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KEYSTONEin: A glycoprotein cue drives predation on mussels and structures rocky intertidal communities

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ABSTRACT: Foundation species provide critical resources to ecological community members and are major determinants of biodiversity. One such species, the California mussel *Mytilus californianus*, is a superior competitor and dominates space on rocky, wave-swept shores (northeast Pacific Ocean). Live mussels secrete a 29.6 kDa glycoprotein (named 'KEYSTONEin'), with expression localized in epidermis, extrapallial fluid, and periostracum (organic shell coating). Hence, KEYSTONEin is available for contact recognition as predators crawl on rocky surfaces. Purple sea stars *Pisaster ochraceus*, whelks (*Nucella emarginata* and *Acanthinucella spirata*), and lined-shore crabs *Pachygrapsus crassipes* are common intertidal carnivores. Spanning 3 phyla and differing substantially in sensory mechanisms and hunting techniques, all are major mussel consumers. Here, we constructed faux prey to mimic both physical and chemical properties of mussels. Purified KEYSTONEin was presented at a typical mussel concentration. All 4 predatory species attacked, and ate, KEYSTONEin-infused faux prey as opposed to the organic enrichment or seawater controls. Sea stars, whelks (both species), and crabs also did not distinguish between KEYSTONEin-laced mimics and their live, intact counterparts. Mechanisms of chemical recognition thus have converged across phylogenetically diverse taxa to promote the exploitation of a valuable, shared, prey resource. By providing critical sensory information, KEYSTONEin drives mussel predation and initiates trophic cascades that shape community structure and function. KEYSTONEin is necessary and sufficient as a predatory cue of considerable ecological consequence.

KEY WORDS: Chemical ecology · Chemical cue · Keystone predation · Foundation species · Mussel · Crab · Whelk · Sea star

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INTRODUCTION

Wave-swept shores are invaluable for developing and testing key ecological principles (Connell 1961, 1970, Dayton 1971, Paine 1974, Menge 2000, Trussell et al. 2002, Navarrete & Berlow 2006). Over the last 2 decades, a profusion of new molecular, biochemical, and biophysical approaches have linked physiological performances to population- and community-wide consequences (Dahlhoff et al. 2001, Somero 2002, 2012, Helmuth et al. 2005). Emerging paradigms recognize the impacts of even seemingly subtle changes in environmental features (such as food, temperature, or oxygen) on species distributions and abundances, as well as on individual growth, reproduction, and fitness (Sanford 1999, Dahlhoff et al. 2001, Tomanek & Helmuth 2002, Helmuth et al. 2002, Menge et al. 2003, Gilman et al. 2006, Sokolova 2013, Kordas et al. 2015).

A synthesis of research on intertidal organisms is nonetheless missing a critical component — the sensory basis for behavioral interactions that determine population- and community-wide attributes. Predator–prey relationships, in particular, can structure rocky

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intertidal communities (Connell 1961, 1970, Murdoch 1969, Paine 1974, Navarrete 1996, Menge 2000, Trussell et al. 2006, Navarrete & Manzur 2008). Almost 50 yr ago, the keystone species concept was proposed and applied to interactions among sea star predators and mussel prey in rocky intertidal habitats (Paine 1969). Until recently, the chemical cues for such species interactions and community-wide impacts were unknown.

Mussels (*Mytilus californianus*, *M. galloprovincialis*, and *M. edulis*) produce and secrete glycoprotein orthologues (named KEYSTONEin) which act, singularly, to cue sea star predatory responses (Fig. 1) (R. K. Zimmer et al. unpubl.). These orthologues are expressed uniquely in the epidermis, extrapallial fluid, and periostracum (organic shell coating) of live, intact animals. Thus, they are available for contact by the chemosensory receptors of sea stars (and other predators) crawling on rocky substrates.

A large conserved, globular domain places KEYSTONEin within the Complement Component 1 Domain Containing (C1qDC) protein family. C1q proteins are part of the classical complement pathway and are critical especially to the innate immune systems of invertebrates (Gestal et al. 2010, Gerdol et al. 2011). For mussels, these compounds are also secreted by the mantle epidermis and serve in shell formation through Ca^{2+} binding and subsequent calcium carbonate deposition (Hattan et al. 2001, Yin et al. 2005). A coiled coil motif promotes the assembly of oligomeric complexes and functions in protein–protein associations as required in formation of the shell organic matrix (Yin et al. 2005, Gerdol et al. 2011).

Collective evidence supports the hypothesis that KEYSTONEin evolved ancestrally in immunological roles, and later in biomineralization roles (Yin et al. 2005, Gao et al. 2015, R. K. Zimmer et al. unpubl.). Presumably, the molecule is subjected to purifying selection, in order to maintain its original function and binding specificity. More recently, this compound has been exploited by sea stars as an honest and predictable contact chemical cue in prey recognition. Here, we tested the generality of KEYSTONEin effects and broadened current understanding on established mechanisms of prey recognition.

Experiments were performed using a phylogenetically diverse (from 3 phyla) suite of common intertidal predators (the purple sea star *Pisaster ochraceus*; 2 whelks, viz. *Acanthinucella spirata* and *Nucella emarginata*; and the lined-shore crab *Pachygrapsus crassipes*). These species differ substantially in sensory mechanisms and hunting techniques. Sea stars and crabs explore the substrate with contact

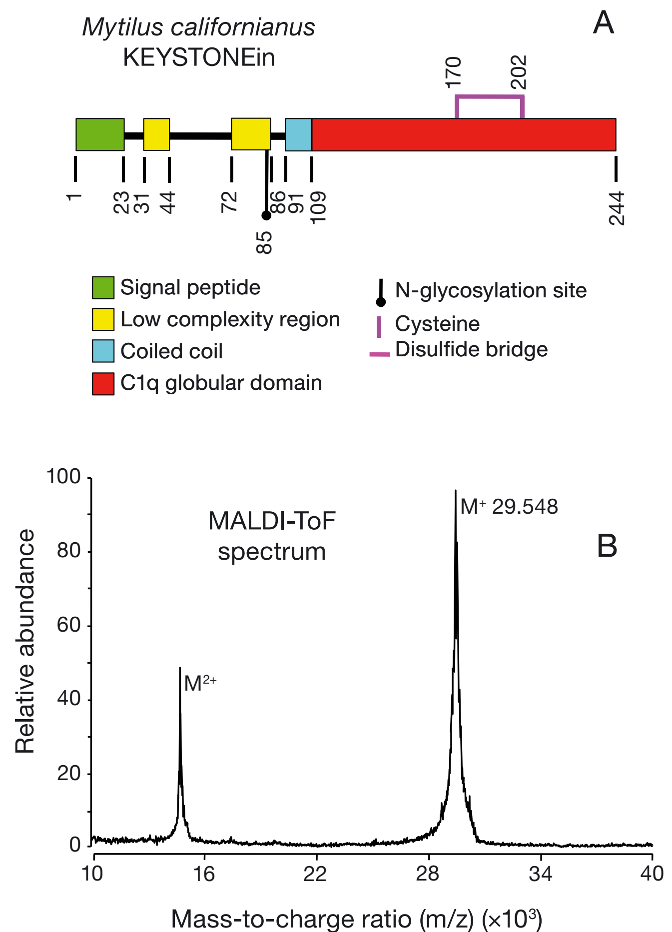

Fig. 1. (A) Structural architecture and domain organization of *Mytilus californianus* KEYSTONEin (R. K. Zimmer et al. unpubl.), a 29.6 kDa glycoprotein with 244 amino acids (1 = amino terminus; 244 = carboxy terminus). A signal peptide and conserved motifs (low-complexity regions, coiled coil, and globular domain) are denoted by color-specific rectangles. The amino acid position of a single *N*-glycosylation site is indicated by a closed circle. Vertical lines (pink) with numbers denote cysteine positions. A disulfide bridge is marked with a horizontal line (pink) that connects the 2 cysteine residues and produces a single 3-dimensional fold towards the carboxy terminus. (B) MALDI-ToF mass spectrum, showing abundant peaks for the singly- and doubly-charged KEYSTONEin ions corresponding to a molecular mass of 29.6 kDa in the intact, fully purified, molecule

chemoreceptors located on tube feet or on walking legs and chelae, respectively (Case 1964, Schmidt 1989, McClintock et al. 1994). In contrast, whelks have receptors distributed diffusely across the foot and concentrated on a retractable proboscis that probes substrate surfaces (Crisp 1971, Hodgson & Brown 1985). Sea stars feed by first protruding their lower stomach into a mussel; whelks penetrate the shell using a drill-like radula; crabs catch, crush, and manipulate a mussel using legs and clawed ap-

pendages. As a consequence of genetic divergence between protostomes (arthropods, mollusks) and deuterostomes (echinoderms) 670 million yr ago (Ayala et al. 1998), molecular processes of chemosensory recognition differ considerably between these 2 groups (Ache & Young 2005, Bargmann 2006, Cummins et al. 2009, Derby et al. 2016). For example, crabs and snails, but not sea stars, express and use ionotropic chemoreceptors. Here, we show that sensory mechanisms have converged across these phylogenetically diverse taxa for glycoprotein detection to promote exploitation of a valuable, shared, prey resource.

MATERIALS AND METHODS

Isolation and purification of KEYSTONEin for use in bioassays

Chemical fractionation and isolation of KEYSTONEin were based on established procedures. Elsewhere, we reported on the translations of complete amino acid and nucleotide sequences for this bioactive protein and its encoding gene, respectively (GenBank accession no. KC152469) (R. K. Zimmer et al. unpubl.). Mussel extrapallial fluid is a major source of KEYSTONEin, secreted by the mantle epidermis before incorporation into tissues along the lateral shell margin. Initial purification steps involved separating extrapallial fluid components on the basis of solubility through stepwise ammonium sulfate precipitation. The fluids (0.1–1.0 ml per mussel) were removed by catheter, without rupturing or piercing mantle tissue. Bioactive compounds in these fluids were separated from other organic molecules starting with 25%, increasing to 50%, 75%, and ending with 100% saturation. Ammonium sulfate was added to the solution and stirred at 4°C for 10 min, then centrifuged at 40 000 × g (4°C for 15 min), before the supernatant was removed. KEYSTONEin was retained as a component in precipitate of the 50 to 75% saturation solution (R. K. Zimmer et al. unpubl.). This material (>80% protein by mass, hereafter called the extrapallial fluid bioactive precipitate [EFBP]) was washed, resuspended in 0.8 ml of 50 mmol l^{-1} tris-hydroxymethylaminomethane-HCl (Tris buffer) at pH 7.5, and dialyzed (Slide-A-Lyzer© dialysis cassettes, 5000 Da molecular weight cut-off [MWCO]; Thermo Fisher Scientific) against, and diluted with, 0.45 µm filtered seawater, as needed.

Additional purification of KEYSTONEin involved protein separation and isolation through preparative SDS-PAGE. The resuspended precipitate was conditioned with 5× Laemmli buffer. Prepared samples (1 ml) were then loaded onto 7.5% acrylamide (acrylamide:bis = 29:1) slab gels (16 × 22 cm). They were run at 180 V, until molecular weight markers had clearly separated. All protein bands were visualized with imidazole-zinc reverse staining. From each gel, the KEYSTONEin band was excised with a sterile scalpel, de-stained in Laemmli buffer, and recovered from the acrylamide via electroelution. Gel fragments, containing KEYSTONEin (a 29.6 kDa protein), were put into Spectra/Por regenerated cellulose dialysis tubing (1 kDa MWCO, 8 ml volume; Spectrum Medical), sealed, and placed between the electrodes of a Western blotting rig. Both tubing and rig were filled with Laemmli buffer, stirred, and cooled on ice. An electrical current (100 V) was applied for 1 h. Isolated KEYSTONEin migrated out of each fragment and into the buffer. Following electroelution, fragments were removed and stained with Coomassie G-250 dye to check protein recovery (which was ~65%). The buffers were exchanged, through dialysis, to 50 mmol l^{-1} Tris-HCl (pH 7.5). Purified KEYSTONEin was concentrated 10-fold through ultrafiltration (MWCO = 10 kDa) and dialyzed (Slide-A-Lyzer© dialysis cassettes, 5000 Da MWCO) against, and diluted with, 0.45 µm filtered seawater, as needed.

Faux prey

Faux prey were fabricated for use in laboratory experiments. Live California mussels *Mytilus californianus* were collected at field sites and transported to the lab for immediate processing. All soft tissues, along with pallial and extrapallial fluids, were removed with sterile instruments. The empty shells were combusted for 12 h at 130°C, bathed for 15 min in a stirred, 5% (v/v) HCl solution, and scrubbed 7 to 10 times with 100 ml aliquots of Nanopure-grade deionized water to eliminate any residual organics. Cleaned, empty shells served as physical replicas of live animals ('faux prey').

Prey chemistry was simulated in flavored gels. Sodium carboxymethylcellulose (11.7% w/v) was mixed with seawater or a prescribed chemical solution and allowed to cure for 10 min at 4°C. Once prepared, each gel had the consistency of mussel flesh and was injected into a cleaned, empty mussel shell for immediate use in an experiment. Held together using a rubber band, shell valves were filled with gel protruding 1 mm beyond the shell margin to simulate

the mantle in a live mussel. The low temperature protected proteins and prevented denaturization. Protein levels were established using the Bradford method with bovine serum albumin (BSA) as a standard (Bradford 1976). Concentrations of proteins (w/w) were carefully matched between live prey extrapallial fluids and gels containing organic inclusions. The purity and concentration of KEYSTONEin were confirmed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF, Voyager DE-STR, Applied Biosystems; Fig. 1B).

Experiments testing KEYSTONEin as a potential predatory cue

Animal maintenance and holding procedures

Laboratory tests took place during May to October (2012 and 2013) when predatory sea stars, whelks, and lined-shore crabs fed actively on rocky, wave-swept shores. Animals were collected at field sites (Broad Beach, Malibu, California, USA; 34° 02′ 04″ N, 118° 51′ 42″ W), held in the lab, and fasted for 3 to 5 d before testing. The lab set-up consisted of a 28 000 l seawater reservoir (oceanic quality, 33 psu salinity) with particle filtration (5 µm cut-off), UV sterilization (as needed), and computer-controlled water/air temperature (±1°C of set-points) and light cycle (12:12 h light:dark, light on at 06:30 h). Each species was held separately at a density of 12 to 15 m^{-2} (whelks), 8 to 10 m^{-2} (crabs), or 3 to 4 m^{-2} (sea stars) in tanks (240 cm × 60 cm × 20 cm, 280 l capacity), with continuously recirculating (45 l min^{-1}) seawater, sand-filtered, aerated, and cooled to site temperatures (18–21°C). Seawater was replaced twice per week.

General experimental procedures

All experiments were performed in Plexiglas chambers (see 'Bioassays' below for more details). These tanks were 60 cm (length) × 60 cm (width) × 15 cm (depth) for sea stars and crabs, or 10 cm × 10 cm × 5 cm for whelks. A constant supply of single-pass, 5 µm filtered seawater (1 l min^{-1}) was delivered to every chamber, held at ambient ocean temperature (18–21°C) and salinity (33 psu). Each tank was washed thoroughly with 1% v/v HCl in Nanopure water after every trial, and then rinsed with Nanopure water and seawater to eliminate any residual organics. All trials were performed between 10:00 and 18:00 h. An individual animal was tested once only in a given experiment before return to the field. During experiments and animal holding periods, lighting (full spectrum, General Electric Daylight Ultra) was maintained at a level (75 µmol m^{-2} s^{-1}) simulating field habitat during late afternoon.

Bioassays

Two experiments were conducted for each predatory species. Expt 1 tested for KEYSTONEin bioactivity. Each trial began when a single sea star (7–12 cm from proximal tip of longest arm to center of oral disc), whelk (both species, 1.5–2.5 cm shell length), or crab (3.0–4.0 cm carapace width) was placed at the center of a chamber and given a choice between 3 faux prey. The treatments consisted of BSA (organic enrichment) control, seawater, and purified KEYSTONEin. Each prey type was assigned using a random number table to 1 of 4 positions, 10 cm (sea stars and crabs) or 3 cm (whelks) away from a corner. Every trial ran for 1 h, or until a single faux prey was chosen and eaten. Faux prey sizes of 4.0–5.0, 1.5–2.5, or 1.2–2.0 cm shell length, respectively, were those preferred by sea stars, whelks, or crabs when feeding on live mussels in the field or lab (G. A. Ferrier & R. K. Zimmer unpubl. data). A minimum of 14 replicate trials was performed for each species.

Expt 2 established the level of bioactivity in KEYSTONEin relative to a live mussel. In this case, 1 live and 3 faux mussels were tested in each trial (chamber). The 3 faux mussels were fabricated from purified KEYSTONEin, seawater, or the whole extrapallial fluid bioactive precipitate. All other bioassay procedures were identical to those described for Expt 1. A minimum of 24 trials was run for each predatory species. Equivalent activity was indicated by predators feeding equally as often on all prey types, but rejecting seawater.

RESULTS AND DISCUSSION

Experimental findings for sea stars, both whelk species, and crabs were consistent and in close agreement. Purified KEYSTONEin was highly effective as a contact cue in prey recognition, selection, and feeding by each predatory species, as compared to an organic enrichment (BSA) control (*G*-test for homogeneity with Williams' and Bonferroni's corrections: $G^2 \geq 9.09$, df = 1, $p < 0.0026$) and a seawater control ($G^2 \geq 9.09$, df = 1, $p < 0.0026$; Fig. 2). This single compound, by itself, was equal in bioactivity to

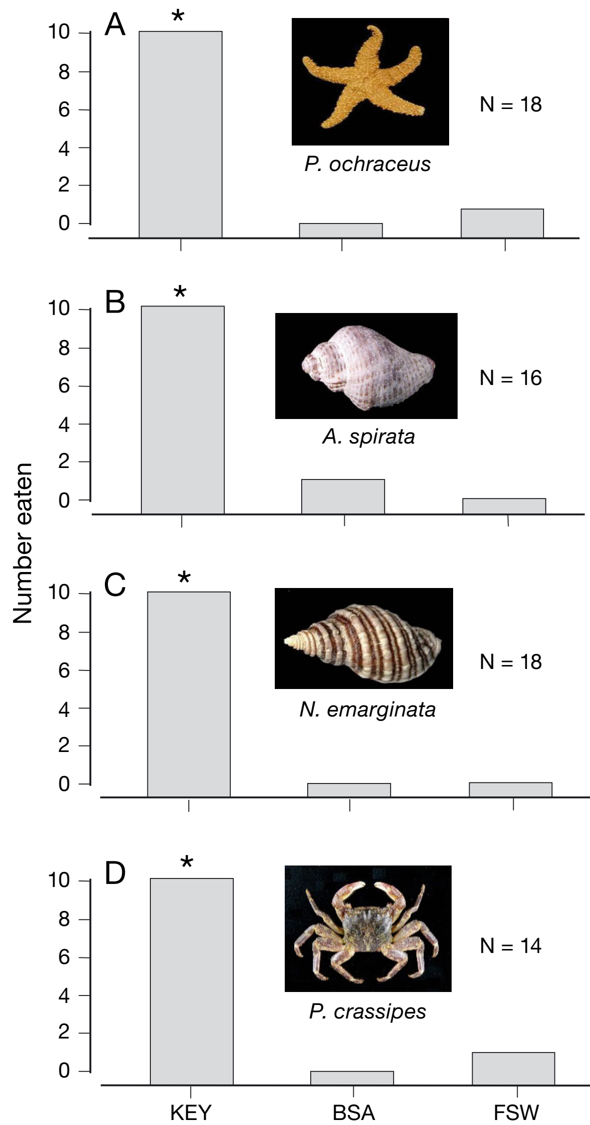

Fig. 2. Expt 1 results: feeding preferences of sea stars *Pisaster ochraceus*, whelks (*Acanthinucella spirata* and *Nucella emarginata*), and lined-shore crabs *Pachygrapsus crassipes* for faux prey. KEY: purified KEYSTONEin-laced faux prey; BSA: bovine serum albumin (same concentration as KEYSTONEin); FSW: 0.45 µm-filtered seawater. The *y*-axis shows the number of trials in which a predator fed on a given treatment or control faux prey. N: number of replicate trials in a particular experiment. * denotes a significant response relative to FSW ($p < 0.0026$)

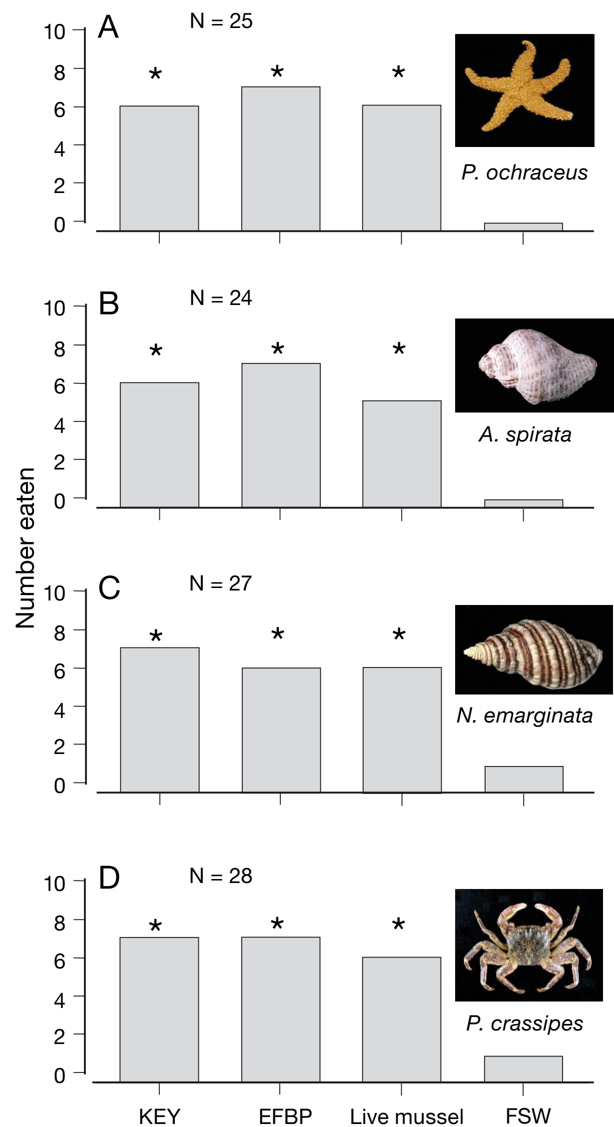

Fig. 3. Expt 2 results: feeding preferences of predators (as in Fig. 2) for faux prey relative to live, intact mussels. KEY: purified KEYSTONEin-laced faux prey; EFBP: extrapallial fluid bioactive precipitate; FSW: 0.45 µm-filtered seawater. Protein concentration in each faux prey treatment was scaled to the level in extrapallial fluids of live mussels. The *y*-axis shows the number of trials in which a predator fed on a given prey type. N: number of replicate trials in a particular experiment. * denotes a significant response relative to FSW (control) ($G^2 \geq 3.89$, df = 1, $p < 0.048$)

the whole EFBP, and to an intact, live mussel (*G*-test for homogeneity with Williams' and Bonferroni's corrections: $G^2 \leq 0.10$, df = 2, $p > 0.752$). When presented at the same concentration (= 67 µg g^{-1} gel) as in mussel extrapallial fluid, sea stars, whelks (both species), and crabs did not discriminate between KEYSTONEin-impregnated faux prey and their live, intact counterparts (Fig. 3). Here, we used extrapallial fluid because of the ease in extraction and purification processes. Comparatively, KEYSTONEin concentrations (w/w) in extrapallial fluid are 2- to 10-times lower than those in periostracum and mantle epidermal tissue. KEYSTONEin, therefore, is necessary and sufficient as a predatory cue of considerable ecological consequence. Mechanisms of chemical recognition are converged across phylogenetically

diverse taxa for use in exploiting a valuable, shared, prey resource.

Requisite for many basic biological functions, glycoproteins are well suited for sensory exploitation. A prime example is the targeting of host-expressed mucin and collagen by pathogenic bacteria for attachment and colony establishment (Roos & Jonsson 2002). Like mussels, the barnacles *Amphibalanus amphitrite* and *Balanus glandula* incorporate glycoproteins into the integument as part of the cuticle, shell, and immunological defense (Wu et al. 2012, Ferrier et al. 2016). Some of these molecules (named SIPC and MULTIFUNCin, in these 2 barnacles, respectively) are exploited as contact-chemical cues by con- and heterospecific organisms alike. MULTIFUNCin and SIPC, for example, induce habitat colonization (settlement) by conspecific larvae (Dreanno et al. 2006, Clare 2011, Zimmer et al. 2016). In addition, MULTIFUNCin acts as a potent feeding stimulant to major barnacle predators, including whelks (*Acanthinucella spirata* and *Nucella* spp.) and sea stars *Pisaster ochraceus* (Zimmer et al. 2016, Ferrier et al. 2016, G. A. Ferrier & R. K. Zimmer unpubl. data). Chemical mediation of such antagonistic interactions likely is not limited to barnacles and their predators. Surface glycoproteins are distributed widely, among diverse species, in every major biome on earth (Gagneux & Varki 1999, Mengerink & Vacquier 2001, Snell 2011).

An extreme environment demands extreme physiological and behavioral innovations, especially in rocky intertidal habitats. When shores are flooded, crashing waves and high shear stresses dictate mechanical limits to organism size (Denny et al. 1985). Intense turbulent mixing rapidly dilutes dissolved chemical concentrations and creates extreme variation in the spatial and temporal distributions of waterborne cues (Weissburg & Zimmer-Faust 1993, Zimmer & Butman 2000, Crimaldi et al. 2002). Under such demanding conditions, submerged predators are unable to navigate successfully via metabolite plumes in finding prey (Jackson et al. 2007, Robinson et al. 2011, Ferrier et al. 2016). Biotic interactions on wave-swept shores thus depend as much — or more — on surface chemistry as opposed to alternative types of sensory stimuli (Feder 1964, Lubbock 1980, Dreanno et al. 2006, Zimmer et al. 2016). The information encoded in contact chemical cues can be detected reliably by predators, even while turbulence degrades odor plumes, disrupts acoustical and vibrational signals through scattering effects, and occludes visual fields due to high concentrations of air bubbles and/or suspended particulate matter. In general, a contact chemical cue becomes critical for resource recognition, selection, and acquisition when extreme fluid-dynamic constraints dominate habitats and impede the transmission of most alternative sensory information.

Mussels are foundation species and are superior competitors for space (Paine 1966, Dayton 1971, Suchanek 1992). Besides supplying food for consumers, these animals live in high-density beds that create significant topography which, in turn, act as shelters for diverse assemblages of invertebrate species. Providing critical chemosensory information, KEYSTONEin cues predation on mussels and significantly impacts community structure and function. Mussel predation not only facilitates habitat colonization by sub-dominant competitors, but it changes the dynamics within spatial refugia, and thereby exerts strong direct and indirect effects on species distributions (Paine 1974, Wootton 1993, Saier 2001, Harley 2011). These changes lead to new 'winners' and 'losers' in a sweepstakes among subordinate or dependent species, thus initiating a cascade of community-wide impacts that determine biodiversity (Petraitis & Dudgeon 1999, Harley 2011).

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