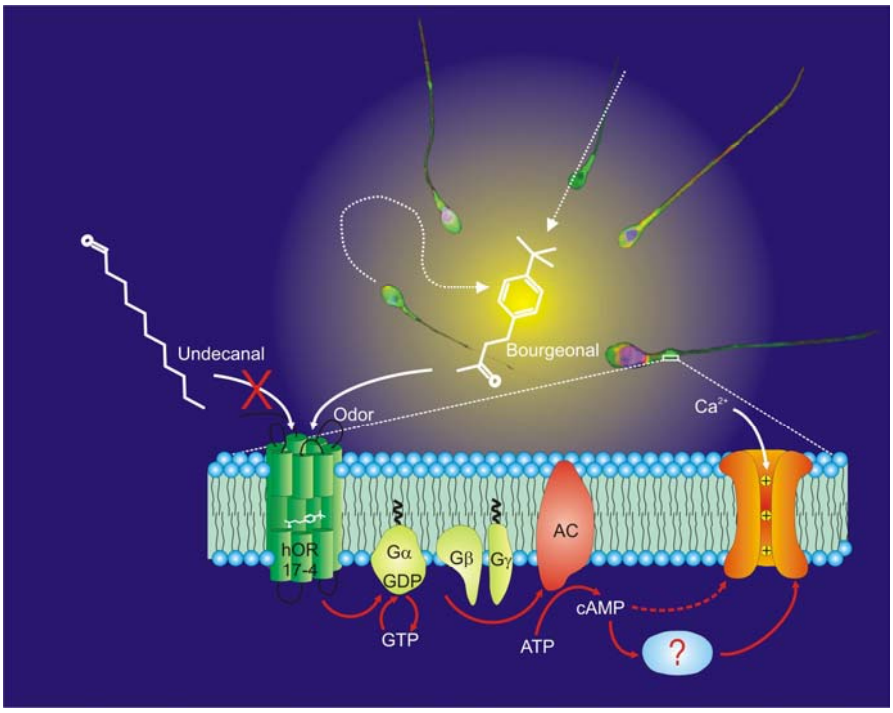

Figure 5. Transduction events leading to chemosensory responses by human sperm. The odorant bourgeonal binds to a G-coupled receptor located on the spermatozoan flagella, leading to cAMP expression and calcium entry into the cell.

E. RESEARCH ACCOMPLISHMENTS

The principle goals of this research grant was to establish the physical and chemical basis for fertilization success. This was accomplished in three components: (1) establish the abalone fertilization kinetics in still water, (2) characterize the natural abalone hydrodynamic environment, and determine the influence of shear on gamete interactions, and (3) determine the chemosensory effects on fertilization using both abalone and human sperm as model systems. Through funding by the UC Environmental Quality Initiative, research on these three components have resulted in 4 publications, one of them in the journal *Science* (detailed below), with an additional 5 manuscripts already submitted or in preparation.

Abalone fertilization kinetics: This manuscript has been recently submitted to *American Naturalist* for review:

Riffell, J.A., and R.K. Zimmer. Egg limitation and the control of fertilization: does the sperm-limitation paradigm really exist? *Submitted to American Naturalist.*.

Influence of shear and sperm chemotaxis on sperm-egg interactions: This component of the research grant will result in 3 publications, which are targeted for the *Journal of Experimental Biology*, and the journal *Limnology and Oceanography*:

Riffell, J.A., and R.K. Zimmer. Numerical modeling and effects of steady shear on attractant plume and gamete motility. *In preparation for Journal of Experimental Biology.*

Riffell, J.A., and R.K. Zimmer. Sperm chemotaxis and the effects of steady shear. *In preparation for Journal of Experimental Biology.*

Riffell, J.A., and R.K. Zimmer. Influence of steady shear on gamete encounters and fertilization success in the red abalone, *Haliotis rufescens*. *In preparation for Limnology and Oceanogragy*.

Effects of sperm chemotaxis on gamete interactions and fertilization: Research in this area resulted in 4 publications and very fruitful collaborations in the disparate fields of genetics and chemical sensing:

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.

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F. COLLABORATORS AND PARTICIPATING INSTITUTIONS

During this project, work was conducted with the California Department of Fish and Game and fellow university researchers. Specifically, we conferred with Dr. Peter Haaker (CDFG, Los Alamitos Office) and participated in a CDFG cruise (April 2001) that censused abalone at the Channel Islands. Our abalone research has involved informal collaborations with both Mia Tegner (deceased) and Paul Dayton at Scripps Institution of Oceanography. Discussions with Dr. Dayton and his students are continuous, and have thus allowed us to launch our field operation in the Point Loma kelp forest from his laboratory. Additional collaborations occurred with Dr. Hanns Hatt at the Ruhr University Bochum, Germany, and Dr. Marc Sphehr, University of Maryland.

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