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Endogenous signaling pathways and chemical communication between sperm and egg

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SUMMARY

Red abalone (*Haliotis rufescens*) sperm detect a waterborne chemical cue released by conspecific eggs, and change their swimming behavior to increase the likelihood of fertilization success. Previously, we isolated the natural sperm attractant by bioassay-guided fractionation and high-performance liquid chromatography, and chemically identified it as the free-amino acid L-tryptophan (L-Trp). In the present study, levels of this ecologically meaningful compound were quantified in various abalone tissues, and in freshly spawned eggs. Tryptophan was the least abundant of 19 dissolved free amino acids (DFAAs) in ovary, testis, foot muscle, gill, stomach and hemolymph. As a proportion of the DFAA pool, however, Trp concentrations were significantly elevated in eggs (three- to seven-times higher) relative to all other sampled tissues. Natural rates of Trp release from eggs also were measured and correlated with fertility. Fertilization success peaked during an initial 30 min period (post-spawn), but decreased to nil over the next 50 min. Closely paralleling these events, Trp accumulated in seawater around freshly spawned eggs for the first 45 min (post-spawn) before decaying rapidly from solution. Older eggs stopped releasing Trp approximately when they became infertile, revealing a critical link between gamete physiology and chemical signaling. This apparent negative feedback loop did not arise from tryptophan oxidation, uptake by bacteria in seawater, or a degrading enzyme released by eggs. As a metabolic precursor critical to development of the larval nervous system, Trp could be an honest indicator of egg fitness for prospective sperm suitors. Our results suggest that endogenous signaling pathways have been co-opted for external communication between gametes, as an adaptation to increase reproductive success by promoting sperm navigation towards fertile eggs.

Key words: fertilization, sexual reproduction, gamete interactions, sperm, egg, attractant, chemotaxis, tryptophan, amino acid, abalone, *Haliotis rufescens*.

INTRODUCTION

Despite a century of intensive research, fertilization remains one of the least understood fundamental biological processes (Vacquier, 1998). Current theory predicts that membrane-bound proteins promote gamete recognition (Palumbi, 1999; Levitan and Ferrell, 2006). However, soluble egg compounds attracting conspecific sperm could also act as pre-zygotic factors maintaining species integrity while increasing fertilization rates (Jantzen et al., 2001; Riffell et al., 2004; Eisenbach and Giojalas, 2006; Kaupp et al., 2008). Fluid-borne sperm attractants would operate upstream of cell-surface proteins prior to gamete contact. Although cell-surface recognition between male and female gametes is critical for sexual reproduction, the roles of soluble egg factors remain largely undescribed. Gamete passage through a fluid medium is a general phenomenon occurring for broadcast spawners as well as for internal fertilizers. Principles of sperm navigation and egg signaling, therefore, may generalize across such distantly related taxa as marine invertebrates and humans (Spehr et al., 2003).

In nature, sperm must locate and bind to the egg surface within a fluid environment. Dissolved signal molecules that cause sperm to orient and accelerate towards an egg could increase gamete encounter rates. Species-specific attractants might be evolutionarily important barriers to hybridization, promoting reproductive isolation among sympatric species (Harrison et al., 1984; Miller, 1985; Miller, 1997). Despite their importance, few sperm chemoattractants have been chemically identified (Ward et al., 1985; Nishigaki et al., 1996; Olson et al., 2001; Riffell et al., 2002; Yoshida et al., 2002; Böhmer et al., 2005), and none tested for their significance for fertilization success under natural conditions.

Membrane proteins involved in sperm–egg interactions are better characterized for abalone (*Haliotis* spp.) than for any other genus, and demonstrate strong selection for species-specific gamete recognition as a pre-zygotic barrier to hybridization (Swanson and Vacquier, 1995; Swanson and Vacquier, 1998; Swanson and Vacquier, 2002a; Swanson and Vacquier, 2002b). This system thus represents a logical choice for investigating the contribution of soluble egg factors and chemosensory-mediated sperm behavior in fertilization ecology and evolution. We have shown that red abalone (*Haliotis rufescens*) sperm detect waterborne signal molecules from conspecific eggs, and change their swimming behavior to increase the likelihood of successful contact (Riffell et al., 2002). As established by bioassay-guided fractionation of natural egg-conditioned seawater, male gamete attraction is dose-dependent and stereospecific for the L-isomer of tryptophan (L-Trp). Closely related metabolites, including serotonin, tyramine and seven other structural analogs, do not affect sperm motility or orientation towards

eggs (Riffell et al., 2002) (and R.K.Z., unpublished data). Consequently, red abalone sperm require only a single, identified compound for gamete attraction.

Improved understanding of how chemical signals interact with the environment should lead to important insights about the natural selective forces driving fertilization. Given that eggs release fluid-borne attractants, investigations are needed to focus on principles of chemical production and transport (Zimmer and Butman, 2000). Structures, concentrations and fluxes of attractant molecules must be identified, and rates of advection and diffusion measured to ascertain chemical distributions over time and in space (Karp-Boss et al., 1996; Visser and Jackson, 2004; Xiang et al., 2005). Knowledge of these factors would enable analysis of the constraints imposed by natural physiochemical phenomena on sperm behavioral responses mediating gamete encounters and determining fertilization success.

Fertilization is a complex interaction among the biological and chemical traits of gametes and physical properties of fluid environments. Red abalone sperm navigate most effectively and fertilization success is maximized under experimental conditions simulating the hydrodynamic environment of adults spawning in nature (Riffell and Zimmer, 2007). Still unresolved, however, are aspects of the dynamics of gamete attractant production and release. Here, we (1) determine concentrations of L-Trp, and 18 other free amino acids, from a wide selection of abalone tissues, (2) measure L-Trp release rates and link them to egg fertility, and (3) identify whether the ovum, egg jelly, or both, are source(s) of L-Trp. Establishing vital connections between gamete physiology and chemical signaling, this study also reveals the degree to which L-Trp is sequestered by eggs (relative to other tissues) and, therefore, functions in communication with sperm.

MATERIALS AND METHODS

Tissue preparations

Specimens of *Haliotis rufescens* Swainson were maintained at Scripps Institution of Oceanography (University of California, San Diego, CA, USA) in a flow-through seawater system. Animals were fed *ad libitum* on fresh kelp (*Macrocystis pyrifera*) and used in experiments 1–2 weeks after procurement from The Cultured Abalone (Goleta, CA, USA). Our first objective was to determine whether sperm attractant (tryptophan) was enriched in eggs, relative to other abalone tissues. To compare the composition of the dissolved free amino acid (DFAA) pool across tissue types, the following tissues (1 g wet mass) were dissected from replicate (N=6), freshly sacrificed specimens: foot muscle, stomach lining and gill (ctenidia). Additionally, hemolymph (0.5 ml) was collected from each specimen. Tissues were added to 100 ml artificial seawater [Marine Biology Laboratory (Woods Hole) formula (Cavanaugh, 1964)], made with ultrapure water and filtered to 0.22 μm (FSW), and homogenized with a Tissue Tearor (BioSpec Products Inc., Bartlesville, OK, USA). Male gonadal tissue (0.5 g) and ovaries (1 g) also were dissected from specimens (N=3 of each gender) and homogenized in 5 ml FSW. Homogenates were immediately strained, filtered to 0.22 μm, and kept frozen (at –80°C) prior to analysis. Negative controls, prepared in parallel with FSW only, yielded no detectable background traces of DFAA or biogenic amines in high-performance liquid chromatography runs.

Egg and jelly coat analyses

Abalone eggs are surrounded by a jelly coat that potentially could serve as a reservoir for sperm attractants. Compositional analysis was therefore performed on (1) freshly spawned eggs with the surrounding jelly coat intact, (2) eggs from which the jelly coat had been removed, and (3) the egg jelly itself. Female red abalone (N=11) were spawned using methods described previously (Riffell et al., 2002; Riffell et al., 2004). Eggs were collected within 1 min of spawning and concentrated over 52 μm Nitex mesh into a suspension of 10^5 eggs ml^{-1}, and subsampled for compositional analysis (this section) and for release rate experiments (see below). For analysis of eggs + jelly coat, each batch of eggs (0.5 g wet mass of concentrated egg slurry; N=4 replicates) was immediately homogenized in 1 ml FSW. The egg jelly was removed from a different lot of eggs (1.0 g; N=4 reps) by incubation in 15 ml of FSW adjusted to pH 5.0 for 15 min; this treatment solubilized the jelly coat while leaving the eggs intact (Glabe and Vacquier, 1977). A portion of the jelly slurry solution (10 ml) was then removed and filtered. De-jellied eggs were washed over Nitex mesh with 15 ml FSW, four times, to remove any residual jelly, and then homogenized in 1 ml FSW. All solutions were frozen (at –80°C) for later analysis. Jelly slurries were concentrated tenfold by centrifugation under vacuum prior to compositional analysis. Total protein was estimated for egg homogenates by comparison with previously published studies using a BCA colorimetric assay, run according to manufacturer's recommendations (Bio-Rad Corp., Hercules, CA, USA). The protein assay was standardized with bovine serum albumin (BSA), and absorbance of BSA-reacted samples and standards was monitored at 562 nm with a microplate reader.

High-performance liquid chromatography analysis of amino acid composition

High-performance liquid chromatography (HPLC) was used to quantify dissolved free amino acids in homogenates of abalone tissues, and from concentrated, egg-conditioned seawater. The method of Onoue et al. (Onoue et al., 1983) was modified to generate fluorescent derivatives of amino acids, prior to separation by reversed-phase chromatography. Samples were treated with *o*-phthalaldehyde (OPA), which reacts with primary amines to generate fluorescent derivatives (Roth, 1971). 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 sample, or a standard mixture of amino acids, was dissolved in HPLC-grade water mixed with the OPA reagent in a 4:1 ratio, by a robotic arm designed for automated, pre-column derivatization (Beckman Coulter Model 508; Fullerton, CA, USA). The reaction proceeded for 1 min prior to column injection.

Fluorescent derivatives were separated by reversed-phase HPLC on an Ultrasphere ODS column (4.6 mm×25 cm, 5 μm particle size; Beckman Coulter), eluting at 1 ml min^{-1}. Before each run, the column was equilibrated in Solvent A for 15 min. One minute after injection, a linear gradient from 0 to 20% of 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; both solvent mixtures were pH 6.8). Fluorescent derivatives were detected after elution from the column with a fluorometer (Jasco Instruments, Easton, MD, USA), monitoring at 453 nm with an excitation wavelength of 332 nm. Measurements were sensitive and reproducible to picomole levels.

Commercial standards (Sigma-Aldrich, St Louis, MO, USA) were used to calibrate retention times, and response factors for integrating peak areas, in Gold Nouveau software (Beckman Coulter). Samples were analyzed with separate HPLC runs for standard amino acids

plus taurine, and biogenic amines. One standard included 18 of the 20 coding amino acids (all but cysteine and proline) plus taurine, a sulfonic amino acid that is an osmolyte in the tissues of many invertebrates. A second set of standards for biogenic amines included dopamine, L-DOPA, tyramine and serotonin. Standards were run at four concentrations: 2.5×10^{-9}, 5×10^{-10}, 1×10^{-10} and 2.5×10^{-11} moles of each analyte per injection. Replicate standard injections were done at the beginning, middle and end of sample runs (i.e. three replicates per concentration level) to control for changes in retention times over the course of a run. Two calibrations were done to maximize accuracy. Calibrations with standards ranging from 5×10^{-10} to 2.5×10^{-11} moles accurately measured analytes present at $<5\times10^{-10}$ moles per injection, but over-estimated more abundant compounds; calibrations using all four standard concentrations were therefore only used to measure compounds present at $>5\times10^{-10}$ moles.

All samples were run at two concentrations because taurine was up to 20 times more abundant than the next most common amino acid, and thus was quantified separately. Prior to derivatization and injection, an aliquot was diluted either 1:10 for quantifying the 18 coding amino acids or biogenic amines, or 1:100 for measuring taurine. The average of two replicate injections for each sample at each concentration was used as the value for that replicate tissue preparation. Co-injection with standards confirmed the identity of critical peaks.

For statistical analyses, the DFAA total was determined for each tissue by summing the 18 coding amino acids. The proportion of Trp in each replicate sample of each tissue was then determined, and the means and standard deviations calculated. A one-way ANOVA was used to test the null hypothesis of no difference in the percentage of Trp in the DFAA pool among abalone tissues. A *post-hoc* Dunnett's *t*-test was then applied to compare the proportion of Trp in egg homogenate against all other tissues.

Taurine and tyramine were quantified for each tissue but were not initially included in total DFAAs used to compute the percentage composition of Trp. Subsequently, tyramine was included in the DFAA total and the proportion of tyramine was computed for each tissue. A one-way ANOVA tested the null hypothesis of no difference in the proportion of tyramine across tissues. A *post-hoc* Dunnett's *t*-test compared the proportion of tyramine in ovarian tissue against all other tissues. We followed the same procedures for taurine, including an ANOVA test but no *post-hoc* comparisons.

Release rate of sperm attractant

To quantify release rates for the abalone sperm attractant, secretion and accumulation of tryptophan from eggs into seawater were measured at ecologically relevant time scales. Female abalone were spawned and eggs concentrated as described above to yield a slurry of 10^5 eggs ml^{-1}. Eggs were immediately transferred to experimental vessels, sterile glass Ehrlenmeyer flasks containing FSW that were held in a water bath at 15°C (a typical field temperature for subtidal abalone populations in southern California) for the duration of the experiment. Based on pilot studies, 3×10^5 eggs (~3 g) were incubated in 25 ml FSW, a sufficient concentration for HPLC measurements of released tryptophan. Five replicate vessels were used for each of the following time points: 1, 15, 30, 45, 60 and 120 min. These intervals were chosen based on preliminary results indicating abalone eggs only remain fertile for an hour after spawning. Egg-conditioned seawater (ESW) samples were immediately filtered at the end of the incubation time and frozen at –80°C prior to HPLC analysis.

For tryptophan measurements, the majority of each ESW sample was desalted and concentrated prior to analysis using Sep-Pak reversed-phase C-18 cartridges. Sep-Paks were initially washed with HPLC-grade methanol (10 ml) to wet the resin, then equilibrated with ultra-pure water (15 ml). Each ESW sample was loaded onto a column by gravity feed, and the column washed with ultrapure water (15 ml) to desalt. Tryptophan was then eluted with 30% methanol (10 ml), which was concentrated under vacuum to 100 µl for HPLC analysis. Replicate standards of tryptophan (2.5 nmol l^{-1} and 0.25 nmol l^{-1}; N=3 per concentration) were added to FSW and concentrated on Sep-Pak cartridges in parallel for calibrating HPLC runs of ESW. This procedure controlled for any Trp loss during the concentration steps.

For the entire dataset, release of Trp over time was modeled by polynomial regression, using a second-order model. Release over the first 45 min (the period of egg fertility) was separately modeled using linear regression. Based on inspection of residual plots, a log-transformation was applied to the response variable (Trp concentration, expressed as fmol egg^{-1}) to improve homogeneity of variances (Quinn and Keough, 2002).

Attenuation of sperm attractant signal

Based on results of the preceding experiment, we investigated quenching of the tryptophan signal produced by freshly spawned eggs at later time points (see *Egg fertility and sperm attractant signal* in the Results section). Three alternative hypotheses were tested: Trp initially accumulates around eggs but is gradually eliminated by (1) oxidation; (2) uptake by bacteria in seawater; or (3) a Trp-degrading factor released by eggs, in a negative feedback loop. To test the first two hypotheses, three sets of standard solutions (N=4 replicates each) were made of 1 µmol l^{-1} Trp in seawater (25 ml). In the first treatment, Trp solutions were placed in conical tubes and floated in 15°C seawater for 3 h. To test for rapid oxidation, controls were prepared in parallel but immediately frozen. In the second treatment, solutions were prepared and incubated for 3 h using seawater to which the following antibiotics had been added: streptomycin sulfate (150 mg l^{-1}), gentamycin (100 mg l^{-1}), and penicillin G (150 mg l^{-1}). A decline in Trp concentrations in the seawater-only incubation, but not in the antibiotic-seawater treatment, would suggest microbial uptake. A decrease in both 3 h incubations, relative to controls, would support rapid oxidation of Trp (a highly reduced molecule) within hours of spawning.

To test the third hypothesis, freshly spawned eggs were concentrated as before, added to replicate containers (N=4) of seawater at 15°C, and incubated for 60 min to produce ESW. This treatment presumably would contain any soluble egg factor that degrades Trp. An aliquot (1 ml) of each ESW preparation was filtered to 0.22 µm, and a Trp standard added to yield a final concentration of 1 µmol l^{-1} Trp in filtered ESW. The control was Trp standard added to FSW only (N=4 reps). Samples were incubated at 15°C for 2 h, then frozen prior to analysis.

Egg fertility and sperm attractant signal

Bioassays established the relationship between egg fertility and tryptophan concentration (in solution) as a function of time. These tests were conducted simultaneously with experiments on tryptophan release and accumulation. Here, 100 freshly spawned eggs from a single female were placed into each of 52 (13 treatments × four replicates) sterile Petri dishes (32 mm diameter) containing FSW. Sperm from a single male were added (10^5 cells ml^{-1}, final concentration) to four replicate dishes at each of 13 time treatments (1, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120 min post-spawn). All dishes were incubated for 3 h (at 15°C) after sperm were added, eggs were fixed in 5% formalin,

and percentage fertilized (cleaved) were counted under a compound microscope (×100; Olympus Model IX70). Because both male and female gametes were the same age when combined, a separate control isolated an egg from a sperm effect. In parallel with the main experiment, four additional dishes contained 120-min-old sperm, from the original male, added to a new batch of fresh eggs from a second female (1 min post-spawn). All other methods were identical. If percentage fertilization in the control is equal to that at 1 min in the main experiment, sperm fertility would be eliminated as a causative factor, and any change over time in reproductive success attributable only to eggs. This entire experiment was repeated four times, using gametes from different males and females in each run.

RESULTS

Amino acid composition of abalone tissues

Tryptophan was the least abundant of 18 coding amino acids quantified in all red abalone tissues except eggs (Table 1). As a proportion of the DFAA pool, Trp concentrations were significantly elevated in eggs relative to all other body tissues (Fig. 1, and results of a one-way ANOVA: $F_{7,23}$=64.75, P<0.0001; *post-hoc* Dunnett's *t*-tests comparing egg against each tissue, P<0.001). By percentage composition, Trp was threefold more abundant in eggs than in ovarian tissue, and five to seven times more concentrated in eggs than in hemolymph, muscle and gill tissue. Tryptophan was a negligible percentage of the DFAA pool in testes and stomach. In eggs, Trp and methionine (Met) were equally abundant, but in other tissues Met was up to fivefold more concentrated. Similarly, Trp was twice as abundant as asparagine (Asn) in eggs, but in other tissues Asn was up to sixfold more abundant. The mean concentration of tryptophan was ~0.8 pmol egg^{-1}, whereas total soluble protein was estimated at 125 ng egg^{-1}.

Arginine, alanine, glycine and glutamic acid were the four most prevalent coding amino acids in all tissues except eggs, which had very low levels of alanine and were enriched in aspartic acid and serine relative to all other tissues (Fig. 2). In eggs, no single amino acid was particularly common, with arginine, aspartic acid and glutamic acid each constituting 14% of the DFAA pool, followed by serine and glycine at 9% apiece. The most abundant amino acid differed between tissues (Fig. 2). Glycine was 35.4±5.4% (±s.e.m.) of the DFAAs in testes, but only about 10% of DFAAs in other tissues. Arginine represented almost half the DFAA pool in muscle (44.4±8.5%) and was elevated in hemolymph (29.4±3.7%), but constituted less than 20% of the DFAAs in all other tissues. Glutamic acid was the most abundant amino acid in ovarian tissue (25.1±0.1%), nearly twice that found in eggs (13.7±0.8%). Taurine

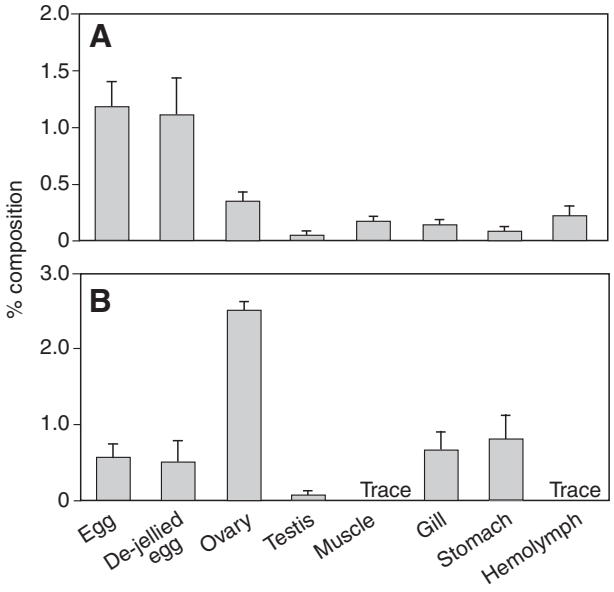

Fig. 1. Percentage composition (means ± 1 s.e.m.) of dissolved free (A) tryptophan and (B) tyramine in tissues of the red abalone, *Haliotis rufescens*.

Table 1. Concentrations of dissolved free amino acids (DFAAs) in tissues of the red abalone, *Haliotis rufescens*

	Concentration (mmol kg^{-1} wet mass tissue)							
Compound	Egg	De-jellied egg	Ovary	Testis	Muscle	Gill	Stomach	Hemolymph
Glutamic acid	1.33±0.83	1.57±0.50	9.33±0.88	7.08±2.25	4.99±1.23	3.00±0.51	5.47±0.24	0.14±0.03
Arginine	1.31±0.69	1.66±0.53	4.23±0.88	6.63±2.37	35.61±8.81	3.50±0.57	5.22±0.47	0.44±0.15
Aspartic acid	1.29±0.72	1.41±0.42	2.11±0.27	3.55±1.40	0.69±0.12	1.30±0.24	1.78±0.15	0.06±0.02
Serine	0.91±0.45	0.90±0.41	2.17±0.21	3.17±1.31	1.59±0.36	0.49±0.09	0.07±0.41	0.07±0.02
Glycine	0.89±0.40	0.99±0.43	4.29±0.46	23.03±4.59	9.91±2.99	1.94±0.36	2.79±0.28	0.16±0.08
Alanine	0.66±0.33	0.50±0.08	5.64±0.53	7.85±2.49	5.44±1.26	3.21±0.56	4.44±0.17	0.21±0.10
Threonine	0.48±0.25	0.42±0.17	1.52±0.15	2.73±1.04	1.78±0.89	0.36±0.06	0.40±0.11	0.04±0.02
Valine	0.35±0.16	0.41±0.19	1.98±0.23	2.66±1.16	0.85±0.17	0.34±0.07	0.81±0.07	0.03±0.01
Histidine	0.33±0.17	0.32±0.15	0.40±0.04	0.81±0.04	0.85±0.20	0.19±0.03	0.29±0.05	0.05±0.02
Isoleucine	0.31±0.14	0.32±0.17	1.17±0.14	1.60±0.67	0.48±0.05	0.18±0.04	0.47±0.07	0.02±0.01
Leucine	0.30±0.16	0.31±0.09	1.13±0.14	1.58±0.55	1.40±1.22	0.20±0.05	0.75±0.39	0.03±0.01
Tyrosine	0.29±0.15	0.30±0.08	0.77±0.07	1.36±0.48	1.26±1.01	0.20±0.04	0.77±0.07	0.02±0.01
Glutamine	0.25±0.07	0.26±0.07	0.71±0.06	1.30±1.14	4.58±1.38	0.17±0.02	0.42±0.16	0.12±0.07
Phenylalanine	0.24±0.08	0.24±0.08	0.43±0.04	0.66±0.21	0.77±0.16	0.07±0.12	0.57±0.33	0.03±0.01
Lysine	0.19±0.16	0.21±0.17	0.56±0.07	2.56±1.43	4.60±1.58	0.90±0.16	1.02±0.29	0.06±0.04
Methionine	0.17±0.17	0.16±0.09	0.22±0.02	0.15±0.03	0.42±0.09	0.09±0.01	0.03±0.03	0.03±0.01
Tryptophan	0.16±0.04	0.16±0.05	0.15±0.01	0.03±0.02	0.15±0.03	0.03±0.00	0.02±0.01	0.00±0.00
Asparagine	0.08±0.03	0.07±0.04	0.32±0.04	0.19±0.17	0.20±0.03	0.11±0.01	0.05±0.03	0.01±0.01
DFAA (total)	9.6±3.9	10.1±4.1	37.1±3.6	67.0±20.3	75.5±16.0	16.3±2.8	25.7±0.9	1.5±0.6

Values are means ± 1 s.d.
De-jellied eggs were pre-treated to remove the surrounding jelly layer prior to homogenization.
DFAA, dissolved free amino acids: DFAA (total) = sum of 18 coding amino acids (excluding taurine and tyramine).

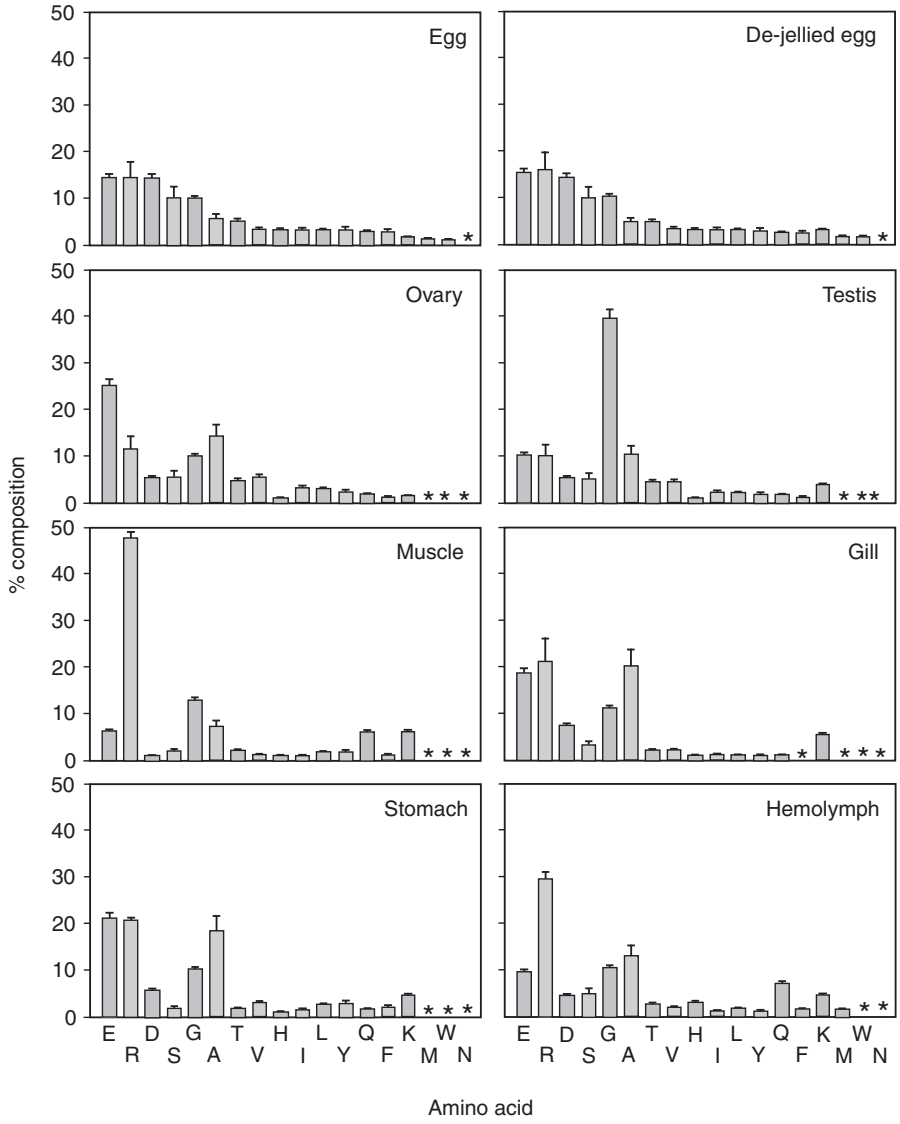

Fig. 2. Percentage composition (means ± 1 s.e.m.) of 18 dissolved free amino acids (excluding taurine and tyramine) in tissues of the red abalone, *Haliotis rufescens*. One letter codes for amino acids: E, glutamic acid; R, arginine; D, aspartic acid; S, serine; G, glycine; A, alanine; T, threonine; V, valine; H, histidine; I, isoleucine; L, leucine; Y, tyrosine; Q, glutamine; F, phenylalanine; K, lysine; M, methionine; W, tryptophan; N, asparagine. Asterisks (*) denote contributions of <1%.

was more abundant in each tissue than the total pool of 18 coding amino acids (Table 2, Fig. 3). Out of all tissues studied, the ratio of taurine to the 18 coding amino acids was greater than 5:1 for eggs and gills and about 4:1 for all other tissues, except testis.

The biogenic amine tyramine was detected in eggs, ovary, gill and stomach tissue (Table 2, Fig. 1). When included in the total DFAA pool, tyramine made up a significantly greater proportion in ovaries, compared with all other tissues (Fig. 1, and results of a one-way ANOVA: $F_{7,23}$=236.71, P<0.0001; *post-hoc* Dunnett's *t*-tests comparing ovary against each tissue, P<0.001). Ovaries and eggs contained a trace of DOPA, but no serotonin was detected. Conversely, muscle tissue contained serotonin and a trace of DOPA, but no detectable tyramine.

There was no difference in the concentration of any DFAA between eggs with an intact jelly layer, *versus* eggs from which jelly was experimentally removed (Table 1, Fig. 2). The small amount of Trp detected in the jelly slurry did not exceed the amount that would be released by eggs over 15 min, the period of incubation for jelly removal (see below). Thus, the jelly layer surrounding fresh eggs did not serve as a reservoir of Trp, or any other amino acid.

Egg fertility and sperm attractant signal

Natural rates of Trp release from eggs were quantified and linked with fertility. Concentration surrounding eggs increased linearly over the first 45 min post-spawn (Fig. 4A, and linear regression of time on log-concentration Trp: $F_{1,18}$=36.77, R^2=0.67, P<0.0001). The rate of release was ~0.2 fmol egg^{-1} min^{-1} during this period. After 45 min, however, Trp dwindled rapidly from solution. Release and accumulation kinetics, therefore, were best modeled overall by a quadratic function, because of the decay of Trp in the second hour (polynomial regression of time on log-transformed Trp concentration: $F_{2,25}$=20.76, R^2=0.62, P<0.0001). Closely paralleling these events, fertilization success peaked during an initial 30 min period (post-spawn), but decreased to nil over the next 50 min (Fig. 4B). Whereas egg fertility diminished over time, sperm fertility did not. Thus, older eggs stopped releasing Trp as they became infertile.

Attenuation of sperm attractant signal

Potential causes were explored for this decay in the Trp signal. No reduction was found in the concentration of a Trp standard after 3 h in filtered seawater alone (1.04±0.05 μmol l^{-1}; mean ± s.e.m.) or in

Table 2. Concentrations of tyramine and taurine in tissues of the red abalone, *Haliotis rufescens*

Tissue	Concentration (mmol kg^{-1} wet mass tissue)	
	Tyramine	Taurine
Egg	0.050±0.025	50.21±23.65
De-jellied egg	0.045±0.040	52.78±12.45
Ovary	1.015±0.110	138.98±18.07
Testis	0.025±0.007	78.75±22.62
Muscle	Trace*	227.06±46.65
Gill	0.108±0.019	80.30±15.62
Stomach	0.194±0.086	101.60±3.07
Hemolymph	Trace*	5.45±1.24

Values are means ± 1 s.d.
*Trace: <0.001 mmol kg^{-1} (wet mass tissue).
De-jellied eggs were pre-treated to remove the surrounding jelly layer prior to homogenization.

FSW + antibiotics (1.08±0.04 μmol l^{-1}), compared with un-incubated controls (1.02±0.04 μmol l^{-1}). Similarly, there was no change in Trp following a 2 h incubation with 60-min ESW, compared with unconditioned seawater controls (unpaired two-tailed *t*-test: d.f.=6, t=0.99, P=0.36). Hence, Trp was neither oxidized nor degraded naturally in seawater during experiments, nor did eggs release soluble factors that digested Trp.

DISCUSSION

Sperm attractant production and release

The dissolved free amino acid L-tryptophan is a sperm attractant in the red abalone *Haliotis rufescens*, increasing fertilization in still water and shear flows (Riffell et al., 2002; Riffell and Zimmer, 2007). Here, we quantified levels of this ecologically important signaling molecule in various abalone tissues, and in freshly spawned eggs. As a percentage of the total DFAA pool, Trp was substantially more concentrated in eggs than in any other tissue. Tryptophan levels were even higher in eggs than in ovarian tissue. Thus, red abalone selectively concentrate a sperm-attracting amino acid in their ova. A higher initial Trp concentration would intensify the odorant plume released by an egg, and thereby, create a stronger signal for sperm in surrounding seawater (Karp-Boss et al., 1996; Visser and Jackson, 2004).

When male gametes of red abalone approach a conspecific egg, they swim faster and navigate directly towards the egg surface (Riffell et al., 2002; Riffell and Zimmer, 2007). Sperm tracks around single conspecific eggs were computer imaged in the presence or absence of the enzyme tryptophanase, which selectively digests free tryptophan (Riffell et al., 2004). In the enzyme treatment, sperm did not orient towards fertile eggs; yet, male and female gametes otherwise were not adversely affected. The overall impacts of eliminating tryptophan were to extinguish sperm chemotaxis, reduce gamete encounter rates, and therefore, lower fertilization success. In the present study, older eggs stopped producing Trp at the point when their fertility dropped below 50%, a key link between gamete physiology and chemical signaling. This observed covariation between signal production and egg fertility was consistent with the ecological role of Trp in promoting fertilization. From combined results, egg fertility does not depend on the presence of Trp in free solution, but Trp release rate is highly dependent on egg age, and hence, fertility (Riffell et al., 2002; Riffell et al., 2004) (and present study).

Several mechanisms might explain the apparent negative feedback loop quenching the sperm signal after 1–2 h. Tryptophan is typically one of the least abundant amino acids in seawater, probably because the aromatic ring system is readily oxidized (Fuhrman and Ferguson, 1986; Coffin, 1989; Keil and Kirchman, 1991; Suttle et al., 1991). We tested whether rapid oxidation caused the observed decline in Trp from ESW by spiking FSW with micromolar levels of a Trp standard. After several hours of incubation, there was no decrease in Trp concentration. Consequently, oxidation is not the cause of signal decay in ESW preparations. Our seawater was filtered to 0.22 μm which removes most bacteria, and no difference was found in Trp levels after incubation with or without antibiotics. It is therefore unlikely that Trp was taken up by suspended microbes. Feasibly, eggs could have secreted a Trp-metabolizing enzyme when they ceased to be fertile. This negative feedback loop could be adaptive if it prevented sperm from being 'distracted' by infertile eggs, decreasing contact rates with longer-lived eggs produced by a given female. When seawater previously conditioned by eggs was spiked with a Trp standard, no digestion of the attractant signal occurred, ruling out a secreted egg factor. We thus hypothesize that microbes on the surface of abalone eggs scavenge amino acids from the surrounding seawater. Amino acid efflux would greatly exceed microbial uptake, but only while eggs are fertile. Although speculative, there are precedents for this phenomenon in other systems (Decho et al., 1998; Hidalgo-Grass et al., 2004).

Among free-spawning invertebrates, gamete signaling has been especially well studied in deuterostomes (echinoderms and ascidians) (Ward et al., 1985; Yoshida et al., 2002; Yoshida et al., 2003; Kaupp et al., 2003). Sea urchin and sea star eggs, for example, release short peptides that bind receptors on the sperm flagellum, activating sperm and altering patterns of motility in a concentration-dependent manner (Ward and Kopf, 1993; Nishigaki and Darszon, 2000; Nishigaki et al., 1996; Böhmer et al., 2005). The cascade of

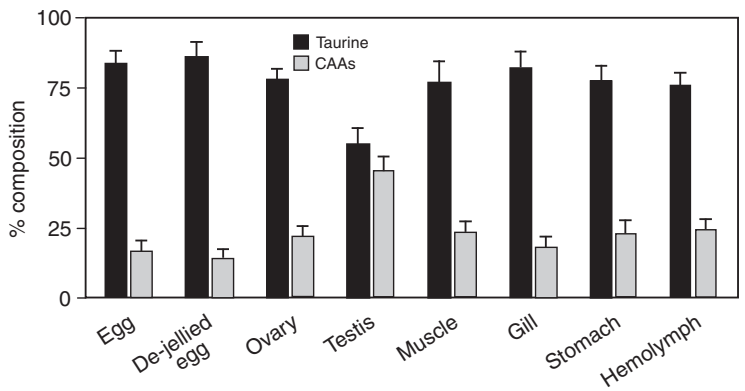

Fig. 3. Percentage composition (means ± 1 s.d.) of dissolved free taurine and 18 coding amino acids (CAAs) in tissues of the red abalone, *Haliotis rufescens*. See text for details.

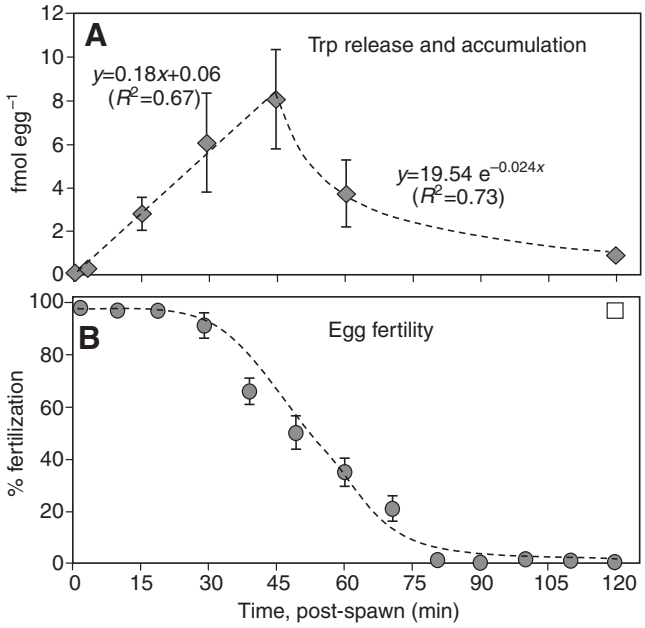

Fig. 4. (A) The accumulation and attenuation of sperm attractant (tryptophan) in solution over time, after release from individual eggs of red abalone, *Halitois rufescens*; values are means ± 1 s.e.m. Tryptophan was released at a constant rate during the first 45 min of each experimental period, and then decayed exponentially until trials were terminated after 120 min. (B) A logistic regression line describing the relationship between time after spawning and percentage of eggs fertilized; values are means ± 1 s.e.m. After the first 30 min, fertilization success was reduced to nil over the next 50 min. No significant difference was found between eggs fertilized within the first 30 min, and eggs freshly spawned at the end of the experiment and mixed with 120-min-old sperm (white square; one-way ANOVA with *post-hoc* Dunnett's *t*-tests: $P>0.99$). Thus, egg fertility, but not sperm fertility, decreased over time during this experiment. Some error bars are smaller than symbol sizes.

internal signal transduction events includes activation of guanylyl cyclase and production of cGMP, followed by an influx of calcium ions that triggers asymmetric flagellar beating (Matsumoto et al., 2003; Neill and Vacquier, 2004; Kaupp et al., 2006; Hirohashi et al., 2008). To date, only a few peptidic egg factors have been shown to induce chemotaxis, and the mechanism by which ionic fluxes lead to directional sperm navigation remains unclear (Ward et al., 1985; Nishigaki et al., 1996; Kaupp et al., 2003; Böhmer et al., 2005; Kaupp et al., 2008). Comparative studies with abalone could reveal if homologous transduction pathways regulate sperm motility and egg recognition among protostomes.

Amino acid profiles of tissues

The profile of soluble amines in ovaries did not mirror that in spawned eggs. Tyramine, for example, was more abundant in ovaries than in any other tissue type. Thus, different biologically active amines can be selectively enriched in various abalone tissues. Red abalone ovary contained ~140 mg tyramine kg^{-1} wet mass, a much higher level than reported for ovaries of the scallop *Pecten maximus* (~10 mg kg^{-1}). Yields from testes were approximately the same for red abalone (25 mg kg^{-1} tissue) and scallop (28 mg kg^{-1} tissue) (Mackie et al., 1997). Future studies should examine whether molecules biosynthetically related to aromatic amino acids are concentrated in eggs of other *Haliotis* species, and if so, whether they function as species-specific sperm signals.

The proportional composition of red abalone eggs differed somewhat from that reported for the blacklip abalone *Haliotis rubra*; levels of Glu and Asp were lower in eggs of *H. rubra*, compared with *H. rufescens* (King et al., 1996; Litaay et al., 2001). Tryptophan was not quantified in *H. rubra* eggs. Total concentrations of DFAAs and soluble protein were ~1.5–2.0 times higher in eggs of *H. rufescens* than *H. rubra*. Per-egg content of protein (~125 ng) and taurine (~500 pmoles), however, showed excellent agreement between the present and a previous study on *H. rufescens* (Wellborn and Manahan, 1995).

The high levels of Arg, Ala and Gly measured in abalone foot are characteristic of muscle from a variety of mollusks (Carr et al., 1996). The same four amino acids (Arg, Gly, Glu and Ala) were proportionally the most abundant in *H. rufescens* and *H. diversicolor* muscle (Chiou et al., 2001). In both species, Arg represented the majority of the DFAA pool in muscle but not in viscera. Taurine is also present in abalone muscle at exceedingly high levels, where it acts as a substrate for a dehydrogenase enzyme that recycles NADH during hypoxia (Gäde, 1988). The high concentration of Arg in *H. rufescens* hemolymph is in agreement with similarly high levels in hemolymph of the sea hare *Aplysia californica.* In both species, the top three DFAAs were Arg, Ala and Gly in that order (Derby et al., 2007). Major patterns of DFAA profiles for select tissues are therefore shared by red abalone and other marine gastropods.

Why tryptophan?

Identifying signal molecules is essential to linking behavior, chemosensory physiology and ecological function. Studies are needed to distinguish unique natural products from more common metabolites that serve a variety of roles. One strategy for producing chemical signals is to biosynthesize a novel substrate for each required cue, as with anthopleurine, a sea anemone alarm pheromone (Howe and Sheikh, 1975), or adrenaline, the mammalian 'fight-or-flight' hormone (Baulieu and Kelly, 1990). Alternatively, ordinary molecules can have extraordinary effects. Cellular mechanisms of chemosensory reception are highly converged (Ache, 1994; Hildebrand and Shepherd, 1997; Bargmann, 2006). The same molecules, therefore, may function in chemical communication by phylogenetically diverse species and among different cell types. Histamine, for example, initiates local immune responses (Jutel et al., 2006; Sugata et al., 2007), regulates receptor-mediated physiological processes in the gut (Filippova and Nozdrachev, 2007), functions as a neurotransmitter (Jones, 2005; Stuart et al., 2007), and acts as an environmental cue of habitat suitability for invertebrate larvae (Swanson et al., 2004; Swanson et al., 2006).

Similarly, the ubiquitous amino acid L-Trp has multiple and varied functions in diverse tissues and organisms. It is an essential amino acid required for protein formation (Yanofsky, 2003; Ladner et al., 2007), acts as a sperm attractant for red abalone (Riffell et al., 2002; Riffell et al., 2004), and serves as a precursor for the biosynthesis of many biogenic signals including plant hormones and brain neuromodulators (Schaechter and Wurtman, 1990; Sarwar and Frankenberger, 1994). One tryptophan metabolite, serotonin, plays an especially critical role in nervous system development, including the apical sense organ (Byrne et al., 2006; Croll, 2006). Larvae of red abalone and other marine taxa use this sense organ to select a suitable site for irreversible metamorphosis into the juvenile form (Hadfield et al., 2000); a critical period in marine life histories (Palmer and Strathmann, 1981; Young, 1990).

Given the importance of serotonin during the larval stage, elevated levels of Trp in red abalone eggs may reflect a redirection

of resources to biosynthetic pathways involved in nervous system functions. If high Trp concentrations are correlated with enhanced serotonin production, neural function, and settlement success, Trp release could be an honest indicator of egg fitness. Likewise, if the intensity of this signal predicts egg quality, sexual selection may favor sperm that respond to Trp. Alternatively, the paucity of Trp in natural seawater may have selected for enrichment of this common metabolite in eggs, producing a sperm cue with no appreciable 'background noise' under ambient conditions. Future work should test whether chemoattractants reflect selection for sperm that recognize fitness cues from potential eggs, or physico-chemical constraints on effective signaling in the fluid medium through which gametes communicate.

Once intractable to manipulation, remote communication between sperm and eggs is now experimentally accessible using abalone as a model system. Recent results have shown species specificity in sperm chemoattraction among congeneric abalone, presenting novel opportunities for comparative study (Riffell et al., 2004). Further research should reveal if Trp and related metabolites function as different dialects of an evolutionarily conserved language that communicate gamete location and fitness, as well as species identity.

This paper is dedicated to the memory of Mia J. Tegner, a leading researcher and tireless advocate for the conservation of endangered abalone populations. We thank Paul K. Dayton and Michael I. Latz for graciously providing laboratory space and facilities, and Cheryl Ann Zimmer for her help in every aspect of this project. This research was supported by awards from the National Science Foundation (IOS 08-20645), NOAA California Sea Grant College Program (R/CZ-197), and the UCLA Council on Research.

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