



Chemosensory Exploitation and Predator-Prey Arms Races

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Thousands of armed predatory species, distributed widely across the metazoan tree-of-life, consume only hard-shell or exoskeleton-bearing organisms (called “durophagy”). Prey armor clearly has evolved in response to selection by predators, but there is little evidence of the contrary, counter-adaptation by predators. Evolved consumer responses to prey, in general, might be more readily expressed in ways other than morphological traits, including *via* sensory cues. Here, we explored the chemosensory basis for durophagy in a model predator-prey system, and identified intimate associations between durophagous predators and their shelled prey. Barnacles (*Balanus glandula* and *Semibalanus cariosus*) bear hard shells and secrete, respectively, a 199 or 201 kDa glycoprotein ortholog (named “MULTIFUNCin”), with expression limited to the body armor (epidermis, cuticle, and live shell). To test for effects of MULTIFUNCin on predators, we constructed faux prey to mimic meaningful physical and chemical characteristics of live barnacles. In separate experiments, each consumer species was presented MULTIFUNCin, purified from either *B. glandula* or *S. cariosus*, at a typical armor concentration. All six predatory species (sea star, *Pisaster ochraceus*; whelks, *Acanthinucella spirata*, *Nucella emarginata*, *N. ostrina*, *N. canaliculata*, and *N. lamellosa*) attacked and ate MULTIFUNCin-infused faux prey significantly more than controls. Akin to barnacles, secretion of glycoprotein-rich extracellular matrices is common among armored prey species—from marine sponges to terrestrial vertebrates. Our results, therefore, suggest that chemosensory exploitation of glycoproteins could be widespread, with notable consequences for life on land and in the sea.

Keywords: chemical cue, feeding stimulant, durophagy, sensory ecology, predation

INTRODUCTION

A myriad of metazoan species have evolved armor in morphological defenses. The scales of fishes and reptiles, cuticles and carapaces of insects and crustaceans, as well as skeletons and shells of corals, mollusks, brachiopods, echinoderms, and sponges, all protect against the “slings and arrows” of a dangerous world (Kruppert et al., 2020). There is an intuitive appeal to viewing struggles among

species as arms races between natural enemies (Brodie and Brodie, 1999). The evolution of armed predators may have led to the evolution of mineralized armor in prey since near the beginning of the Cambrian (Vermeij, 1989; Marshall, 2006; Wood and Zhuravlev, 2012).

The arms race analogy has been applied to a wide array of natural enemies, even though the degree of reciprocity and details of selection vary among antagonistic encounters. Predator-prey interactions, especially, may be characterized by asymmetrical selection that precludes arms races (selection on prey is thought to be stronger than on predators) (Dawkins and Krebs, 1979; Brodie and Brodie, 1999; Whitenack and Herbert, 2015). Elaborated jaws, and other weapons, increase the ability of consumers to capture prey. Such innovations also evolve in response to selection pressures imposed by higher-order predators and by competitors for food, space, and mates (Vermeij, 1994, 2013; Bicknell and Paterson, 2018; Aristide and Morlon, 2019). Enhanced capacity to capture prey can be an unintended consequence of escalation in a particular anti-predator, or anti-competitor, morphological or behavioral trait.

Prey armor clearly has evolved in response to selection from predators, but there is little evidence of the contrary, counter-adaptation by predators (Harvell, 1984; Vermeij, 1994, 2013; Kosloski and Allmon, 2015; Klompmaker et al., 2017). Evolved predator responses to prey might be more readily expressed in ways other than morphological traits, including *via* sensory cues. Essentially every living organism has a chemical sense to exploit valuable resources and ward off danger. Prey flee, hide, or elaborate armor in response to infochemicals emitted by durophagous consumers (Zimmer et al., 2006; Poulin et al., 2018; Weiss et al., 2018). Morphologically defended prey also release alarm substances when attacked. These compounds cue escape or defense in other, threatened individuals (Ono et al., 2003; Jacobson and Stabell, 2004; Smeets and Weissburg, 2006). Surprisingly, little is known about the reciprocal interaction: do durophagous predators exploit molecules associated with armored prey? If so, are these cues species-, or taxon-specific, and are they expressed uniquely in prey body armor?

Hard-shelled barnacles are superior competitors for space within the higher-reaches of wave-swept shores. As foundation species, they provide resources to community members and act as determinants of biodiversity (Trussel et al., 2003; Maggi et al., 2015). Durophagy by sea stars (*Pisaster ochraceus*) and whelks (*Acanthinucella spirata*, *Nucella emarginata*, *N. ostrina*, *N. canaliculata*, and *N. lamellosa*) shapes barnacle (*Balanus glandula* and *Semibalanus cariosus*) populations, thereby impacting intertidal communities along the Pacific Ocean coast of North America (from the Aleutian Islands to central Baja, **Table 1**; Connell, 1961, 1970; Murdoch, 1969; Dayton, 1971; Navarrete, 1996). Sea stars explore the substrate with contact chemoreceptors located on tube feet (McClintock et al., 1994). In contrast, whelks have contact chemoreceptors distributed diffusely across the foot and concentrated on a retractable proboscis that probes substrate surfaces (Crisp, 1971; Hodgson and Brown, 1985). Whereas sea stars feed by first protruding their lower stomach into a barnacle, whelks penetrate the shell using a drill-like radula.

TABLE 1 | Tidal heights and geographical ranges of study species along the North America Pacific Ocean coast.

Species	Tidal height	Geographical range
Predators		
<i>Acanthinucella spirata</i>	Mid to high	Bodega Bay, CA to San Quintin, Baja, MX
<i>Nucella emarginata</i>	Mid to high	Half Moon Bay, CA to Punta Eugenia, Baja, MX
<i>Nucella ostrina</i>	Mid to high	Yakutat, AK to Point Conception, CA
<i>Nucella lamellosa</i>	Low to mid	Aleutian Islands, AK to Santa Cruz, CA
<i>Nucella canaliculata</i>	Low to mid	Aleutian Islands, AK to Piedras Blancas, CA
<i>Pisaster ochraceus seignis</i>	Low to mid	Point Sal, CA to Ensenada, Baja, MX
Prey		
<i>Balanus glandula</i>	Mid to high	Aleutian Islands, AK to San Quintin, Baja, MX
<i>Semibalanus cariosus</i>	Low to mid	Aleutian Islands, AK to Morro Bay, CA
<i>Mytilus californianus</i>	Low to mid	Aleutian Islands, AK to Socorro Is, MX
<i>Tegula funebralis</i>	Mid to high	Vancouver Island, BC to Comondú, Baja, MX

Species used in each set of experiments is shown in **Figure 2**.

Each barnacle species (*B. glandula* and *S. cariosus*) secretes, respectively, a 199 or 201 kDa glycoprotein ortholog (named “MULTIFUNCin,” in recognition of its multifunctional properties), with expression limited to the body armor (epidermis, cuticle, and live shell materials) (Ferrier et al., 2016a; **Figure 1**). To test for effects of MULTIFUNCin on consumers, we constructed faux prey to mimic meaningful physical and chemical properties of live barnacles. In separate experiments, MULTIFUNCin, purified from either *B. glandula* or *S. cariosus*, was presented alone at a typical armor concentration. All six predatory species attacked and ate MULTIFUNCin-infused faux prey significantly more than controls. These results are consistent with a chemosensory basis for durophagy, and suggest that mechanisms for glycoprotein recognition have converged across phylogenetically diverse taxa (sea stars and whelks) to promote the exploitation of a valuable, shared prey resource.

MATERIALS AND METHODS

Isolation and Purification of MULTIFUNCin Proteins

Overview

Chemical fractionation and isolation of MULTIFUNCin followed established procedures (Ferrier et al., 2016a,b). Elsewhere, translations of complete amino acid and nucleotide sequences are described for each molecule and its encoding gene (GenBank accession numbers: KC152471 for *B. glandula* MULTIFUNCin, and KC152472 for *S. cariosus* MULTIFUNCin) (**Figure 1**; Ferrier et al., 2016a). For each barnacle species (*Balanus glandula* and *Semibalanus*

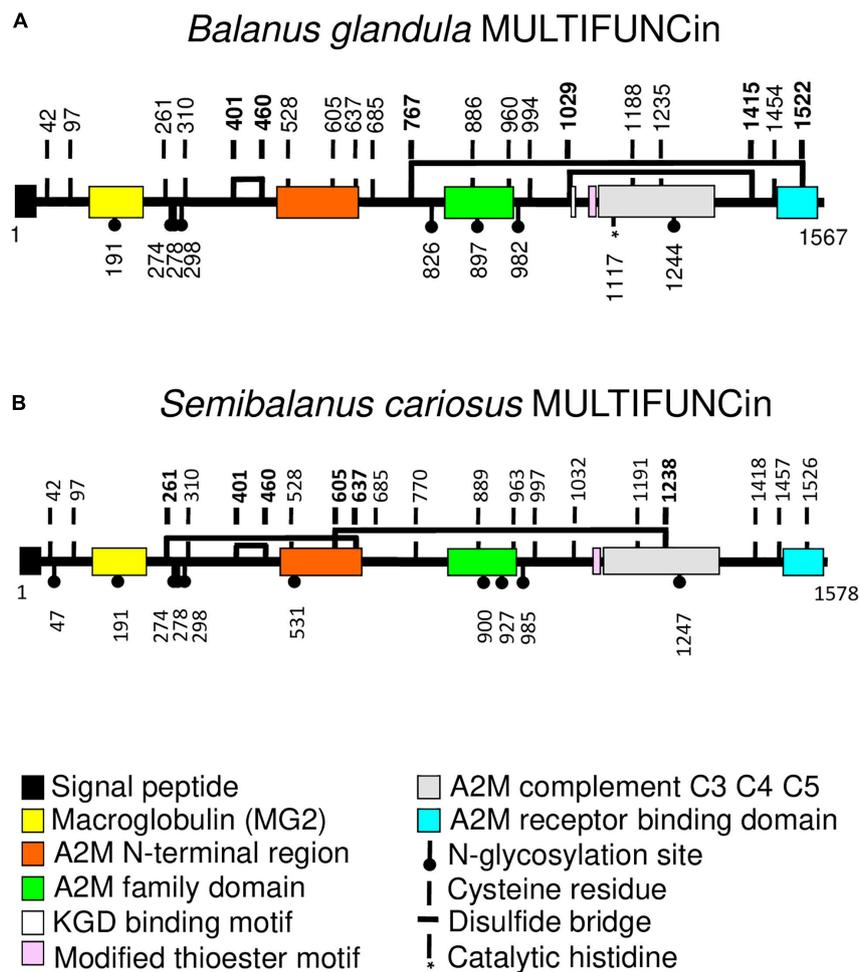


FIGURE 1 | Structural architectures and domain organizations of **(A)** *Balanus glandula* MULTIFUNCIn and **(B)** *Semibalanus cariosus* MULTIFUNCIn. These 199 and 201 kDa glycoprotein orthologs, respectively, are expressed uniquely in the armor (epidermis, cuticle, and new shell material) of each species (Ferrier et al., 2016a). The two compounds and their encoding genes share 82% amino acid and 83% nucleotide sequence homologies. Several, shared, conserved domains place both substances within the α_2 -macroglobulin subgroup of the thioester-containing protein family. A signal peptide, modified thioester motif, and other notable domains and regions are highlighted by color-specific rectangles. Predicted amino acid positions of N-glycosylation sites are provided (closed circles), as well as the site of a putative catalytic histidine (*) in *B. glandula* MULTIFUNCIn. Vertical lines with bold numbers denote cysteine positions. The estimated locations of disulfide bridges are marked with horizontal lines connecting bold cysteine residues. A2M, α_2 -macroglobulin; KGD, lysine-glycine-aspartic acid; C3, C4, and C5, complement component factor proteins 3, 4, and 5. Each MULTIFUNCIn molecule consists of 1,567 or 1,578 amino acid residues (1 = amino terminus; 1,567 or 1,578 = carboxy terminus).

cariosus), MULTIFUNCIn purification steps comprised, in succession, (1) extraction of animal shells and tissues with tris(hydroxymethyl)aminomethane-HCl (hereafter, tris-HCl)-buffered saline, (2) ammonium sulfate ($[(\text{NH}_4)_2\text{SO}_4]$) protein precipitation, (3) size-exclusion chromatography, (4) lentil-lectin (LCA) affinity chromatography, and finally, (5) SDS-PAGE protein separation with electroelution of purified compounds. Purity and concentration of MULTIFUNCIn were confirmed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Voyager DE-STR, Applied Biosystems, Foster City, CA) (Ferrier et al., 2016a).

Tissue Extraction and Protein Precipitation

Live barnacles (0.4–1.0 cm shell height) were collected at field sites, flash frozen in liquid nitrogen, and either transported

directly (*Balanus glandula* from Malibu, CA), or shipped on dry ice *via* overnight courier (*B. glandula* from Cattle Point, WA, and *Semibalanus cariosus* from Bodega, CA), to our UCLA laboratory. Individuals from each collection site were separated, crushed, and homogenized at high speed (using a Waring blender) in a 1:1.5 (v/v) of 50 mmol L⁻¹ tris-HCl (pH 7.5) buffer. Resulting homogenates were stirred on ice for 120 min, rough filtered through gauze (nominal 80 μm pore diameter), and centrifuged at 40,000 $\times g$ (for 30 min at 4°C). Supernatants were filtered to 0.45- μm , and eluates served as “crude extracts.”

Initial purification steps involved separating eluate components on the basis of solubility through stepwise ammonium sulfate precipitation. Bioactive proteins were isolated from other, organic, molecules starting with a 35%, increasing to 70%, and ending with 100% $(\text{NH}_4)_2\text{SO}_4$ saturation.

Ammonium sulfate was added to each barnacle crude extract and stirred at 4°C for 10 min, then centrifuged at 40,000 × g (4°C for 15 min), before the supernatant was removed. MULTIFUNCin was retained as a component in precipitate of the 35–70% saturation solution (Ferrier et al., 2016a). Each pellet was resuspended in 2 mL of 50 mmol L⁻¹ tris-HCl (pH 7.5) and dialyzed [Slide-A-Lyzer™ dialysis cassettes, 5 kDa molecular weight cutoff (MWCO, hereafter); Thermo Fisher Scientific, Waltham, MA] against and diluted with 0.45 μm-filtered seawater, as needed.

Size-Exclusion and Lentil-Lectin Affinity Chromatography of Barnacle Bioactive Precipitate

Resuspended precipitates were fractionated using size-exclusion (SEC) and lentil-lectin (LCA) affinity chromatography. A HiPrep 16/60 high resolution S-200 sephacryl gel size-exclusion column (SEC, GE Healthcare, Waukesha, WI) was eluted at 0.8 mL min⁻¹ with 50 mmol L⁻¹ Tris-HCl (pH 7.5) buffer on a Biologic™ fast performance liquid chromatography (FPLC) system (Bio-Rad, Hercules, CA). MULTIFUNCin was retained in the 150–530 kDa SEC peak (Ferrier et al., 2016a).

Each peak was collected, and divided into two samples based on the presence/absence of glycan groups. A 16/40 column (GE Healthcare) was packed with LCA sepharose 4B beads (GE Healthcare), and equilibrated with a FPLC system running buffer [0.5 mol L⁻¹ NaCl, 25 mmol L⁻¹ tris-HCl (pH 7.5)] at 0.8 mL min⁻¹. Concentrated (1 mg mL⁻¹) 150–530 kDa peak material was washed through the LCA beads with an additional 150 mL of equilibrated buffer. MULTIFUNCin, bound to the LCA beads following the wash, was removed with 150 mL of elution buffer [0.2 mol L⁻¹ methyl α,D-mannopyranoside, 0.5 mol L⁻¹ NaCl, 25 mmol L⁻¹ tris-HCl (at pH 7.5)] increasing linearly from 0 to 100% over 30 s at the same flow rate. It was then concentrated using ultrafiltration (MWCO = 10 kDa).

SDS-PAGE Protein Separation and Electroelution of Purified MULTIFUNCin

Final purification involved protein separation and isolation through preparative SDS-PAGE. Each sample (1 mL), containing MULTIFUNCin, was conditioned with 5X Laemmli buffer and loaded onto a 7.5% acrylamide (acrylamide:bis = 29:1) slab gel (16 × 22 cm). It was run on ice at 180 V, until clear separation of molecular markers. All protein bands were visualized with imidazole-zinc reverse staining. From each gel, the MULTIFUNCin band was excised with a sterile scalpel, destained in Laemmli buffer, and recovered from the acrylamide *via* electroelution. Each gel fragment, containing MULTIFUNCin, was put into a Spectra/Por regenerated cellulose dialysis tubing (1 kDa MWCO, 8 mL volume; Spectrum Medical Corp., Long Beach, CA), 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 electric current (100 V) was applied for 1 h. Isolated MULTIFUNCin migrated out of each fragment and into the buffer. Following electroelution, selected fragments were removed and stained with Coomassie G-250 dye to check protein recovery (which averaged 65%). The

buffers were exchanged, through dialysis, to 50 mmol L⁻¹ tris-HCl (pH 7.5). Purified MULTIFUNCin was concentrated 10-fold through ultrafiltration (MWCO = 10 kDa), dialyzed (Slide-A-Lyzer™ dialysis cassettes, 5,000 Da MWCO) again, and diluted with 0.45 μm-filtered seawater, as required for bioassay. When not used immediately in bioassay, MULTIFUNCin was stored at –80°C in tris-HCl buffer.

Bioassays of MULTIFUNCin as a Contact Cue to Predation

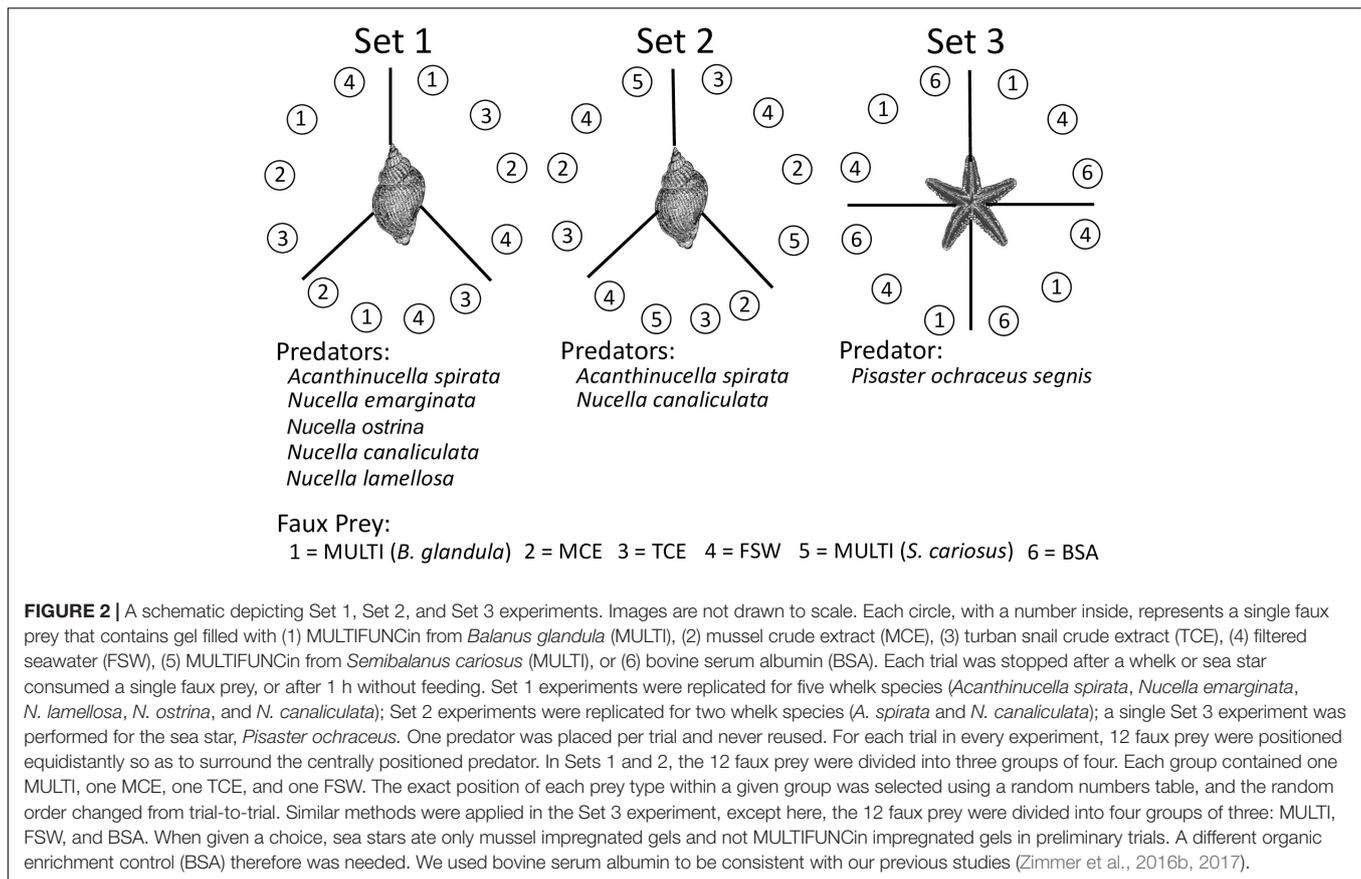
Overview

Experiments tested for MULTIFUNCin effects on durophagy. Six, armed, consumer species were chosen for bioassays, based on their important roles in structuring rocky-intertidal communities (Connell, 1961, 1970; Paine, 1966, 1974; Murdoch, 1969; Navarrete, 1996). Three of these species are more abundant in northern latitudes (*Nucella ostrina*, *N. canaliculata*, and *N. lamellosa*), whereas the other three prevailed in southern latitudes (*Acanthinucella spirata*, *N. emarginata*, and *Pisaster ochraceus segnis*) (Table 1). Each selected consumer species naturally preys on dense patches of barnacles (*Balanus glandula*, *Semibalanus cariosus*), mussels (*Mytilus californianus*), turban snails (*Tegula funebris*), and, less often, other invertebrate taxa (Feder, 1959; Paine, 1966; Murdoch, 1969; Connell, 1970; Dayton, 1971; Navarrete, 1996).

Faux Prey

Faux prey were fabricated for use in laboratory experiments. All soft tissues, along with cuticles, were removed with sterile instruments from barnacles (*Balanus glandula* and *Semibalanus cariosus*, 0.4–0.6 cm shell heights for experiments on whelks; 0.8–1.0 cm shell heights for sea stars). The empty shells were combusted for 12 h at 150°C, bathed for 15 min in a stirred, 5% (v/v) HCl solution (in tris-HCl buffer), and scrubbed thoroughly with Nanopure-grade deionized water to eliminate any residual organics. Cleaned, empty, barnacle shells served as physical replicas (“faux”) of the live animals.

Prey chemistry was simulated in flavored gels. Sodium carboxymethylcellulose (11.7% w/v) was mixed with 0.45-μm filtered seawater and purified MULTIFUNCin (from *B. glandula* or *S. cariosus*), then cured for 10 min at 4°C. Gels also were made using crude extracts of mussels or turban snails (see section “Materials and Methods” in “Tissue extraction” for barnacles), bovine serum albumin (BSA, 4 × 10⁻⁷ molar), or 0.45-μm filtered seawater alone. These alternative extracts and BSA served as organic enrichment controls in bioassays on chemical prey preferences. Once prepared, each gel had the consistency of prey flesh, and a 0.3–0.6 mL aliquot of gel was injected into a cleaned, empty, barnacle shell for immediate use in experiments. Concentrations of MULTIFUNCin (75 μg mL⁻¹, about 4 × 10⁻⁷ molar) were the same across all prepared gels and live barnacle shell materials. Similarly, molar concentrations of BSA and MULTIFUNCin were the same across all prepared gels. Total protein levels in mussel and turban snail crude extracts were established using the Bradford method with bovine serum albumin as a standard (Bradford, 1976).



Relative to MULTIFUNCin gels, total protein concentrations (w/v) were approximately 50 times higher in mussel and turban snail crude extract gels.

Animal Maintenance and Holding Procedures

Bioassays were performed over 2 years during June–September, when sea stars and whelks fed most actively in native habitats. Predators were collected from locations in Northern California (Horseshoe Cove and Mussel Point, Bodega Bay), Southern California (Broad Beach, Malibu), and Washington (Cattle Point, San Juan Island). These consumers were collected and either transported directly (*Acanthinucella spirata*, *Nucella emarginata*, and *Pisaster ochraceus* from Malibu, CA), or shipped on ice via overnight courier (*N. lamellosa* from Cattle Point, and *N. ostrina* and *N. canaliculata* from Bodega) to our laboratory. Consumers were fed an *ad libitum* diet of barnacles, mussels, and turban snails for 1–2 weeks, and then fasted for 3–5 days before testing.

The experimental 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 ($\pm 1^\circ\text{C}$ off set-points) and light cycle (L:D 14:10, light on at 0630 h). Animals from Malibu, Bodega, and Cattle Point were held and tested, respectively, at water temperatures of 19–21, 13–15, or 9–11 $^\circ\text{C}$. During animal holding periods and experiments, lighting (full spectrum, General Electric Daylight Ultra) was maintained at a level (75 μmol

$\text{m}^{-2} \text{s}^{-1}$) simulating field habitats during late afternoon. Each species was held separately at a density of 12–15 m^{-2} (whelks) or 3–4 m^{-2} (sea stars) with continuously recirculating seawater (45 L min^{-1}), replenished twice per week.

General Experimental Procedures

All bioassays were performed in Plexiglas arenas (see section “Results” below for more details). These tanks were 60 cm (length) \times 60 cm (width) \times 15 cm (depth) for sea stars, or 10 cm \times 10 cm \times 5 cm for whelks. A constant supply of single-pass, 5- μm filtered seawater (1 L min^{-1}) was delivered to every arena, held at ambient ocean temperature and salinity (see “Animal maintenance” above). 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.

Chemical Prey Preference Tests

For consistency and comparison of results to previous studies (Ferrier et al., 2016a,b; Zimmer et al., 2016a), we performed each experiment as a preference test (Figure 2). The suite of experiments, conducted here, provided the most meaningful information in the fewest number of trials. Three sets of experiments were performed. In Set 1, we compared the consummatory responses of five whelk species. A trial began

when a single whelk (1.5–3.0 cm shell length) was placed at the center of an arena and presented with 12 faux prey. The 12 faux prey were divided into three groups of four that encircled the whelk. Each group contained one MULTIFUNCin (prepared using *Balanus glandula* from Malibu), one mussel crude extract, one turban snail crude extract, and one filtered seawater treatment. The exact position of each prey type within a given group was selected using a random numbers table. Each trial lasted until the predator selected, attacked and consumed a single faux prey, terminating after 1 h regardless.

Set 2 experiments were performed identically to set 1 with two notable exceptions. Here, (1) trials were limited to two whelk species (*Acanthinucella spirata* and *Nucella canaliculata*), and (2) MULTIFUNCin was purified from *Semibalanus cariosus*. These tests were truncated because of the considerable time and effort needed in purifying large amounts of MULTIFUNCin for meaningful bioassay. Consummatory responses in Set 1 tests were nearly identical across all five whelk species (see section “Results,” below), rendering the re-testing of each whelk species redundant.

Set 3 consisted of a single experiment. In initial trials ($n = 5$), sea stars preyed only on mussel-crude-extract gels, and not on MULTIFUNCin-gels (Binomial test: $P = 0.031$). This result was not surprising, given that sea stars prefer mussels over all other tested prey (Paine, 1966, 1974; Landenberger, 1968; Dayton, 1971). Consequently, we repeated chemical preference tests for sea stars, substituting bovine serum albumin (BSA, an organic enrichment control) for crude extracts, together with filtered seawater and MULTIFUNCin (purified from *Balanus glandula* collected at Malibu) faux prey. The use of BSA was consistent with our previous studies (Zimmer et al., 2016b, 2017). Similar to Sets 1 and 2, a trial began when a single sea star (length: 7–12 cm from distal tip of longest arm to center of oral disc) was placed in the center of an arena and presented with 12 faux prey. The 12 faux prey were divided into four groups of three that encircled the sea star. As in Sets 1 and 2, the exact position of each prey type within a group was determined using a random numbers table. Each trial lasted until the predator selected, attacked and consumed a faux prey, terminating after 1 h regardless.

RESULTS

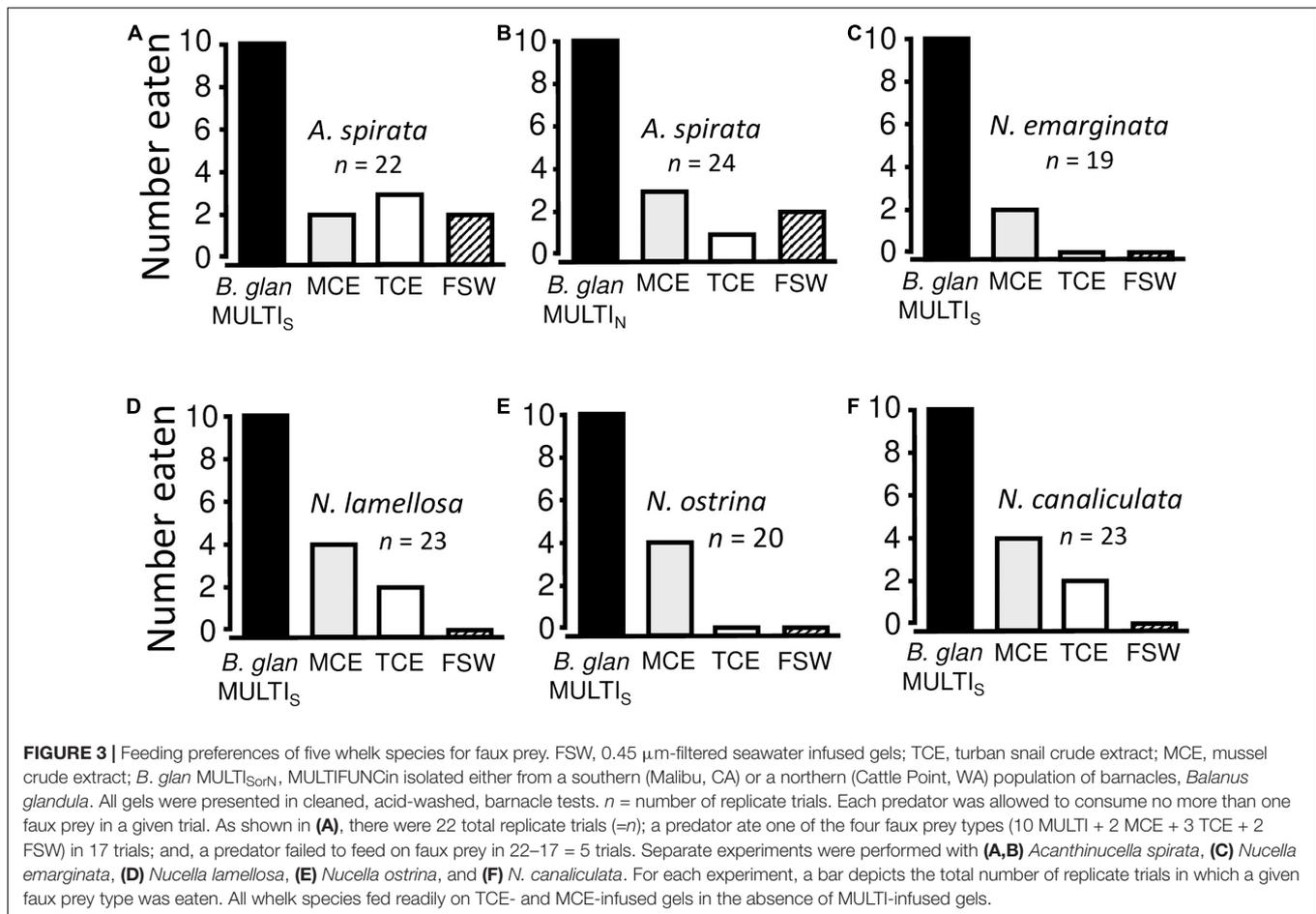
When faux prey were prepared using *Balanus glandula* from Malibu populations, feeding preferences were not significantly different among the five whelk species (Chi-square test for heterogeneity with Williams’ correction for small sample sizes: $\chi^2 = 8.896$, $df = 16$, $P = 0.928$) (Figures 3A,C–F). In performing this analysis, we included a category for null responses, as well as separate categories for positive responses to each of the four experimental treatments (MULTI, MCE, TCE, and FSW). Given no significant difference, data were subsequently pooled across all five species before further analyses. This last procedure greatly increased statistical power in multiple comparisons among the experimental treatments. Combined,

whelk consumers fed significantly more often on faux prey with MULTIFUNCin as opposed to seawater [Sign test with Bonferroni’s correction ($\alpha = 0.05/6 = 0.008$): $P < 0.00001$], turban snail crude extract ($P < 0.00001$), and mussel crude extract ($P < 0.0001$). Each whelk fed only on one faux prey per trial. In addition, whelks attacked and ate significantly more faux prey with mussel crude extract than with seawater ($P = 0.0018$), but not with turban snail crude extract ($P = 0.067$). There was no significant difference in whelk reactions to turban snail crude extract and seawater ($P = 0.193$). Thus, overall, attack and feeding by whelks were paramount in response to the single molecule, MULTIFUNCin. As conservative tests of selectivity for this glycoprotein, MULTIFUNCin concentration ($75 \mu\text{g mL}^{-1}$; about 4×10^{-7} molar) was approximately 50 times lower than total concentrations (w/v) of protein in crude extracts.

A strong genetic cline separates southern (Monterey Bay, southward) from northern (Half Moon Bay through Alaska) populations of *B. glandula* (Wares and Cunningham, 2005). The experiment with *Acanthinucella spirata*, therefore, was repeated using faux prey with purified MULTIFUNCin from barnacles collected at our northernmost locale, Cattle Point, WA. Chemical preferences of *A. spirata* were independent of the MULTIFUNCin source population (Chi-square test for heterogeneity with Williams’ correction for small sample sizes: $\chi^2 = 1.838$, $df = 4$, $P = 0.772$) (Figures 3A,B). Consequently, all other experiments were performed using only the southern barnacle variant in order to save time and purified material.

MULTIFUNCin also was isolated from a second barnacle species, *Semibalanus cariosus*. Results were non-significantly different between bioassays using the *Semibalanus cariosus* MULTIFUNCin and each of two whelk species (*Acanthinucella spirata* and *Nucella canaliculata*) (Chi-square test for heterogeneity with Williams’ correction: $\chi^2 = 2.772$, $df = 4$, $P = 0.621$) (Figures 4A,B). Combined, whelks significantly preferred *S. cariosus* MULTIFUNCin over mussel and turban snail crude extracts, and over seawater (Sign test with Bonferroni’s correction, $\alpha = 0.008$: $P \leq 0.0061$, all comparisons). In addition, findings were similar for *S. cariosus* MULTIFUNCin and *Balanus glandula* MULTIFUNCin (compare Figures 3A–4A, Figures 3F–4B) (Chi-square tests for heterogeneity with Williams’ correction: $\chi^2 \leq 5.480$, $df = 4$, $P \geq 0.286$, both comparisons). Each whelk species did not discriminate between faux prey containing MULTIFUNCin from one or the other barnacle species (unpublished data). The two compounds, therefore, are likely quantitatively equivalent with respect to chemosensory recognition.

Bioassays with sea stars were limited in scope. The number of trials was restricted by system failures of our aged FPLC, which occurred after whelk and during sea star experiments. When substituting BSA for crude extracts, sea stars fed significantly more often on MULTIFUNCin-laced faux prey (Figure 5). Here, sea stars preferred MULTIFUNCin over both organic enrichment and seawater (absence of organics) controls (Sign tests with Bonferroni’s correction, $\alpha = 0.0167$: $P \leq 0.0059$, both comparisons).



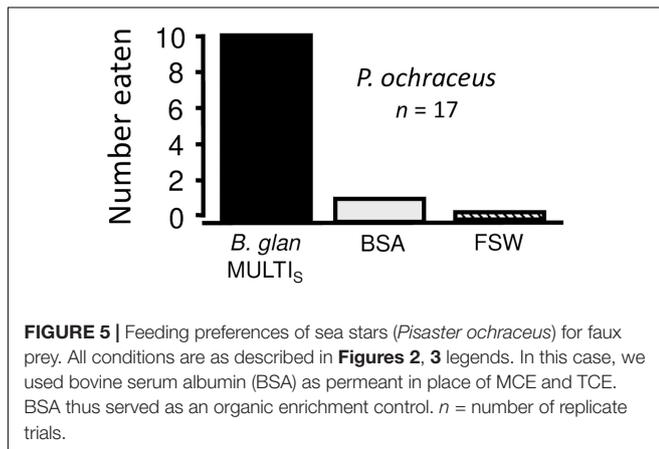
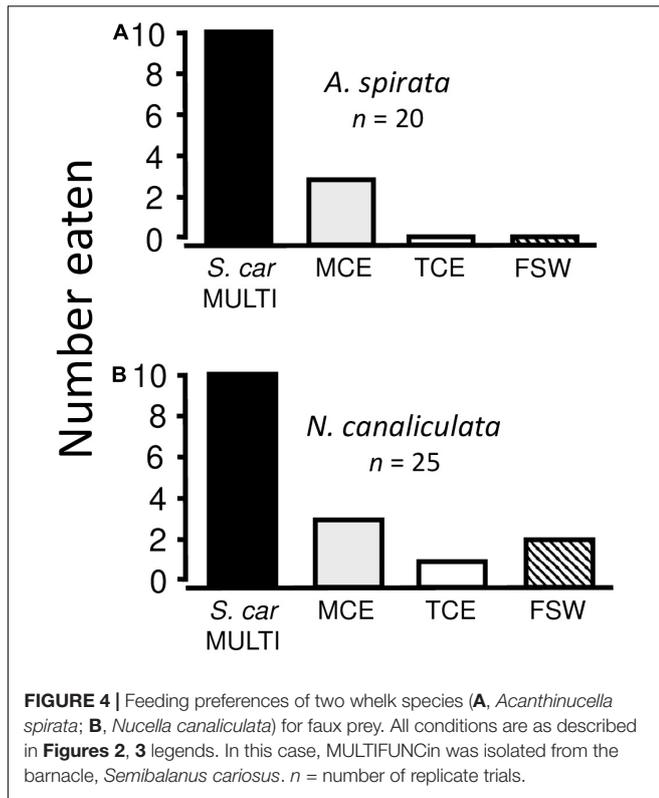
DISCUSSION

Many armed predatory species have specialized diets and feed only on hard-shell or exoskeleton-bearing organisms. The degree to which these exploiters and victims have evolved in response to one another has been difficult to discern. This problem has been especially challenging in regard to the predators. For example, development of specialized weaponry may enhance the likelihood for prey capture and subjugation. Yet, such innovation commonly evolves as a consequence of selection pressures imposed, not by the prey, but by competitors and/or higher-order consumers (Dietl and Kelley, 2002; Vermeij, 2013). In addition to morphological traits, species-, or taxon-specific chemical cues would provide a meaningful axis for evaluation of relations among enemy combatants. It has been well established that armored prey exhibit strong behavioral and morphological responses to unique infochemicals released by specific (and deadly) durophagous consumers (Poulin et al., 2018; Rittschar et al., 2020). In contrast, until now, there has been little evidence for the reciprocal interaction.

This study identifies chemosensory exploitation by armed consumers as an evolved predatory response to armored prey. Specifically, our results show that all six tested consumer

species attacked and ate MULTIFUNCin-infused faux barnacle prey significantly more than controls. In fact, MULTIFUNCin alone was sufficient to trigger a predatory response. After 670 million years of genetic isolation (Ayala et al., 1998), molecular mechanisms for chemical recognition differ considerably among protostomes (including mollusks) and deuterostomes (including echinoderms) (Bargmann, 2006; Cummins et al., 2009; Derby et al., 2016). Yet, for sea star and whelk consumers, glycoprotein detection has converged across the protostome-deuterostome division to promote durophagy on a valuable, shared, prey resource.

Resistant (or tolerant) consumers have evolved the ability to recognize toxins and poisons using chemosensory mechanisms. Thus, select predatory species can dine on specific, chemically defended prey species (Bernays et al., 2002; Hwang et al., 2007; Wink, 2018). Such biotic interactions play seminal roles, shaping community structure and function and, in some cases, triggering arms races and co-evolution especially among plants and herbivores (Anderson and Mitchell-Olds, 2011; Bruce, 2015). Unlike chemical defense compounds, molecules uniquely associated with armored defenses would impact ecological relationships without the need for toxin resistance or tolerance in source and/or consumer species. Given freedom from these evolutionary constraints, chemosensory exploitation of prey



body armor is likely widespread, acting as a critical ecological determinant.

Barnacles are widely distributed on wave-swept shores along the entire North America Pacific Ocean coast (see Table 1). Here, we identify a potential community role for chemosensory exploitation by armed consumers. In response to a glycoprotein cue (MULTIFUNCin), predation by sea stars, and especially by whelks, could limit the abundances and distributions of prey (barnacle) populations, thereby altering the availability of community resources, including food and space (Feder, 1959; Connell, 1961, 1970; Dayton, 1971). Such changes would lead to new “winners” and “losers” in a sweepstakes among subordinate and dependent species, thus

initiating cascades of community-wide impacts (Connell, 1972; Maggi et al., 2015).

Shared, conserved domains place MULTIFUNCin in the α_2 -macroglobulin (A2M) subgroup of the thioester-containing protein (TEP) family (Ferrier et al., 2016a). The A2Ms are vital to structural defenses (Armstrong and Quigley, 1999) and for the attack and subjugation of surface-colonizing microbial pathogens (Chu and Pizzo, 1994; Blandin and Levashina, 2004; Budd et al., 2004; Armstrong, 2010). MULTIFUNCin orthologs also cue habitat colonization by conspecific larvae and possibly act as pheromones (Dreanno et al., 2006; Zimmer et al., 2016a). To maintain these critical, ancestral, functions, we propose that MULTIFUNCin has been subjected to purifying selection in barnacles. Purifying (or “negative”) selection would eliminate deleterious mutant alleles in order to maintain stringent binding affinities and other, essential molecular properties. Accordingly, MULTIFUNCin would evolve as an honest and reliable cue in chemosensory exploitation by armed predators.

Chemosensory exploitation extends beyond barnacles and their predators on rocky, wave-swept shores. Akin to barnacles, mussels (*Mytilus californianus*, *M. edulis*, and *M. galloprovincialis*) dominate space and act as foundation species on wave-swept shores (at intermediate and/or low tidal levels) (Paine, 1966; Dayton, 1971; Suchanek, 1992). Each mussel species produces and secretes a glycoprotein ortholog (named KEYSTONEin, molecular weights: 28.1–29.6 kDa) which acts, singularly, to cue sea star, crab, and whelk predation (Zimmer et al., 2016b, 2017). These orthologs are uniquely associated with mussel body armor (extrapallial fluid, epidermis, periostracum, and shell materials). A large, conserved, globular domain places KEYSTONEin within the Complement Component 1 Domain Containing (C1qDC) protein family (Nonaka and Kimura, 2006; Carland and Gerwick, 2010). 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 act in shell formation through Ca^{2+} binding and subsequent calcium carbonate deposition (Hattan et al., 2001; Yin et al., 2005).

Chemical recognition *via* surface glycoproteins is not limited to species on wave-swept shores (Wyatt, 2014). The molecules serve, for example, as signals when bound in the integuments of rotifers, crustaceans, sea hares, squids, insects, amphibians (newts), and mice (Snell and Carmona, 1994; Painter et al., 2004; Dreanno et al., 2006; Snell et al., 2009; Woodley, 2010; Cummins et al., 2011; Matsuura, 2012; Roberts et al., 2012). They also act in communication between metazoan sperm and egg, and function as stringent pre-zygotic barriers that block hybridization among gametes of different species (Vacquier, 1998; Wilburn and Swanson, 2016).

Often requisite for internal (cell level) or external (organismal level) communication, glycoproteins are well suited for chemosensory exploitation. A prime example is the targeting of host-expressed mucin and collagen by pathogenic bacteria for attachment and colony establishment (Roos and Jonsson, 2002). In fact, thousands of armored metazoan species—from marine sponges to terrestrial vertebrates—secrete glycoprotein-rich extracellular matrices (Sarashina and Endo, 2006;

Ehrlich, 2010; Wang and Nilsen-Hamilton, 2013). As an evolved counter-adaptation by durophagous predators, chemosensory exploitation of glycoproteins may well have notable effects across the metazoan tree-of-life.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material; further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

RZ, GF, and CZ: conceptualization, methodology, formal analysis, investigation, writing—review, and editing. RZ:

writing—original draft. RZ and CZ: project administration, funding acquisition. All authors contributed to the article and approved the submitted version.

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