Title
Identification of Natural and Synthetic Peptides for Controlling Marine Larval Set

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TECHNICAL NARRATIVE

Identification of Natural and Synthetic Peptides for Controlling Marine Larval Set
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Overview

Colonization of the benthic environment requires planktonic larvae to settle from the water column and metamorphose to a juvenile stage. For many species, planktonic larvae can discriminate between settlement sites; the larvae not only choose consistently between substrates in the laboratory, but cluster on preferred substrates in the field. Chemical properties of benthic environments are known to provide essential cues for larvae actively colonizing habitat sites. The requirements for chemical inducers to trigger settlement and metamorphosis can be exploited for ecological management and commercial benefits.

This project has advanced new methods for isolating and for designing inducer compounds. Applications for these inducers specifically targeted the mariculture and fishery industries for California red abalone (*Haliotis rufescens*) and eastern oysters (*Crassostrea virginica*). Although they are highly valued animals, harvested wild populations of *C. virginica* have recently reached historic lows while abalone populations have crashed in all but a few locations off the southern and central California coasts. Moreover, we worked to identify the inducers for barnacle larvae (*Balanus amphitrite*), as a critical step towards engineering potent antagonists. Because barnacles cause serious economic damage through biofouling, antagonists to settlement and metamorphosis by barnacle larvae have long been sought.

Our Sea Grant sponsored research included additional investigations on chemical signals that facilitate sperm-egg interactions in *Haliotis* species. Southern California historically supported commercial fisheries for five species of abalone, which once commonly occurred in high-density aggregations. All of these species have seen significant declines in population densities in the past decade, and despite a moratorium on commercial harvesting, natural populations have not recovered. We hypothesized that adult densities may have been lowered to such an extent that free-spawned sperm and eggs are diluted too rapidly for appreciable fertilization to occur. It is therefore crucial to understand the factors that mediate abalone sperm-egg interactions, in order to improve population management and recovery efforts for these vulnerable and commercially valuable species**.**

Oyster and barnacle research: Larval settlement and chemical inducers

Laboratory studies. The natural inducers for oyster larvae were characterized through hollow fiber dialysis, selective enzyme and chemical oxidations, and high-performance liquid chromatography to be low-molecular-weight peptides (< 1000 g mol^{-1}) with arginine at the carboxy-terminus. Structure-activity relationships were determined between the physicochemical properties of peptides and settlement induction. Responses of oyster larvae were tested to solutions containing one of 53 analogs. Of these, only 18 small peptides induced

responses at the submicromolar concentrations found in natural estuarine environments. Glycyl-glycyl-L-arginine (GGR) was the most potent compound examined. Replacing the C-terminal amino acid of GGR with lysine, glycine, histidine (GGH), aspartic acid, or leucine left little, if any, activity. Inverting the sequence (RGG) or emphasizing arginine (RRR) stifled all activity indicating that the presence of arginine in the molecule is insufficient to induce settlement. While adding glycine to the amino terminal (GGGR) caused only a slight decrease in activity, removing a glycyl monomer (GR) resulted in an inactive compound. Peptides with five or more amino acids retained little activity even when they contained an arginine at the C-terminus. Thus, only peptides in a very narrow range of molecular masses (250-600 g mol^{-1}), or amino acid lengths (3-4), were effective inducers of settlement.

Numerical modeling studies. Previous investigations have shown that barnacle (*Balanus amphitrite*) larvae settle like oysters in response to low-molecular-weight peptides with arginine or lysine at the C-terminal. We therefore used a multivariate partial least squares algorithm to investigate composite properties for the hydrophilicity, size and charge of each amino acid and the sequence position to oyster and barnacle settlement patterns. From the information in these QSAR models, the apparent variability in amino acid sequences eliciting settlement responses was explained in each case, and more potent peptide analogs were hypothesized on the basis of untested amino acid sequences. Remarkably, these peptide signals were all structurally related to the carboxy terminal sequences of mammalian C5a anaphylatoxin, a potent white blood cell chemoattractant. Through the physicochemical properties of amino acids, the QSAR models clearly differentiated between the optimal sequences for eliciting oyster and barnacle settlement. Thus, QSARs provided a novel and powerful method not only for relating the physicochemical properties of molecules of settlement but also for differentiating responses to chemicals by individuals of different species.

Field studies. Field experiments were conducted to evaluate the potencies and performances of one of these peptides (glycyl-glycyl-L-arginine, GGR). Using the mathematical principles of solute diffusion from a porous material, polyacrylamide gels were designed through numerical modeling for controlled chemical release from larval collectors in North Inlet Estuary, South Carolina. These trials were intended to determine the responses by oyster and barnacle (*Balanus amphitrite*) larvae, because GGR effectively stimulated settlement in both species in laboratory tests. Repeated plankton tows indicated that oyster larvae were not in the water column during the course of this field investigation. Nevertheless, significantly more barnacle larvae colonized collectors emitting trace amounts of GGR (5×10^{-8} to 5×10^{-10} *M*), than those releasing either seawater or glycyl-glycyl-L-histidine (GGH), an organic enrichment control. Because GGR and GGH were basic peptides with nearly identical molecular masses and chemical functionalities, settlement induction could not be attributed to stimulation by organic compounds in general, but was due, specifically, to the arginine moiety at the carboxy-terminus of the peptide. Thus, larval delivery to the seabed likely resulted from hydrodynamic transport of larvae that also swam (or sank) downward in response to dissolved signal molecules. The potent effects of subtle changes in seawater chemistry on larval behavior therefore warrant careful attention as putative agents mediating habitat colonization.

Abalone research

Larval settlement. The bioactive components of red algal extracts were originally described as high molecular weight proteins, but were subsequently postulated to be low-molecular-weight compounds containing GABA-mimetic structural features. No pure molecules were ever isolated. In the current investigation, we found significant settlement activity by abalone larvae in response to crude extracts of the red algae *Lithothamnium californica*, *Plocamium cartilaginum*, and *Laurencia pacifica*. Research was performed to isolate bioactive low-molecular-weight fractions from these algal extracts. Crude aqueous extracts were subjected to size-exclusion chromatography on Sephacryl S-200 gel. The major protein and carbohydrate peaks co-eluted in the low molecular weight fractions for both *Plocamium* and *Lithothamnium* extracts. The bioactivity of the crude extracts principally co-eluted in these fractions as well, as determined by both metamorphosis and the suppression of larval swimming. Further chromatography of the low molecular weight fractions on Bio-Gel P2 resin only achieved partial separation of the component molecules, and bioactivity could not be consistently followed after size-exclusion, reversed phase, and anion exchange HPLC. We therefore focused on chemical and physical mediation of gamete encounter as a more promising avenue of investigation.

Sperm chemoattraction. 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 increase gamete encounter rates and thus enhance fertilization success. Soluble egg factors that attract only conspecific sperm might also function as pre-zygotic agents maintaining species integrity, acting upstream of membrane-bound proteins to promote species-specific gamete recognition. We considered the behavioral responses of sperm of the red abalone (*Haliotis rufescens*) to soluble factors released into seawater 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 HPLC. Chemical characterization by NMR indicated that the free amino acid L-tryptophan was the natural sperm attractant in *H. rufescens*. L-tryptophan was released by eggs at concentrations that triggered both activation and chemotaxis in conspecific sperm, exhibiting significant activity at levels as low as 10^{-9} *M*. The D-isomer of tryptophan was inactive, indicating that the sperm response was stereospecific, and enzymatic treatment with tryptophanase removed all bioactivity from egg-conditioned sea water. L-tryptophan is therefore necessary and sufficient to promote recruitment of sperm to the surface of eggs in the red abalone. An improved understanding of gamete signaling processes should provide insight into the selective forces affecting fertilization and speciation in marine organisms. As many abalone populations are currently endangered, elucidating the chemical basis for sperm-egg recognition may also aid in management and restoration efforts for these threatened species.

Characteristics of natural habitats where gametes are spawned. During the past year we conducted a field study of adult abalone distributions, including flow measurements in their microhabitats. Sites were chosen that historically supported large abalone populations at Point Loma (San Diego) and Harris Point (San Miguel Island). Efforts focused largely on red and pink abalone, because of their high abundances relative to other species, with more limited observations for green and black abalone. Although densities of reds and pinks ranged from 0.03 - 0.76 individuals/m^2, aggregations of 3-7 adults/m^2 were found at local "hot spots" within crevices and particularly under ledges.

Flow speeds were measured at these hot spots using an acoustic Doppler velocimeter (ADV) firmly mounted on the articulating arm of a stable tripod. The small size and sample volume (1.0 ml) of our custom-built Doppler probe allowed high-speed (30 Hz) measurements ~1 cm above abalone living in crevices and under ledges, and in adjacent open areas several meters away from all rocky ledges and boulders. Steady, longshore currents were exceedingly weak, but oscillatory, cross-shelf currents were strong in open habitats. Water flow in the hot spots was 5- to 10-times slower than in exposed areas, with smaller Reynolds stresses, eddy dissipation rates, and flow oscillations (amplitudes only; periods were longer). These eddy dissipation rates are similar to those measured in the surface mixed layer of the open ocean (e.g., 10^{-1} cm^2/s^3), as opposed to the very strong turbulence measured in coastal tidal channels (10^2 cm^2/s^3). Thus, abalone aggregated at sites where water motion was substantially retarded. Moderate flow and turbulent mixing can enhance contact rates between sperm and eggs, thereby increasing fertilization rates over both still water and energetic flow conditions.

Basic properties of gametes and fertilization kinetics. As a prelude to future investigations on fertilization dynamics in flow, we performed laboratory experiments in still water to establish critical properties of gametes. These experiments were run only for red abalone, because gravid adults were readily available through commercial suppliers and because both males and females could be easily spawned using existing methods. An initial set of trials was conducted to enumerate the percentage of eggs fertilized while varying gamete age. These tests showed that sperm remained viable without significant reduction 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 apparently 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 on other free-spawning invertebrates have shown, for example, that fertilization can be limited by gamete dilution within minutes of gamete release, even within low energetic settings. 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 suspension, respectively. Crosses were performed for a wide range of egg (10^0-10^4 gametes/ml) and sperm (10^1-10^8 gametes/ml) density combinations, over interaction intervals of 5 to 2,400 s. Results indicated, first, that percent fertilization was dependent on the ratio of sperm-to-eggs but not on the density of either gamete type. As the ratio of sperm-to-eggs increased from 10 to 1000, percent fertilization increased monotonically from 0% to 100%. Second, contact time mattered little. The asymptotic percent fertilization was achieved within 30 s of initial gamete contacts. Hence, fertilization was extremely rapid, even in still water.

Additional measurements assessed the basic material properties of gamete suspensions that are relevant to their transport in turbulent flows. Density of fresh spawn from males (1.07 g/ml) and females (1.12 g/ml) was considerably greater than seawater (1.02 g/ml). The mean gravitational fall velocity of individual eggs was 0.45 mm/s. However, spawned gametes from females typically consisted of 50-125 eggs stuck together in aggregates that sank at 3.8 mm/s, almost ten times faster than individual eggs. In contrast, the sperm solution was almost neutrally buoyant. Sperm and egg suspensions were much more viscous than seawater (of order 10^{-2} g/cm

s), and their viscosities varied as a function of shear rate indicating that they are non-Newtonian fluids. For abalone eggs, viscosity dropped from 143 g/cm s in still water to 79 g/cm s at a shear rate of 2/s. Likewise, sperm viscosity decreased from 16 g/cm s in still water to 9 g/ cm s at shear rate of 2/s. Our maximal viscosities are about a factor of 3 higher than those measured for non-Newtonian sea urchin gamete suspensions.

Abalone gamete suspensions will not track like a conservative tracer (e.g., dye) in the flow, and thus, their transport characteristics must be determined empirically. In contrast, all previous studies of gamete transport for free-spawning benthic invertebrates have assumed (without direct confirmation) that gamete suspensions can be adequately described by passive flow tracers, for example, using Gaussian transport models. The practice of diluting gamete suspensions to satisfy model assumptions, may favor logistics but defies nature. Gamete viability is so short that natural dilution is likely to be minimal, and it is the highly viscous solutions described above that are mixed by the flow on time scales of fertilization.

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