UC San Diego
Research Theses and Dissertations

Title
The Sensory Basis for Ecological Paradigms on Wave-Swept Shores

Permalink
https://escholarship.org/uc/item/5c10784j

Author
Ferrier, Graham A.

Publication Date
2010

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

THE UNIVERSITY OF CALIFORNIA

Los Angeles

The Sensory Basis for Ecological Paradigms on Wave-Swept Shores

A dissertation submitted in partial satisfaction of the

requirements for the degree of Doctor of Philosophy in Biology

by

Graham Andrew Ferrier

2010

UMI Number: 3452132

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

UMI 3452132
Copyright 2011 by ProQuest LLC.
All rights reserved. This edition of the work is protected against
unauthorized copying under Title 17, United States Code.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

© copyright by

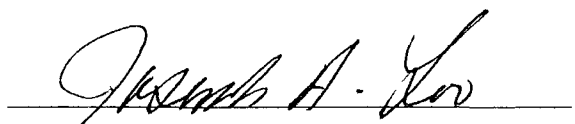
Graham Andrew Ferrier

2010

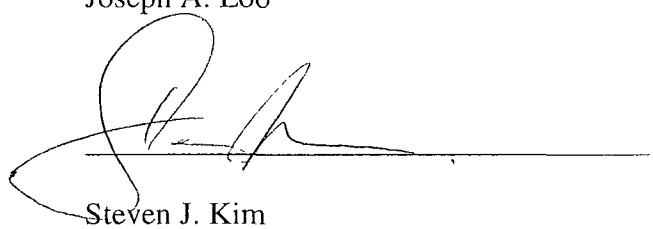
The dissertation of Graham Andrew Ferrier is approved.

Paul Barber

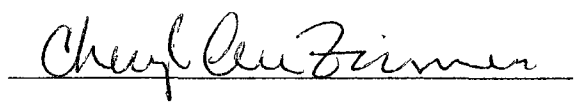
Steven D. Gaines



Joseph A. Loo



Steven J. Kim



Cheryl Ann Zimmer, Committee Co-chair

Richard K. Zimmer, Committee Co-chair

University of California, Los Angeles

2010

DEDICATION

To Carrie, Nayla, Mom, and Dad, for your unwavering support and belief in me for all these years, it has been a long time.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

LIST OF TABLES

Chapter 1

Chapter 3

ACKNOWLEDGEMENTS

The findings of this dissertation are not the single accomplishments of one person, but rather the product of numerous minds coming together to solve a problem. I would like to recognize those people who have contributed to this work over the years and who are partly responsible for its findings. Furthermore, I would like to thank those individuals who helped me through this long journey in science.

First, I would like to acknowledge my family. It was not long ago that I struggled just to stay in school. Through it all my mother and father never lost hope in me and knew all along that a day would come for me to find my own path. Moreover, a lot of my interest in the marine environment stems from my early days sailing with my grandfather, mother, and father in New England. Those days obviously influenced me and I am very grateful to have had such an introduction. Second, I would like acknowledge my wonderful wife, Carrie Schneider Ferrier, whose love and support in even the toughest times keeps me going. She has put up with a level of work and near-poverty for 5 years that many others would have run from. Carrie is my rock, and my constant inspiration that life is too short to not follow something you are passionate about, thank you Carrie. Finally, I must recognize my intrepid dog, Nayla, who follows me where ever I go, even in the roughest of intertidal conditions. She helped collect countless organisms and endured numerous hours in rough intertidal water retrieving settlement plates at all hours of the day. Your webbed feet came in handy on more than one occasion.

Members of the Zimmer lab, Ryan Ferrer, Julie Himes, and Sammi Ali contributed valuable insights and generated thoughtful discussion with respect to research of all sorts. Kevin Peng, Riley Jondle, Ian Roberts, Aleskey Korolyov, Jenny Wang, Michelle Li, Noelle Bridigainberry, Heather Johnston, Patrick Green, Leo Varzi, and Emily Admanson tirelessly conducted field surveys, produced crude extracts and ran bioassays. Don Conestro of the UC Rancho San Marino Reserve was always available and numerously provided a warm meal and good conversation while working the Central California coast.

My committee members, Drs. Joe Loo, Steve Gaines, Paul Barber, and Steve Kim provided expert guidance, comments, and advice throughout the course of my dissertation. Steve Kim took the time to work directly with me in the lab teaching the fundamentals of biochemistry. With his support I learned more biochemistry in two short years than many learn with a Bachelors degree. Joe Loo took the time to teach proteomics and mass spectrometry in a way that could be easily incorporated into ecology. Paul Barber, and the Barber lab, allowed me to use their space and equipment and were always there to answer the questions of a sequencing- and phylogenetics-novice. Finally, Steve Gaines provided critical ecological insights which were instrumental in tying sensory biology, proteomics, and ecology together into a compelling story.

Finally, I am indebted to the support Drs. Dick and Cheryl Ann Zimmer have provided. They gave time, energy, expertise, money, and most importantly an opportunity for me to explore science, and for that I am eternally grateful. Dick "Z"

Zimmer coaxed from me a level of work that I believed I could not do myself and constantly pushed for more. Although he set his bar quite high, his guidance always managed to get me over it. His insight has allowed me to broaden my scientific horizons and from them my mind typically follows ideas from the level of proteins up through their impacts on community dynamics. I owe him much more than I can write here. From Cheryl Ann I learned the critical, but often neglected, importance of scaling in experiments, a lesson I will take with me everywhere. Here ability to convey concepts and messages with enthusiasm is a skill I hope to match when I teach and during times when I felt stuck with courses I often thought of her style and I managed to get through them. Furthermore, Cheryl Ann took the time to assist me with writing. She has patiently gone through countless revisions and taught me how to communicate as a scientist, a skill I lacked before my valuable interactions with her. From Dick and Cheryl Ann's guidance I feel I have accomplished something scientifically meaningful I can not thank them enough for this.

VITA

September 11, 1980	Born, Morristown, New Jersey
2002	Bachelor of Science, Marine Resources and Development, University of Rhode Island Kingston, RI
2005	M. S., Biology, California State University Northridge. Northridge, CA
2005-2010	Teaching Assistant University of California, Los Angeles Los Angeles, CA
2007	Mia Tegner Award in Ecology Western Society of Naturalists 88th Annual Meeting
2010	Division of Ecology and Evolutionary Best Student Research Award Society for Integrative and Comparative Biology Annual Meeting

PUBLICATIONS AND PRESENTATIONS

Ferrier, G.F., and R.C. Carpenter. 2009. Subtidal benthic heterogeneity: Impacts on the flow environment, and marine algal community structure and morphology. *Biological Bulletin* 217:115-129.

Ferrier, G.A., C.A. Zimmer, and R.K. Zimmer. Chemical Cues and the Keystone Species Hypothesis. Talk presented at the 2007 Western Society of Naturalists Meetings.

Ferrier, G.A., C.A. Zimmer, and R.K. Zimmer. The Sensory Basis for and Ecological Paradigm: Predator-Prey Interactions on the Rocky Intertidal. Talk presented at the 2007 Benthic Ecology Meetings.

Ferrier, G.A., C.A. Zimmer, S.J. Kim, and R.K. Zimmer. Chemical signaling in turbulent environments. Talk presented at the 2008 AChemS International Symposium on Olfaction and Taste.

Ferrier, G.A., C.A. Zimmer, and R.K. Zimmer. Keystone Molecules and the signals that structure natural communities. Talk presented at the 2010 annual Society for the integrative and comparative biology.

ABSTRACT OF THE DISSERTATION

The Sensory Basis for Ecological Paradigms on Wave-Swept Shores

by

Graham Andrew Ferrier

Doctor of Philosophy in Biology

University of California, Los Angeles, 2010

Professor Richard K. Zimmer, Co-chair

Professor Cheryl Ann Zimmer, Co-chair

Sensory systems provide critical filters that enable organisms to detect and recognize valuable resources. Trophic cascades, structuring populations and communities, are determined to a large degree by trait-mediated interactions that rely on sensory inputs. Certain molecules serve as chemosensory stimuli and play keystone roles in determining outcomes of predator-prey dynamics at multiple trophic levels. Here, a surface-adsorbed glycoprotein signal molecule, MULTIFUNCin, is fully identified and its role in wave-swept rocky intertidal community dynamics investigated. Barnacles (*Balanus glandula*), are constrained to produce MULTIFUNCin for cuticle/shell formation. This compound evoked settlement by conspecifics larvae in field assays, and thus, operates as seminal

cue for recruitment. Moreover, the same substances triggered predation by a numerically dominant whelk species (*Acanthinucella spirata*) on barnacle juveniles and adults in lab and field experiments. This glycoprotein, therefore, balances simultaneous demographic processes that enhance, and diminish, barnacle populations. As dominant competitors for space, the relative equilibrium between barnacle recruitment and predation mortality has strong, cascading direct and indirect effects on community dynamics. Hence, MULTIFUNCin plays a keystone role within rocky intertidal habitats.

Chapter 1: Whelk-barnacle interactions, the energetics of prey choice, and the sensory basis for an ecological paradigm

ABSTRACT

The rocky intertidal has provided stunning examples of prey stabilization by predators, but the mechanisms used by predators for prey location remain elusive. In a seminal work, Murdock (1969) suggested that predators can stabilize prey populations via density-dependent switching to alternate species. At sites along the southern California coast, we revisited this hypothesis and found the predatory whelk *Acanthinucella spirata* exhibits a strong preference for barnacle prey (*Balanus glandula*) regardless of the densities of alternate prey (*Mytillus spp.* and *Tegula Funebralis*). Experiments showed that, of the three prey species, barnacles yield the greatest net energy gain per unit handling time thus, *A. spirata* forages optimally, switching only after prolonged searches. The efficiency with which whelks find live barnacles in a bed is explained by their ability to locate contact cues associated with *B. glandula*. Feeding assays using carboxymethyl cellulose gels infused with barnacle extracts showed that *A. spirata* is attracted to, and induced to feed on, a surface adsorbed protein of low solubility found in the barnacle. Finally, these results allow for predications of the relative importance of waterborne vs. contact cues in the wave-swept rocky intertidal from which a model describing a general, and ecologically important, pattern of directed motion and foraging is developed.

INTRODUCTION

Identify the consequences of species interactions on community dynamics has been a perennial theme of ecology. Accordingly, studies aimed at defining interaction strengths and outcomes have proliferated in the literature, the results of which have shaped current ideas of how communities are structured. In terrestrial, marine, and aquatic habitats, biological factors such as competition, predation, and parasitism have been identified as critical determinants of community organization, which singularly, or collectively, set species distributions (Connell, 1961a,b, 1970; Dayton, 1971; Paine 1974; Chase *et al.*, 2002), regulate population density, (Murdoch, 1969; Paine, 1974; Menge and Sutherland, 1976; Lively and Raimondi, 1987; Murdoch, 1994; Schmitz, 2003; Trussel *et al.*, 2006a), and direct settlement and recruitment (Gaines *et al.*, 1985; Menge and Sutherland, 1987, Bertness *et al.*, 1992). Despite this wealth of information, there are lingering questions regarding underlying mechanisms and relative contributions of direct and indirect species interactions on the formation and maintenance of natural communities.

Competition and predation are two factors which have been implicated as critical drivers of community structure. In many ecosystems, dominant competitors exclude subordinates from resources, thus decreasing rates of consumption and colonization. Such unidirectional interactions may result in the formation of monospecific communities and the relegation of subordinate species to sub-optimal niche habitats (Connell, 1961a; Carpenter *et al.*, 1987; Petraitis, 1995). Selective predation on the dominant competitor,

however, can ease resource pressure on subordinates indirectly increasing their populations in specific habitats (Paine, 1966; Murdoch, 1969; Connell, 1970). From this generalized example, the consequences of predation are both direct (i.e. death to prey) and indirect (i.e. reduced competition) with effects that often ripple through communities. Extraordinary at that time, and now, these studies demonstrated how interactions among relatively few, select, species can disproportionately affect community structure. Completely unexplored, though, are the mechanisms responsible for driving predation, for example: how do predators find and choose prey?

One habitat in which predator-driven trophic cascades have been well studied is the rocky wave-swept intertidal. At the interface between land and sea, this environment is characterized by steep gradients in temperature, evaporation, and hydrodynamic forces. Thus, it is among the most physically challenging environments on earth (Tomanek and Helmuth 2002). Despite these strenuous conditions, or perhaps due to them, the intertidal exhibits high biodiversity with numerous organisms occupying unique niches, with considerable overlap between species in physiological tolerances, and hence shared spatial distributions. This feature creates a vibrant setting that offers exceptional opportunities to examine the consequences of species interactions as they are defined by events determined at the cellular, whole-individual, population, and community levels.

Community dynamics have been primarily studied in terms of direct, or consumptive, effects of predators on prey population and the corresponding trophic cascade. On rocky intertidal shores, as in many environments, top-down influences of a single, or a few, predators determine densities of second- and third-tier consumers. A

classic example is the selective consumption of competitively-dominant species by whelks (predatory gastropods) which releases a limited resource- space –for use by subordinate species. Here, predation is an obvious means by which a predator influences species distributions. The actions of predators, however, are not simply limited to feeding. To the contrary, predators must also forage, find shelter and mates, all of which may broadcast chemical, visual, and/or tactile signals throughout the environment. The recognition of predatory cues can induce changes in prey behavior that may have wide-ranging community effects (Abrams, 1995; Peacor and Werner, 2000; Trussell *et al.*, 2003; Schmitz *et al.*, 2004). These indirect, non-lethal, species interactions have been termed "trait-mediated", and some have argued these are equally, if not more important, than direct, consumptive, effects in shaping community dynamics (Peacor and Werner, 2001; Trussell *et al.*, 2006a,b; Peckarsky *et al.*, 2008). Correctly defining the relative strengths of consumptive and non-consumptive effects of predators requires experiments that are dynamically scaled in all aspects. No where is this more true than when sensory cues have been suggested to play a role.

The theory behind trait-mediated interaction strengths involves the concept that predator presence influences lower-tier consumer behavior, but many experiments examining this are inappropriately scaled (chemically, physically, and/or biologically), leading to an over-estimation of non-consumptive effects. This problem is particularly relevant for studies on wave-swept rocky intertidal shores. For example, mesocosms are routinely used in which intertidal predators (crabs, sea stars, fish) are placed in an upstream portion of a flow-through tank separated, via a mesh barrier, from downstream

second-tier consumers (whelks, littorine snails, urchins). The purpose of these experiments is to determine if a cue released by the predator influences the behavior (i.e. refuge seeking, feeding, and/or growth rates) of second-tier consumers. The results of such experiments often find that in the presence of a "predator cue" consumers seek refuge habitats more frequently, and consume less of a given resource. Thus, in a three-tier food web the non-consumptive effect of predator presence apparently affects the densities of basal species (primary producers and consumers) by changing the behavior of the next trophic level (Trussel *et al.*, 2003, 2006a,b, 2008; Cotton *et al.*, 2004; Freeman, 2006). Yet, the strength of the non-consumptive effects observed in this experimental design are often artificially elevated, resulting in one or more of the following artifacts: 1) Predators were often continuously fed a diet of second-tier consumers without regard to the potential for alarm cues originating in injured conspecifics, as opposed to signals for the predator. Both energetics and sensory biology play a critical role in determining second-tier consumer response. Many predators have hierarchical prey preferences that may not include a large proportion of the secondary consumer in question. Furthermore, little or no attempt was made to identify the sensory cue associated with the observed behavior, or its natural concentration, which would facilitate accurate representations in experiments. 2) With some exceptions, the predator, and to a lesser extent the next tropic level consumer, are highly-mobile organisms. Many mesocosm experiments simply cage a predator next to the consumer species, thus, artificially forcing an interaction (chemosensory driven or otherwise) for an unrealistically extended length of time. Many predators, and intertidal predators in particular, are very mobile and do not remain in one

area for long periods of time. Prolonging the exposure of second-tier consumers to predators, therefore, artificially increases the time spent in refuge habitat. 3) Finally, for intertidal habitats, the wave-swept shore is a physically dynamic environment. Water flow must be scaled to represent intertidal turbulence, which could dilute a waterborne chemical signal to below recognizable levels (Smee *et al.*, 2008). Miscalculations in scaling any of these parameters will likely introduce artifact(s) into experimental mesocosms. Although the results of these experiments suggest that non-consumptive effects are equally, if not more, important than direct consumptive effects in intertidal habitats, there is a high likelihood that the strength of these interactions had been artificially increased. Realistic studies of the cascading effects of biological interactions (including both density- and trait-mediated interactions) must incorporate meaningful aspects of community ecology, with emphasis on sensory biology (identifying and characterizing active signals that drive behavior), individual and species-level behavior (in response to an active signal), energetics of prey selection (likely a driving force in foraging behavior), and utilizing correctly scaled experiments (e.g. signal concentrations, flow speeds, space and time).

Chemical signals are used by organism in myriad ways including habitat colonization (Matsumura *et al.*, 1998, Raffa, 2001; Steinburg *et al.*, 2002: Koehl and Hadfield, 2004; Dreanno *et al.*, 2006), mate recognition (Snell and Carmona, 1994; Kelly and Snell, 1998), defense (De Moraes *et al.*, 1998; Wolfe 2000; Pohnert *et al.*, 2007), and feeding (Zimmer-Faust, 1993; Nevitt *et al.*, 1995; Zimmer *et al.*, 1999; Ferner and Weissburg, 2005). Historically, chemical ecology has focused on the role of

secondary metabolites as active molecules that have singular functions, but recent studies have shown that chemical signals can significantly influence animal behavior at multiple trophic levels, with community-wide implications (Ferrer and Zimmer, 2007; Zimmer and Ferrer, 2007; Shabani *et al.*, 2008). Feeding initiation, or suppression, signals are at the core of these interactions as the chemistry (smell and taste) of food is a basic functional-response relationship between predators and prey, irrespective of habitat type. Whereas studies once asked only if a given predator consumed a given prey, contemporary theory, technology, and experimental capabilities facilitate studies of what exact, compound(s) trigger foraging, feeding, initiation of competition and, thus, what sensory determinants drive density-dependent and trait-mediated interactions.

MATERIALS AND METHODS

Study species and field setting

Our experiments focused on a species of whelk, *Acanthinucella spirata* (Gastropoda, Muricidae), widely distributed on Eastern Pacific rocky shores from Bodega Bay (CA) to San Quintin (Baja Norte, Mexico) throughout the mid- and high-intertidal. With a diverse diet that includes two competitively dominant foundation species, barnacles and mussels, *A. spirata* is an ecologically important predator. The effects of whelk predation cascade through the mid- and upper-intertidal, and so *A. spirata*, as well as other whelk species, may be considered keystone predators (Paine 1969; Power *et al.*, 1996).

As a direct developer, *Acanthinucella spirata* lays its eggs on, or around, the dominant prey item of a respective beach. As a result the distribution potential of this whelk is low, and populations tend to be variable throughout their range (~1-10 whelks/m^2). Murdoch (1969) described this species as a general predator which feeds on the numerically dominant species of a given beach. Furthermore, these whelks utilize different strategies for consuming a variety of prey (drilling, use of paralytic venom, and the use of labial spines to prop open valves of prey) suggesting that this species switches prey with relative ease. A high degree of seasonality exists for this whelk in southern California, with active foraging limited to April-November and a relatively dormant period from December-March (author's personal observation).

The California wave-swept community is dynamic, and species dominance within each zone can change dramatically over relatively small spatial scales (Gaines *et al.*, 1985). Whelk prey preferences, hence, are dependent on the abundances of available quarry at a given site, and so three test sites were established along the central and southern California coastline that have markedly different abundances of the barnacle, *Balanus glandula*, the mussel, *Mytilus californianus*, and the herbivorous black turban snail, *Chlorostoma funebralis*.

Our first site, Latigo Canyon (Malibu, CA; 34° 1.573 N, 118° 45.341 W), is a sheltered point made up of large boulders (≥ 1m diam.) covered by extensive beds of *M. californianus*. Although *B. glandula* is common at this site, the relative abundances of this barnacle are significantly less than that of mussels and so whelks are most often observed consuming mussels there. Sea surface temperatures at Latigo Canyon vary

between 13 °C (February) to 22 °C (August), and air temperature varies between a low of 8°C (January) to a high of 30°C (August) (NOAA Buoy # 46025, Santa Monica Basin; data from 2007). Santa Monica Bay, and Southern California as a whole, experiences a mixed tidal cycle in which the greatest tidal fluctuation is ~2.65 m. During the warm months of summer the lower intertidal zone is exposed during the early morning hours and generally escapes the heat of midday. However, the mid and high intertidal is exposed in the morning, and again in the early afternoon where it is subject to relatively high summer atmospheric temperatures.

A few miles northwest of Latigo Canyon, Broad Beach (Malibu, CA; 34° 2.074 N, 118° 695 W) is an exposed rocky point characterized by large slate and sandstone formations dominated by extensive beds of *B. glandula* in the mid intertidal and *M. californianus* in the low intertidal. The sea surface and air temperatures at this site are near-identical to those found at Latigo Canyon, which is typical of the northern portion of the Santa Monica Bay. Although the environmental conditions are similar, the distribution of prey species between the two sites is not. With a larger population of *B. glandula*, whelks of Broad Beach feed near-exclusively on barnacles.

The third site, on the central Californian coastline, Rancho San Marino (Cambria, CA; 35° 31. 999 N, 121° 5.107 W) is a boulder-strewn point (boulder size: 0.25- >1 m diam.) dominated by algae (~95% cover) in the lower intertidal and large populations of the turban snail, *C. funebralis*, from the mid to high intertidal. Densities of turban snails in these upper zones can be as high as 200/m^2, and algal cover is often < 5 %. Barnacle

and mussel cover throughout the tidal range of Rancho San Marino is orders of magnitude less than either site in Malibu, and is normally < 5% for both *B. glandula* and *M. californianus*. Despite the low density of barnacles and mussels, *A. spirata* is relatively abundant and can be observed consuming *C. funebralis* exclusively. Both sea surface and air temperature remain cooler throughout the year at Rancho San Marino. Typically sea surface temperature range from 9 °C (May) to 17 °C (September), and air temperatures range from 6 °C (January) to 18 °C (September) (NOAA Buoy # 46028, Cape San Martin; data from 2007). The tidal range on the central California coast is comparable to southern California (~2.6 m maximum difference), and occurs ~ 40 minutes later in the day. Therefore the exposure to warm atmospheric summer conditions is comparable between southern and central Californian sites.

Distributions of Acanthinucella spirata *and prey species*

In order to characterize the natural environment, as well as to determine if this reflects prey choice, field surveys were conducted every other month for two years (2006-2008) at Latigo Canyon (Malibu, CA), Broad Beach (Malibu, CA), and Rancho San Marino (Cambria, CA). At each site the densities and distributions of the whelk, *Acanthinucella spirata*, and its primary prey species (*Balanus glandula*, *Mytilus californianus*, and *Chlorostoma funebralis*) were quantified along three permanent 20 m transects at equivalent tidal heights (above mean low water) at each site: 1.2 m (high intertidal, barnacle zone), 0.75 m (mid intertidal, mussel zone), and 0.35 m (low, algal-mussel zone). The percent cover of *B. glandula* and *M. californianus* were visually

estimated (Deither *et al.* 1993), and the number of *A. spirata* and *C. funebralis* counted, in 0.25 m^2 quadrats at alternating meter markings along each transect. Furthermore, the size frequency distribution of each prey species was quantified quarterly by placing a 0.25 m^2 quadrat with 10 randomly assigned points on a prey patch at alternating meter markings along each transect and measuring the height and width of individuals found below each dot.

Our study locations were picked because the resident whelks exhibited discreet prey preferences. In order to determine if these preferences remained static through time we surveyed feeding whelks every other month during our surveys. This was done by removing the first ten whelks found actively feeding. Once removed the prey type was recorded and its dimensions measured.

Quantifying prey size preferences of Acanthinucella spirata

Preferences for prey size was examined for *Acanthinucella spirata* collected from each site along the coast of California in choice experiments so they may be compared to feeding selections observed in the field. During the summer of 2006, twenty whelks from each location were collected and transported to the S.E.A. Laboratory (Redondo Beach, CA) in aerated coolers containing fresh sea water. Groups of 20 whelks were housed in aquaria (0.27 x 0.1 x 0.17 m) supplied continuously with fresh seawater and acclimated to the light and temperature conditions of the lab (12 h light : 12 h dark cycle) for a period of one week prior to tests. Every three days, whelks were fed fresh barnacles, mussels, or turban snails throughout the duration of their captivity.

To examine whelk prey size preferences, experimental arrays were created in circular choice tanks (n=10 tanks; 0.333 O.D.). Three representatives of each of three size classes (barnacle size classes: small <1-3mm, intermediate 4-6mm, large 7-10mm; mussel size classes: small 10-20mm, intermediate 30-40mm, large >50mm: turban snail size classes: small 5-10mm, intermediate 11-15mm, large >16mm) were randomly affixed to the bottom of each tank using inert silicone grease (barnacles and mussels), or 1 cm of monofilament tether line secured to the bottom (turban snails), at one of nine points 2 cm away from the tank wall and each other. Separate water supply lines continuously fed each replicate tank fresh seawater at a rate of 1 $L \cdot m^{-1}$. Excess water exited the tanks as overflow. Trials were initiated by placing one whelk in the center of each tank. Whelks then foraged until a prey item of a given size was consumed, or 24 hours passed, after which the whelk and prey were removed, the tanks cleaned, and a new trial was initiated. Prey items, and whelks, were used in only one trial, and experiments were repeated until 10 food items of a single size class had been consumed. Maximum likelihood analysis was used to determine if whelks exhibited a size preference for prey.

Energy gained per unit time handled

Preferences for one prey type over another are often driven by the energy value (energy gained • unit time $handled^{-1}$) of each respective prey. To determine if whelk prey preference reflects the energy values associated with a specific prey item the caloric content of barnacles, mussels, and turban snails of varying sizes was estimated. Adult barnacles were collected from Broad Beach, mussels from Latigo Canyon, and turban

snails from Rancho San Marino (n=20/species). Measurements of shell length, width, and height were taken on each prey before they were completely removed from the shell and freeze-dried in preparation for caloric estimation using a 1425 Semimicro bomb calorimeter (Parr Instruments, Moline Il.).

Prior to caloric estimation, freeze-dried soft tissues of each barnacle, mussel, and turban snail were individually ground into a homogenous powder using a mortar and pestle. The whole animal, or 200 mg if it weighed more than this, was then pressed into a pellet before being placed into the bomb calorimeter. A 7 cm length of fuse wire was cut and fitted to the ignition lid and laid to rest just on top of the pellet. The fuse was then ignited burning the pellet and the wire completely in the bomb. The heat of combustion results in an increase in water bath temperature which was measured to the nearest 0.0001 °C. Temperature change was converted to the net change in energy (Q) using the equation (Rossini, 1956):

$$Q = (\Delta T_c)^*(\varepsilon)$$

In which ΔT_c is the change in water bath temperature and ε is a standardization parameter determined by obtaining the heat change of a known amount of benzoic acid. To convert this to "biologically meaningful" values, the gross heat of combustion (ΔH_t) was determined using the equation (Rossini, 1956):

$$\Delta H_t = (-Q + f)/m$$

Where Q is calculated as above, f is the fuse correction in calories (2.3 calories/cm of fuse wire), and m is the mass of the sample. This provided an estimate of joules per gram

of sample, which when multiplied by the total mass of the prey gave an approximation of the energy content available to a predator when all the soft tissue is consumed.

A highly significant linear correlation was found between shell length (for mussels), or shell height (for barnacles and turban snails), and freeze-dried weight. Additionally, a highly significant linear correlation also was found between freeze-dried weight and caloric content of all prey species tested. Using the equations that describe these relationships, we were able to predict the freeze-dried weight of each prey consumed in a feeding trial and, thus, approximate the total available energy to a whelk from a completely consumed prey item of a given size in each experiment.

Using the total available energy of a given prey item, we next determined the energy gained per unit time handled ($E \bullet T_h^{-1}$) (joules/minute) for barnacles, mussels, and turban snails of different sizes. The length, width, and height of barnacles, mussels, and turban snails (n=14, 12, 10 respectively) was measured before one prey of a given size was presented to one whelk in a circular tank (0.333 O.D.). Whelks were placed in the center of each tank to forage until feeding commenced. The period required to consume the prey in its entirety was determined using a time-lapse digital camcorder recording for 20 seconds of each minute until the prey was consumed in its entirety.

Using the shell measurements, the total caloric content of each prey was estimated by means of the equations previously stated. This energy value was then divided by the time required to consume each item in its entirety, yielding an estimate of the energy gained per unit time handled ($E \bullet T_h^{-1}$). Although this metric for net energy gain does not take into account the energy loss due to foraging or consumption, it provides an accurate

estimation of the caloric energy provided to a predatory whelk by prey of a given type and size. Furthermore, it offered a means by which predictions could be made as to what a whelk should prefer if they forage in a means that optimizes energy intake.

Preferences for prey type

At each of the sites chosen in this study *Acanthinucella spirata* typically consumes a single prey species. We tested the consistency of this preference in the lab where abundances of prey could be kept equal both in number and in surface area. Three intermediate sized barnacles, mussels, and turban snails were fixed randomly to the bottom of circular tanks and supplied continuously with fresh sea water as previously described. One whelk was placed in the center of each tank and permitted to forage until a choice for prey was made, or 24 hours had elapsed. Whelks and prey were removed after each trial and the tanks were cleaned and a new trial initiated. As before, whelks and prey were used only once and trials continued until 10 of one prey item were chosen.

Although we were primarily interested in the choice of a single prey item over another, the probability of contacting a prey with a larger surface area could bias the results of single choice experiments. To address this, the relative surface areas of prey were matched by grouping smaller prey together to equal the exposed size of the largest prey in a trial (mussels). Whelks were again placed in the center of each tank to forage until a choice was made for a certain type of prey. Trials continued until 10 of one species had been chosen. All prey choice results were analyzed using maximum likelihood statistics.

Role of Ingestive Conditioning

In his seminal work, Murdoch (1969) found that "training" *Acanthinucella spirata* on alternate diets had a significant effect on future prey selection. In a similar test, we examined whelk preferences following a period of ingestive conditioning. Thirty whelks found feeding on the established preferred prey of each site were collected and transported to the S.E.A. Laboratory. Snails were separated into groups of ten and were fed a diet of only barnacles, mussels, or turban snails for one week under laboratory conditions to acclimate them. Following acclimation, a single whelk was placed in a single tank (10 x 10x 4 cm) supplied continuously with flowing sea water. These tanks were then placed into feeding blocks of thirty tanks (n=90 tanks) and the solitary whelk inside was fed a diet of barnacles, mussels or turban snails for eight weeks. After complete consumption, or after three days, each prey item was replaced. The number of days into the ingestive conditioning that each whelk began feeding was recorded as well as the total number of prey each had eaten throughout. Following the completion of the conditioning, whelks were once more assayed for prey preference in the same choice experiment described above.

Waterborne vs. surface-bound cues

Critical ecological interactions are mediated by chemistry. Time-honored processes, such as predation and trophic cascades, inevitably involve the broadcast of chemical information. For whelks, and other intertidal predators, signals are likely produced and broadcast by energetically-valuable prey in a waterborne and/or surface-

bound state. To examine the role of waterborne signals whelks were tested using barnacle bed water in bioassays conducted under natural conditions at Broad Beach (Malibu, CA). Each trial was performed by placing a single whelk into the downstream section of a portable flow-through acrylic pipe chamber (30 cm long x 5 cm O.D). Seawater was plumbed into each of five replicate chambers by a 12 v marine pump from either a test (1 mm above a dense barnacle patch) or control (no prey, sand bottom) habitat. Chamber flow speed (4 cm/s, measured 2.5 cm above bottom), and turbulent mixing (shear velocity: 0.1 – 0.2 cm/s), were maintained to minimize dilution of any waterborne chemical stimulus by a head tank (kept at constant volume) that fed each test pipe. Sixty replicates trials were run for each treatment over 1-mo intervals in 2007 and 2008, and seawater input was switched among prey/sand patches after every trial. Prior to the start of each run, whelks were acclimated to the conditions of the chambers for 30 minutes by separating the snails from the source with mesh screening and running control (sand patch) water continuously through the chamber. After acclimation, the source water changed, the mesh screen was removed, and the whelk was free to forage around the chamber for 15 minutes. Trial dates and times were logged and oceanographic data from that time period were obtained from instrumented buoys situated adjacent to study sites (Coastal Data Information Program, operated by Scripps Institution of Oceanography). As indicated by buoy data, calm sea states and minimal wave exposures (significant waves heights: 0.2-0.5m) prevailed during the entire study period, thereby minimizing prey odor extinction and maximizing the likelihood of a positive snail response. To serve as a positive response control, 15 barnacles were collected, crushed,

and rendered into a paste. At the end of each trial, this barnacle paste was placed at the source of each chamber and the trials were run for an additional 15 minutes for both the barnacle bed and the sand patch. The number of whelks reaching the source was recorded and compared between all treatments. Tests for the activity of surface-bound signals were done using bioassays and are discussed below.

Whelk feeding bioassay

A new assay was developed to determine if purified barnacle compounds initiated feeding in foraging whelks once contacted. Whole adult barnacles (4-6 mm test height) were collected from Broad Beach (Malibu, CA) and the soft tissues of the animals were dissected out. The empty barnacle tests were then scrubbed with pipe cleaners in deionized water before being bathed, and stirred, in a 5% hydrochloric acid solution for 15 minutes. Each barnacle test was then rinsed again with deionized water and any remaining tissue was taken off before each was placed in a 130°C oven and baked over night. Finally, burned material was carefully removed, and the resulting calcium carbonate served as a clean structure to be filled and coated (< 0.5 mm) with gel creating "faux barnacle prey".

Artificial prey was made by mixing treatment (barnacle extracts) or control (mussel extracts, turban snail extracts, or filtered sea water) solutions with carboxymethyl cellulose sodium salt (MP Biomedicals) at 4°C. Carboxymethyl cellulose (herein referred to as CMC) was the matrix of choice, because gel pore diameter is smaller than compounds ostensibly acting as prey cues (Zimmer-Faust, 1993). Furthermore,

carboxymethyl cellulose does not require heat to polymerize and can be made on ice. Protein concentrations of intermediate sized barnacles, mussels, and turban snails were determined by the method of Bradford (1976) from crude extractions of a known number of each organism. Crude extractions were repeated ten times and protein levels averaged so extract concentrations could be scaled to ecologically realistic levels. Faux prey were made by mixing 1 ml of ecologically scaled test extract, control extract, or filtered sea water (FSW) with 0.117 g of CMC . This amount of CMC represented the optimal weight for polymerization of 1 ml of solution. Furthermore, 1 ml was the smallest volume for which gel production could be done successfully. The average volume of intermediate barnacles was ~0.333 ml so 1 ml of faux prey gel was used to make 3 barnacle mimics (concentrations were scaled to fit this). Once thoroughly mixed, barnacles were filled completely with the gel and a thin coat (<0.5 mm) was smeared along the outer surface before they were placed on ice to harden. Faux barnacles (n=12; 3 faux barnacles per treatment) were than randomly arrayed in a circle in each flow-through test tank (10 x 10 x 3 cm) with fresh seawater continuously plumbed in from above with excess exiting each tank as overflow. A single whelk was placed in the center of each tank and acclimated to the conditions for 10 minutes before the 50 minute observation period. Each faux barnacle visited and eaten was recorded. The resulting choices were compared by maximum likelihood tests.

It is often difficult to recreate field conditions in the lab, and results from these trials may not reflect the behavior of animals under natural conditions. We sought to determine if the foraging and selections observed in the laboratory would translate to the

field. To do this, five experimental plots (10 x 10 cm) were established at Broad Beach in which all but 12 randomly assigned barnacles were removed. Theses remaining 12 barnacles had the live animals removed and the shells scrubbed and cleaned with a 5% HCl solution. The plots were left to the natural conditions for 2 weeks (newly settled species were removed every three days) before trials commenced. For these field trials each barnacle of each plot was randomly assigned a treatment using a random number generator. Faux prey gels were prepared on site, and the individual barnacle shells filled, as the tide was coming in. As the water began to flood the plots 10 whelks were placed around the periphery and permitted to forage as normal with the incoming tide. Many whelks simply moved away from the plots, those that entered were tracked by snorkeling and the barnacles visited and eaten were recorded over 1 hour. As with the laboratory experiments choices were compared using maximum likelihood analysis.

Extraction and purification of biologically active barnacle compounds

Whole adult *Balanus glandula*, collected from Broad Beach (Malibu, CA), were crushed and homogenized in 1:1.5 (w/v) of 50 mM tris-HCl, pH 7.5, and stirred for 120 minutes. This slurry was next rough-filtered through gauze, and centrifuged at 40,000 g for 30 minutes. The resulting supernatant was filtered using Whatman no. 2 filter paper, and this solution served as the barnacle "crude extract" (Matsumura *et al.* 1998). All extraction procedures were conducted at 4° C, and any crude extract not immediately used was stored at -80° C. Protein concentrations were monitored by the Bradford method with bovine serum albumin (BSA) as a standard. Prior to tests crude extracts

were dialyzed against filtered sea water using Slide-a-lyzer © dialysis cassettes (5,000 Da molecular weight cut-off (Thermo Scientific)).

Crude extracts of *Mytilus californianus* and *Chlorostoma funebralis* were prepared as described above for *B. glandula*. In separate assays both crude extracts induced whelk feeding when compared to gels infused with filtered sea water (FSW). These extracts were subsequently used as rigorous positive controls for compound addition to the carboxymethyl cellulose gels. FSW served as the negative control. All *M. californianus* and *C. funebralis* protein concentrations were monitored by the Bradford method, and crude extracts were diluted to pre-determined barnacle protein concentrations used in a particular trial. Due to the limitations associated with the purification of compounds from macro-organisms, *M. californianus* and *C. funebralis* were kept as crude extracts for controls in all trials.

Ammonium sulfate precipitation

A series of ammonium sulfate ($(NH_4)_2SO_4$) precipitations were preformed on adult barnacle crude extract to separate the compounds therein on the basis of solubility. Initially a 35% saturation cut was used in which 0.21 g/ml of $(NH_4)_2SO_4$ was added to the barnacle crude and stirred for one hour at 4° C. The slurry was centrifuged at 40,000 g for 15 minutes (Matsumura *et al.*, 1998). The resulting supernatant was separated for additional precipitations, and the pellet was resuspended in 50 mM Tris-HCl, pH 7.5. The remainder of the 35% supernatant was subjected to a 35% increase in $(NH_4)_2SO_4$ concentration (70% saturation total). Again, the slurry was centrifuged at 40,000 g for 15

minutes, the supernatant was separated and the pellet resuspended. This step was repeated to100% $(NH_4)_2SO_4$ saturation. All re-suspended pellets (and the 100% supernatant) were dialyzed against filtered sea water using Slide-a-lyzer © dialysis cassettes (5,000 Da molecular weight cut-off (Thermo Scientific)) before being tested against one another for activity in laboratory whelk bioassays. The active precipitate was next tested against control solutions (mussel, turban snail, and FSW) to ensure activity remained. Furthermore, the active precipitate was tested under natural conditions in the field bioassays described above. All protein concentrations were monitored by the Bradford method, and precipitates were concentrated or diluted to biologically relevant protein concentrations at that solubility level.

Identifying the biochemical nature of the active compound

Numerous biochemical components are found in both the barnacle crude extract and the active ammonium sulfate precipitate. Barnacles are, however, incredibly protein-rich (~50% total compound composition), so a logical starting point existed in exploring proteins as signal molecules. The proteins in each sample were enzymatically digested using Proteinase K (Promega Corp.; Madison, WI). As a broad-spectrum serine protease, Proteinase K predominantly cleaves the peptide bonds between the carboxylic sides of aliphatic, aromatic, and hydrophobic amino acids. This results in the breakdown of the native protein into peptide fragments which vary in length. Samples were concentrated to 5 mg of protein and exchanged into a 1 ml reaction buffer of 7 M urea, 50mM tris-HCl (pH 8), 3.5 mM DTT. The digestion proceeded following the specification of the

producer and was terminated using 5 mM phenylmethylsulfonyl fluoride (PMSF). To control for the addition of Proteinase K to our samples, we ran the same reactions only inhibited the enzyme with PMSF prior to exposure with barnacle samples; this prevented the digestion of the proteins but kept the enzyme present. Protein digestion, and Proteinase K inhibition, was confirmed using SDS polyacrylamide gel electrophoresis (SDS-PAGE). Treated and inhibited samples were dialyzed against filtered sea water using Slide-a-lyzer © dialysis cassettes (5,000 Da molecular weight cut-off (Thermo Scientific)) before being tested for activity in feeding assays. Choices were compared using maximum likelihood analysis.

RESULTS

Rocky Intertidal Distributions of Whelk and Prey

Our field surveys were conducted to characterize the natural environment, as well as to determine if species distributions, reflects whelks prey choice. Throughout the two year period, barnacle, mussel, and turban snail abundances, as well as whelk populations, remained remarkably stable at each established tidal height at all three sites. Major difference, however, were found in the percent cover of barnacles and mussels, and the populations of turban snails, between sites. For example, at Rancho San Marino (RSM) turban snail abundances were over an order of magnitude greater than at either Latigo Canyon (LC), or Broad Beach (BB). Conversely, barnacle and mussel cover at RSM

were both ≤ 5 %/m^2, whereas at both sites in Malibu barnacles and mussels dominated the high and mid to low intertidal, respectively (Fig. 1).

As with prey abundance, whelk choice remained stable throughout the survey period, with whelks of BB consistently consuming barnacles, those of LC mussels, and those of RSM turban snails. Prey size selection also remained consistent. For example, ~70% of whelks at BB were found consuming barnacles of an intermediate size (5-7mm shell height). This size class, however, does not represent the most common encountered suggesting that barnacle-feeding whelks are selective in their choice of barnacle size (Fig. 2). Unlike for barnacle-feeding whelks, those of LC where observed consistently consuming mussels of 40-50 mm (shell length), which were the most common size class encountered (Fig 3). Finally, whelks of RSM where found consuming turban snails of various sizes near-equally suggesting this population does not exhibit a size preference at this location (Fig 4). That each population of whelks consistently consumed the same prey type at each site, and that the prey chosen was the species most commonly found, is consistent with previous work suggesting whelks selectively consume the dominant basal organism of a given locality.

Prey Size Preferences- When All Things are Equal

Preference for prey of specific dimensions can be variable and depend on the dimensions of the predator. To determine if prey size selection was consistent when equal numbers of prey of differing sizes classes were presented, we used the most common size whelks found in the field (17-22 mm shell height). In laboratory choice

trials, whelks from BB exhibited a distinct and consistent preference for barnacles between 4-6 mm (G^2 = 9.19, D.F. = 2, P = 0.0101; Fig. 5). All but two whelks in the barnacle size preference trials foraged within the tanks making multiple contacts with most of the prey offered until consuming an intermediate-sized barnacle. Mussel-feeding whelks also exhibited a significant preference for intermediate mussels size (30-40 mm shell length) (G^2 = 8.51, D.F. = 2, P = 0.014, Fig. 5), although 40% chose to feed on small mussels. This result is in slight contrast to what was observed in the field suggesting preferential feeding on 30-40 mm mussels may limit the density of this size class forcing whelks to switch to a slightly larger size class. Finally, no whelks in any of the mussel feeding tests chose to eat the largest size class which may suggest large mussels reach a size refuge from predatory whelks. In contrast, whelks from RSM did not display a clear size preference and typically consumed the first turban snail contacted (G^2 = 1.38, D.F. = 2, P = 0.5; Fig. 5). Although no significant difference was found for turban snail-feeding whelks, 50% did consume the largest size class which may have resulted from an increased likelihood of contacting a larger turban snail over a smaller one.

Larger prey are more likely to be contacted by whelks in trials that keep constant the number of prey available due simply to the fact that these present a greater surface area. To correct for this, trials were run in which the surface area of prey were approximately matched. For barnacle and mussel predators, the adjustment in area available for contact did not change the preference for prey size. These whelks continued to consume significantly more intermediate sized prey (barnacle feeders: G^2 = 11.96,

D.F. = 2, P < 0.005; mussel feeders: G^2 = 9.76, D.F. = 2, P = 0.008). However, available surface area was important to turban snail predators. Whelks of RSM, in the matched surface area trials, again consumed most often the first turban snail contacted, but within these treatments even proportions of turban snail sizes classes were consumed (G^2 = 0.194, D.F. = 2, P > 0.5).

Energetics of Choice: Energy gained per unit time handled

A significant relationship between prey size and energy content (j) was found for all three species tested. Caloric content increased linearly with shell size for barnacles (r^2=0.67, n=40), mussels (r^2=0.91, n=28), and turban snails (r^2=0.77, n= 20). Similarly, handling times (H_t) for barnacles, mussels, and turban snails varied predictably. For example, the smallest of the three prey types, barnacles, took significantly less time to consume than either the mussels or turban snails (on the order of hours as opposed to days). However, small barnacles took longer to consume than intermediate barnacles, but less than the largest. This increase was due to the inability of the labial spine of barnacle-feeding whelks to effectively hold open the opercular plates of the smallest barnacles tested. For these prey, whelks resorted to drilling the sides of barnacles to reach the soft tissue, significantly increasing H_t.

A similar pattern of H_t, although not as pronounced, was found for mussel shell length. For the smallest mussels H_t was slightly greater than it was for intermediate mussels, but still less than for the largest sizes. As with barnacles, a switch in feeding strategy was observed, and those consuming the smallest mussels (< 15 mm) did so most

often by drilling, as opposed to envenomation at the site of the abductor muscle, before consumption. The distance between the open valves of the smallest mussels (while filtering) is considerably smaller than that of the intermediate or large one. In one feeding trial, the whelk repeatedly attempted to fit its proboscis between the valves but was unable to slip it in unnoticed; after many failed attempts it changed tactics and drilled the mussel.

Unlike barnacle and mussel prey, H_t for turban snail prey increased linearly with the height of the spire. In 100% of attacks whelks used drilling to access the soft tissues, and in all cases turban snails were attacked either at the second whorl or just above the operculum. Subsequent shell thickness measurements found these areas were significantly thinner than the lateral area of the first whorl.

Organisms which forage optimally choose prey that maximizes net energy gain. To determine which of the alternate whelk prey provided that greatest net energy we considered the caloric value of each prey in terms of the length of time handled. By nearly an order of magnitude barnacle prey provided the greatest energy per unit time handled (Fig. 6). The shape of the curve suggests a substantial energetic benefit is found in consuming intermediate-sized prey. This result is in agreement with our previous findings that barnacle-feeding whelks actively search out prey 4-6 mm in size under natural field conditions. Similarly, the energy gained per unit time handled for mussels revealed a trend that maximizes energy intake in the intermediary size range (25-35 mm) although substantially less energy is gained when compared to barnacles (Fig. 6). Despite this trend, the observed size chosen in the field (40-50 mm) was larger than

would be predicted based on our energy curve. It should be noted, however, that the difference between energy gains of these two mussel size classes is not substantial (~1 j•min^{-1}). Regardless of size, turban snails yielded approximately the same prey value (Fig. 6), which was slightly less than the range for mussels, but also an order of magnitude less than barnacles. These results may explain what appeared to be random feeding on turban snails in the field and lab. From the estimated prey energetic values a hierarchy in prey preference follows that barnacles >> mussels > turban snails. Furthermore, in using these energy predictions we can explore prey choice as it is observed in the field to determine if selection is done in a manner that optimizes energy intake and thus, foraging output.

Based on our energy calculations, *Acanthinucella spirata* should preferentially consume barnacles when they are available. Furthermore, to forage optimally these snails should not only go after barnacles, but should be selective in the sizes they choose so long as this size class is fairly well represented in the population. We examined, in detail, the prey selection observed at Broad Beach and found that whelks there not only preferentially feed on barnacles, but will also attack individuals of the optimal size class more frequently. These results suggest that *A. spirata* may be a more specialist predator than was previous thought, but only when prey populations allow for selection of energy-optimized quarry (Fig. 7).

Preference for Prey Type

In examining the energetic value (E/H_t) of three of the primary prey species consumed by *Acanthinucella spirata* we predicted that whelks should choose barnacles over mussels and turban snails if the net energy intake is maximized through selective foraging. In choice experiments whelks were offered equal numbers barnacles, mussels and turban snails. Whelks found consuming barnacles in the field (Broad Beach whelks) exhibited a strong barnacle preference in laboratory trials as well ($G^2 = 43.94$, D.F. = 2, $P < 0.0001$; Fig. 8). The preference for barnacles also was highly significant for whelks that consume mussels (Latigo Canyon whelks) and turban snails (Rancho San Marino whelks) ($G^2 = 34.94$, D.F. = 2, $P < 0.0001$; $G^2 = 8.49$, D.F. = 2, $P = 0.014$, respectively; Fig. 8). Furthermore, in feeding trials where whelks were offered non-optimal barnacle sizes (1-3 mm or >7 mm) along with energy-maximizing sizes of alternate prey the strong preference for barnacles remained consistent. These results suggest that predatory whelks are able to identify prey types, and when offered a choice will consume that food which offers the highest energy return.

Role of Ingestive Conditioning

Prior and consistent food choices have been known to affect current and future selections in a variety of organisms. We designed an experiment to determine if a consistent diet of one prey item would affect future choice. Our results for *Acanthinucella spirata* suggest that it does not. Feeding began on barnacles within the first four days for all whelks that were presented this food type, and continued at a rate of ~1 intermediate

sized barnacle every 4 days (time to consume 1 barnacle = ~0.31 days). Whelks that had fed consistently on a diet of barnacles (Broad Beach snails) were the most resistant to switch to the alternate prey type. It took approximately 2 weeks for these snails to switch to mussel prey, and 3 weeks to switch to turban snail prey, a result that is consistent with our prey hierarchy. In all tanks these whelks were observed to forage extensively for extended periods of time without selecting the alternate food, considerably longer than was observed for the other populations. Feeding initiation for whelks that eat mussels in the field (Latigo Canyon snails) took on average 7 days, but remained consistent throughout the period of conditioning at a rate of ~ 1 mussel every 20 days (time to consume one mussel = ~3.12 days). Although these whelks readily consumed barnacles and mussels within the first week of conditioning, feeding on turban snails did not begin until the middle of the 2nd week. Feeding rates of Latigo Canyon whelks on turban snails averaged approximately 1 turban snail every 12 days (time to consume one turban snail = ~4 days). Finally, whelks that feed on turban snails in their natural habitat took on average only 4 days to initiate feeding on turban snails, but resisted feeding on mussels for 2 weeks. Feeding on mussels remained constant at a level of ~ 1 mussel every 19 days (time to consume one mussel = ~3.75 days), which was consistent with the other two populations of whelks.

The established preference for barnacle prey remained consistent and significant after 8 weeks of ingestive conditioning with alternate prey for all whelks originally found to be barnacle-feeders. In 100% of trials these whelks continued to eat, or switched back to, barnacle prey as soon as it was available again further supporting the strong

preference for this prey type observed before the ingestive conditioning (barnacle-feeders fed barnacles: G^2 = 10.99, D.F. = 2, P = 0.004; barnacle-feeders fed mussels: G^2 = 10.99, D.F. = 2, P = 0.004; barnacles-feeders fed turban snails: G^2 = 10.99, D.F. = 2, P = 0.004; Table 1). A difference in foraging behavior was observed for all members of the group fed only turban snails. The foraging time of this group was markedly reduced, and each whelk was observed to consume the first barnacle contacted as opposed to foraging in the tank and contacting each available prey item. A similar pattern was found for mussel-feeding whelks collected from Latigo Canyon, in that 100% of these whelks continued to feed on, or switched to, barnacle prey after the 8 weeks (mussel-feeders fed barnacles: G^2 = 10.99, D.F. = 2, P = 0.004; mussel-feeders fed mussels: G^2 = 10.99, D.F. = 2, P = 0.004; mussel-feeders fed turban snails: G^2 = 10.99, D.F. = 2, P = 0.004; Table 1). The only group of whelks which did not feed exclusively on barnacle prey post-ingestive conditioning was those collected from Rancho San Marino, although barnacles were chosen significantly more than either of the alternate prey. Those turban snail-feeders conditioned with barnacles consumed this prey type in 80% of the trials and turban snails in 20% (G^2 = 5.98, D.F. = 2, P = 0.05; Table 1), values which are equivalent to what was observed in the initial prey choice experiments. Turban snail-feeders conditioned with mussels switched to barnacle prey when offered a choice 100% of the time (G^2 = 10.99, D.F. = 2, P = 0.004; Table 1), and those which were conditioned with turban snails chose barnacles significantly more than the alternate food items (G^2 = 5.98, D.F. = 2, P = 0.05; Table 1).

Foraging Response of Whelks to Waterborne Signals

A primary focus of our study was to determine if whelks use a chemical signal to find barnacle prey. The rocky intertidal environment of exposed coasts is often a turbulent habitat, and to maximize the likelihood of getting a response from the *Acanthinucella spirata* our trials were run on a series of hydrodynamically-benign days. Despite the calm sea state whelks did not track to source water originating 5 mm above an actively feeding barnacle bed any more than they did water originating from 5 mm above a control sand patch ($G^2 = 0.076$, D.F. = 1, P = 0.78; Fig. 9). In 60 trials only 8 whelks (13.3%) reached the source when it was barnacle water and 7 (11.6%) when the source water was from a control sand patch. Conversely, significantly more whelks responded, 24 out of 60 (40%), when crushed barnacles were placed in the forward portion of each chamber and combined with barnacle bed water when compared to barnacle bed water alone ($G^2 = 29.836$, D.F. = 1, $P < 0.001$). A similar response was observed with whelks when crushed barnacles were added to control sand patch water. In these trials 19 out 60 (31.6%) whelks reached the source, which was significantly more than for sand patch water alone ($G^2 = 43.375$, D.F. = 1, $P < 0.001$). No significant difference, however, was found when the responses of whelks from barnacle bed water and crushed barnacles were compared to that of whelks from sand patch water and crushed barnacles ($G^2 = 9.08$, D.F. = 1, P = 0.34) indicating the whelks were responding to the crushed barnacles rather than a waterborne signal.

Response of Whelks to Contact Cues

In order to determine if whelks would respond to contact signals we used a purified barnacle crude extract imbedded in carboxymethyl cellulose (CMC), creating "faux prey", to be tested in our bioassays. Examination of the constituent compounds of the barnacle crude extract revealed that it was principally composed of protein (50 % protein, 25.6 % carbohydrate, 4.8% lipid; protein, ash, acid hydrolysis determination respectively (Eurofin Corp. Des Moines IA)) and so proteins content was used scale faux prey. Additional, from 10 crude protein extractions it was determined that one intermediate barnacle contained, on average, 1.289 mg of total protein; all feeding experiments on the crude extract were scaled to this protein concentration.

In all feeding trials whelks were observed foraging in the tanks contacting the majority of faux barnacle prey before making a selections and feeding. Of the whelks that fed, 100% chose to feed on, and consumed in its entirety, faux prey imbedded with barnacle crude extract (Fig. 10). Although we designed our feeding trials to be as free from artifact as was possible selection under laboratory conditions may not reflect what would happen under natural field conditions. When feeding trials were performed at Broad Beach under natural conditions whelks still chose to consume 100% of our faux prey imbedded with crude barnacle extract (Fig. 10).

The strong results obtained in our contact feeding bioassay prompted us to begin searching for the active molecule that underlies recognition. As is typical in initial biochemical purifications, we chose to separate our active barnacle crude extract using ammonium sulfate ($(NH_4)_2SO_4$) precipitation. Separations were preformed in which

$(NH_4)_2SO_4$ was added in increasing concentrations (0-35%, 36-70%, 71-100% saturation) causing compounds to precipitate based on their solubility. Each resulting precipitate and supernatant was then tested against our control solutions in bioassay until the activity was isolated in single precipitation. The active compound that induces whelk feeding was found in our 35-70% $(NH_4)_2SO_4$ pellet cut, and whelks presented these gels consumed significantly more faux prey than the controls in both the lab and the field ($G^2 = 22.00$, D.F. = 3, $P < 0.001$; $G^2 = 30.00$, D.F. = 3, $P < 0.001$, respectively; Fig. 11). Additionally, the concentration of $(NH_4)_2SO_4$ that precipitated the active cue is indicative of a compound that has a low solubility and, thus, would be surface-bound further suggesting that whelks utilize a contact cue rather than a waterborne signal. From 10 replicate precipitations it was determined that 42% of the total crude proteins precipitated out in this cut. Feeding trials were scaled to reflect this concentration.

Biochemical identity of the active compound

Compositional analysis of our active ammonium sulfate ($(NH_4)_2SO_4$) precipitate revealed that this fraction was made almost entirely of protein (96.2% protein, 3.7% carbohydrate, < 1% lipid; protein, ash, acid hydrolysis determination respectively (Eurofin Corp. Des Moines IA)) strongly suggesting that our active whelk feeding cue was going to be proteinaceous in nature. To test this we used a broad-spectrum serine protease, Proteinase K, to break apart all the proteins found in our active crude extract. The effectiveness of each reaction was monitored using SDS-PAGE, and protein bands in the treated crude extract subsequently did not appear in gels after Proteinase K treatment

(peptide smears where identified, as would be expected). In feeding bioassays whelks presented with Proteinase K denatured crude were unable to identify faux prey as food and thus failed to consume a single one. With no preferred barnacle prey proteins detectable in these feeding trials whelks fed on control gels, but failed to consume one type significantly more than another (Fig. 12). To control for the addition of Proteinase K we subsequently ran feeding trials in which the reactions were carried out in the same manner except that the Proteinase K was inhibited using PMSF prior to the reactions. The results of these reactions were monitored using SDS-PAGE as before, and protein bands appeared at the same molecular weight as non-treated crude extract. Following this, we presented faux prey imbedded with the inhibited Proteinase K treated crude to whelks alongside controls. Whelks consumed significantly more faux barnacles made with inhibited Proteinase K then they did controls ($G^2 = 16.40$, D.F. = 3, $P < 0.001$; Fig. 12). These results strongly suggest that foraging whelks utilize a protein signal of low solubility (surface-bound) to identify preferred, energy-rich, barnacle prey, and that the detection of this cue drives prey choice.

DISCUSSION

The energetics of prey choice

Throughout the course of our experiments the densities of each prey species examined (barnacles, mussels, and turban snails) remained relatively unchanged in each area where whelks forage. Furthermore, whelks consistently chose the dominant prey

species available at a site. This result is consistent with that of Murdoch (1969) who determined that *A. spirata*, as a general predator, will consume the dominant prey item at a location, switching only when prey densities fell below a given encounter rate. Despite this consistency, the present study showed significant differences in prey preference when whelks were offered prey choices.

A strong preference for barnacle prey was identified for all whelks regardless of collection site or previous diet. This is best explained by the fact that whelks appear to optimize energy intake by choosing barnacles which offer an order of magnitude more energy per unit time handled in comparison to mussels or turban snails (Fig. 6). From these experiments a strict hierarchy in prey selection was established for *A. spirata* in which barnacles >> mussels > turban snails. In contrast, Murdoch found that *A. spirata* would consume only the prey they had been conditioned with and exposed to (e.g. *Mytilus edulis* or *Balanus glandula*). In the present study, the mussel species consumed by *A. spirata* was universally *Mytilus californianus*, whereas the mussel predominantly used by Murdoch was *Mytilus edulis*. *Mytilus californianus* is typically found on the open coast, exposed to large waves (and the projectile objects within them), as well as to predation by crabs and other snails, therefore this species will grow thicker shells than *M. edulis*, which inhabits more sheltered environments (Shanks and Wright, 1986; Rabenheimer and Cook 1990; Blanchette *et al.*, 2007). As an inducible defense, these thicker shells may be an effective selection deterrent relative to other, more easily handled, prey. The thinner shells of *M. edulis* may be easier to penetrate or crack than

those of *M. californianus*, potentially explaining the relatively equal preference of whelks for barnacles and mussels in Murdoch's study.

The large difference in available caloric content per unit time handled between each prey species likely drives the foraging pattern of predatory whelks. In most cases whelks were observed to randomly move around the tanks and sample most of the available quarry before making a choice on what prey to consume. In addition to a general preference for barnacles, a discrete preference for the intermediate size class, which further maximized the energy gained, suggested that whelks search for prey yielding the greatest energy for the least effort. A single variable accounts for this result- it takes substantially longer (on the order of days as opposed to hours) to consume a mussel than it does a barnacle. As second-tier consumers, whelks must also limit their exposure to other predators such as crabs and sea stars. Although a whelk may get more calories from a single mussel than it would from a barnacle, in doing so it increases its predation risk. Therefore, there is a fitness consequence, in addition to energetic one, associated with consuming barnacles over mussels. With dietary preferences that maximize energy intake and reduce predation risk, equal encounter and success rates, and what appear to be instant recognition of energy-optimizing prey, whelks appear to be optimally foraging (Hughes, 1979; Burrows and Hughes, 1990).

Hierarchical preferences, as found for *Acanthinucella spirata*, are often strongly influenced by the availability of prey at a given location. When a preferred prey is not available, the predator switches to a prey of lesser value. Murdoch (1969) found that switching in whelks would take place when the density of the preferred species dipped

below that of a less-preferred species because encounter rates of the second species would be higher. This scenario is likely for predators which do not exhibit strong prey preferences and may induce community stability (Post *et al.*, 2000). Results from our ingestive conditioning experiments, however, indicate that whelks do not readily switch to a sub-optimal food source. For example, barnacle-feeding whelks presented with either mussels or turban snails were highly resistant to prey switching. These individuals continuously foraged for the preferred prey for a period of time (up to (in some cases) 3 weeks) before switching to an alternative food. Thus for innate barnacle feeders, these results are consistent with Murdoch's in that previous diet appeared to influence the foraging behavior of whelks. Unlike Murdoch's findings both mussel- and turban snail-feeding whelks readily switched to barnacles when presented, further establishing barnacles as the prey of choice for *A. spirata*. When barnacle populations are low *A. spirata* will switch to an alternative prey, however, that threshold density of barnacles is yet to be determined.

A sensory basis for prey selection and the role of contact cues

The recognition of a chemical signal(s) often underlies and influences foraging for a preferred prey item. Thus, a main goal of this study was to determine if whelks use a cue to identify live barnacles in their environment. A cursory examination of foraging whelks revealed a telling behavior– as whelks search for food they periodically pat the substrate with their tentacles. Furthermore, as whelks move forward they sweep side to side in order to sample a swath of substrate. These behaviors are characteristic of an

organism searching for a chemical stimulus which is likely to be adsorbed on the substrate. Subsequent experiments were aimed at identifying a signal recognized by whelks that indicates the presence of an energy-maximizing barnacle. A logical starting point in this examination was to explore the role of waterborne versus contact cues.

The rocky wave-swept intertidal of exposed coasts is a hydrodynamically turbulent environment which can experience water velocities that exceed 25 m s^{-1} (Denny *et al.*, 2003). Although this figure almost certainly represents an extreme, it none-the-less highlights the potential of near-complete signal dilution in close proximity to an odor's source. Weissburg and Zimmer-Faust (1993), for example, experimentally demonstrated that the success of blue crab chemo-orientation was diminished during periods of relatively high flow speeds. Their experiments, designed to mimic estuarine habitats, found that at a free-stream velocity of 14.4 cm s^{-1} predatory success was only 10%, conditions for which prey may find a refuge from odor plume-tracking predators. Free-stream flow speeds frequently exceed 14.4 cm s^{-1} in the rocky intertidal and so dilution of any waterborne signal would happen very close to a point source. To test this hypothesis, foraging whelks were exposed to water originating from dense barnacle beds. These experiments were conducted on a series of calm summer days to reduce the likelihood of signal dilution due to turbulent mixing. Nevertheless, whelks were no more inclined to track to the barnacle bed source water than control sand-patch water. These experiments strongly suggest that whelks are not reliant on waterborne signals to orient and/or find preferred barnacle prey.

The lack of response to waterborne signals, as well as the characteristic sampling of the substrate exhibited by foraging whelks, indicated the likely role of contact signals in prey identification. Extracted barnacle compounds, at ecologically realistic concentrations, induced whelk feeding in significantly more trials than did filtered sea water, extracted mussel- or turban snail compounds. Because the pore size of carboxymethyl cellulose gel is ostensibly smaller than the compounds embedded in it, these results indicate that whelks use surface-bound contact signals to identify live adult barnacles. Furthermore, when active barnacle crude extract was subjected to ammonium sulfate precipitation and fractionation, all activity was retained in the 35-70% saturation level demonstrating that the active compound is of low solubility. The strong feeding response exhibited by whelks to these two treatments is a clear indication that this predator is reliant on surface-adsorbed signals to locate preferred prey.

The activity of contact- as opposed to waterborne cues in the rocky intertidal is consistent with the hydrodynamic environment. However, an obvious question remains in terms of foraging– how, if removed from barnacle beds, do whelks then re-locate their prey patches? The answer to this question may have been indirectly identified in work preformed by Vadas *et al.* (1993) with their vertical linear array (VLA)– an experimental set-up designed to simulate the vertical environment of many dog whelks (*Nucella lapillus*). Much like *N. lapillus*, *Acanthinucella spirata* is a vertical migrator. Once removed from a barnacle patch, *A. spirata* crawl in a seemingly random pattern until a vertical wall is contacted at which point the whelk crawls up (author's personal observation). From this behavior patterns of motion were identified in which whelks

respond negatively to geotaxis when submerged for periods > 6 hours, and positively to geotaxis if exposed to air for > 6 hours. These behaviors were noted in the study by Vadas *et al.* (1993) and were the reasoning for using the VLA; these same behaviors were frequently observed win *A. spirata* in our holding tanks. Furthermore, the use of these patterns of motion allows whelks to attain a vertical position within the intertidal that will place them in the zone most populated with a given prey.

The combination of energy-based barnacle preferences, active feeding when surface-bound protein signals are contacted, and geotactic responses, supports a model describing predatory whelk search behavior on wave-swept shores. For foraging whelks, patterns of motion are governed by vertical migration mediated by geotaxis. Reliance on this migratory pattern ultimately places whelks within the vertical zone of barnacles. Local patterns of motion then are dictated by contact chemoreception in which a hungry whelk will begin feeding once the active barnacle protein is contacted. Furthermore, a satiated whelk could thus utilize the contact signal to align itself in refuge habitat that is in, or in close proximity to, a food source patch, minimizing its exposure to other predators. This model explains, in simple terms, the observed migratory and foraging behavior of whelks in both the lab and the field, and may be applicable to other contact cue-driven predators.

The role of proteins in whelk-barnacle interactions

Many interactions of ecological significance are mediated by chemistry. The identification of the signal molecules that drive these relationships provides valuable

insight into the underlying process that couple the external environment to the central nervous system, and finally to the behaviors which are the foundation of species interactions. Initial surveys of the active crude barnacle extract revealed that it was ~50% protein. Furthermore, whelks were shown to consume prey mimics when ecologically realistic concentrations were detected. Using Proteinase K, the protein was successfully digested into small peptide fragments which were used in feeding assays. Results of these trials revealed that whelks did not recognize or consume the faux prey containing digested proteins, unequivocally showing the active signal is an intact protein. This is one of only a handful of examples in which a protein has been identified as a feeding cue.

The essential building blocks of all organisms are proteins. However, the vast majority of proteins remains within cells and so are not readily available for detection by predators. Barnacles, like other shelled organisms, produce proteins that are requisite to biomineralization of shell and cuticle (Marin and Luquet, 2004). These proteins (many of which belong to macroglobulin families) would be a good target for predator recognition. Furthermore, with barnacles actively producing cuticle as they grow, these signals would be available for contact on the outer surfaces of adult barnacles where they could be readily detected by foraging whelks. In fact, Dreanno *et al.* (2006) identified a glycoprotein complex found in the barnacle, *Balanus amphitrite*, as the settlement cue for conspecific larvae. They suggest that this glycoprotein is associated with the production of shell and cuticle, but it is also found in significant concentrations adsorbed to the shell where it may act as a contact signal. The glycoprotein complex exhibits most of the traits identified in our whelk feeding cue (i.e. low solubility, contact-recognized) and so may

be a similar protein to what was found in *Balanus glandula*. Our subsequent research is focused on the full identification, characterization, and role of the active glycoprotein (or homolog) within an ecological context.

The ecological and experimental implications of signal recognition

The top-down role of predators (especially those which play keystone roles) in influencing the distribution of other species within a habitat has been a perennial theme in ecology. Despite the importance of predation to community structure, little work has examined the underlying signals that allow marine predators to find prey. Without this piece of the puzzle ecology lacks a critical component that links environmental stimulus space to cellular recognition mechanisms that, in turn, drive individual behaviors. Here, we have identified a protein signal produced by barnacles that whelks utilize as an indicator of preferred, energy-rich, prey. This signal serves as the underlying mechanism that elicits prey selection, thereby driving a critical ecological interaction, and its cascading consequences, between a foundation species (barnacles) and a keystone predator (whelks).

In addition to the role this surface-bound protein plays in whelk-barnacle interactions, it also provides insight into the dynamics of signal production and detection within the rocky wave-swept environment. With flow speeds often exceeding 1 m s^{-1}, waterborne signals may play a role in only those species interactions that occur over very short spatial scales, leaving many organisms heavily dependent on contact cues. Therefore, environmental context needs to be heavily considered when looking at the

responses of organisms to waterborne stimuli. A prime example of this is seen in the work by Smee *et al.* (2008) when they showed that increased turbulence diminished the response of clams to blue crab effluent. They noted that the response of organisms to waterborne chemical stimuli is contingent upon the species interacting, the sensitivity of those species to a specific signal, and the risk/reward associated with response. With few exceptions, however, contact cue recognition is likely to be prevalent in wave-swept environments and should be considered when exploring density- and trait-mediated effects of species interactions.

Recent studies aimed at trait-mediated indirect interactions in which a chemical stimulus induced behavioral responses that changed rates of growth, feeding, and/or refuge habit seeking of primary producer/basal species density may represent over estimations (Peacor and Werner, 2000; Trussel *et al.*, 2003; Trussel *et al.*, 2006). The basic experimental design of these studies, in which predators, second-tier consumers, and foundation species have been placed inside mesocosms, may force a response that is ecologically irrelevant. This artifact may be especially true for studies examining these responses in intertidal habitats. Keeping predators caged upstream of consumers for extended periods of time, as is common in mesocosm experiments, likely leads to an artificial increase in predator effluent. Furthermore, feeding those predators (especially if they are general predators) a constant diet of second-tier conspecifics ultimately would induce a response of foragers downstream. Having shown that whelks do not readily respond to waterborne signals under natural conditions provides an alternative interpretation of these results. It should be noted, however, that there likely exists trait-

mediated responses to higher-order predators in the intertidal, but that these probably occur on small spatial scales and over short time periods (e.g. only when that predator is present and close by).

Species interactions are the foundation of community dynamics. Investigations leading to the better understanding of the ways in which these interactions occur, the mechanisms that drive them, and the community-level consequences are critical to understanding ecological dynamics. Future studies, especially in marine environments, should focus on interactions as they progress from stimulus space, to cellular level recognition, to behavior driven response, and finally to the cascading effects of that behavior. From this approach, accurate models can be developed in which there is a further understanding of how species interactions are driven, as well as what elicits them. The present study attempted to do this by examining a species relationship known to structure communities (whelk-barnacle), as well as to establish a prey preference rooted in maximizing energy intake per unit time handled. In doing so a protein signal was identified that rests at the heart of an important ecological interaction. Future examination into signals of this sort will likely uncover many more.

Table 1: Results of prey choice trials after 8 weeks of ingestive conditioning. Despite forced feeding, preferences for barnacle prey remained for each whelk type.

Post-Ingestive Conditioning Prey Choice

Predator Type	Fed Only	Proportion Barnacles Consumed	Proportion Mussels Consumed	Proportion Turban Snails Consumed
Barnacle Feeders	Barnacle	**1**	0	0
	Mussel	0	0	0
	Turban Snail	0	0	0
Mussel Feeders	Barnacle	**1**	0	0
	Mussel	0	0	0
	Turban Snail	0	0	0
Turban Snail Feeders	Barnacle	**0.8**	**0.2**	0
	Mussel	**0.9**	**0.1**	0
	Turban Snail	0	0	0

Figure 1

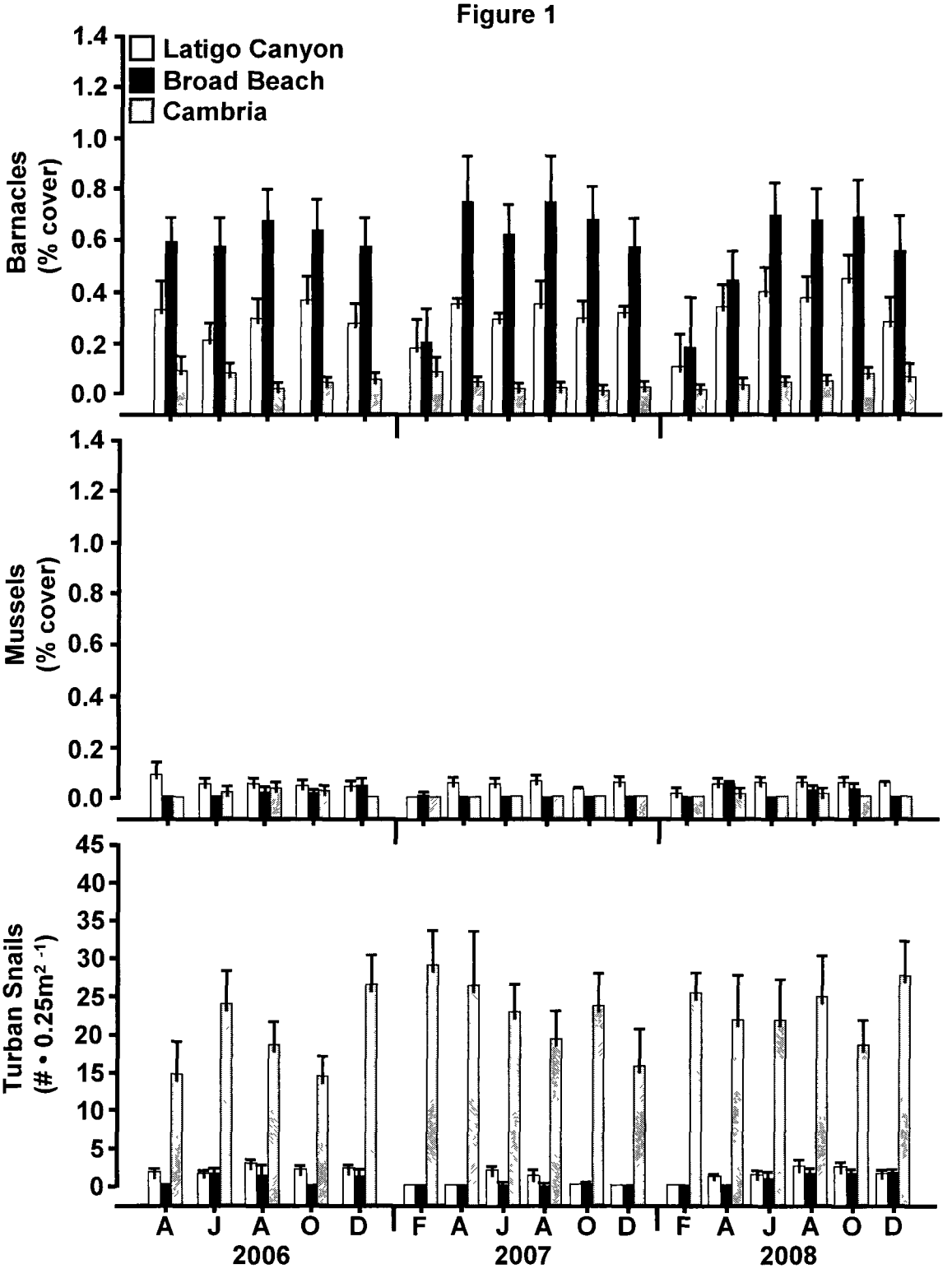

Latigo Canyon
Broad Beach
Cambria
Barnacles (% cover)
Mussels (% cover)
Turban Snails (# • 0.25m^{2} $^{-1}$)
A J A O D F A J A O D F A J A O D
2006
2007
2008

Figure 2

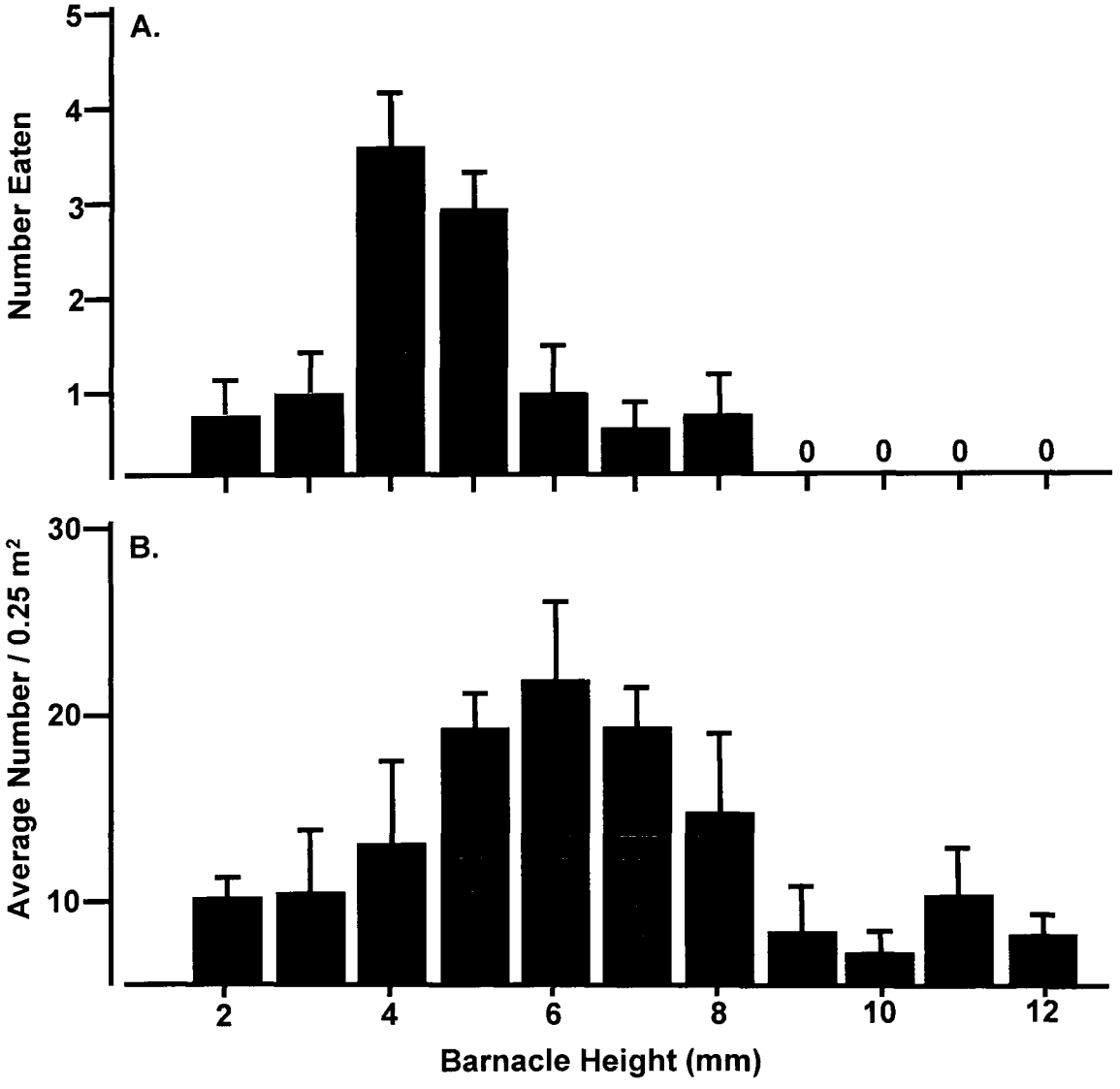

A.
Number Eaten
5
4
3
2
1
0
0
0
0
B.
Average Number / 0.25 m²
30
20
10
2
4
6
8
10
12
Barnacle Height (mm)

Figure 3

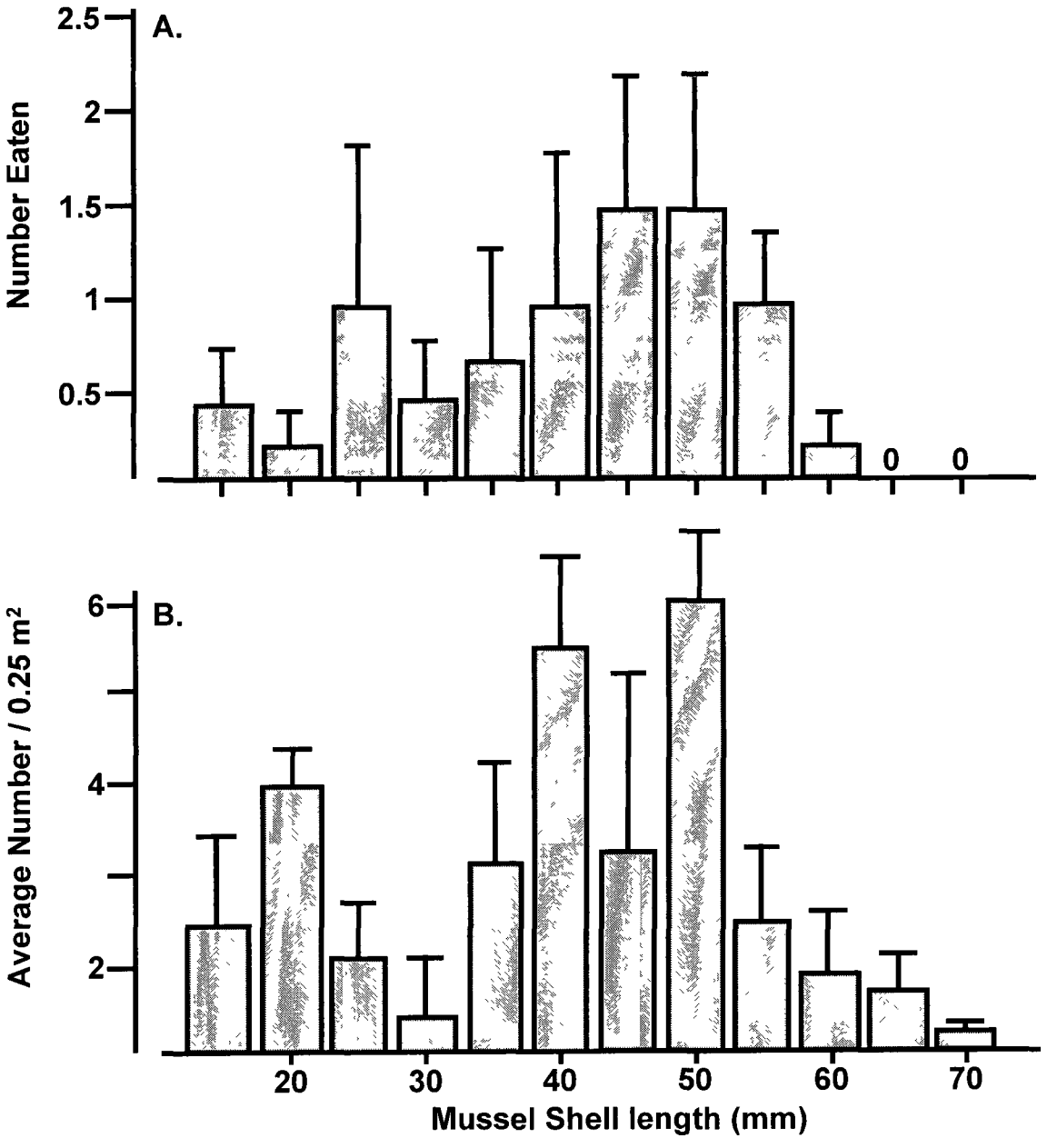

A.
Number Eaten
2.5
2
1.5
1
0.5
0
0
B.
Average Number / 0.25 m²
6
4
2
20
30
40
50
60
70
Mussel Shell length (mm)

Figure 4

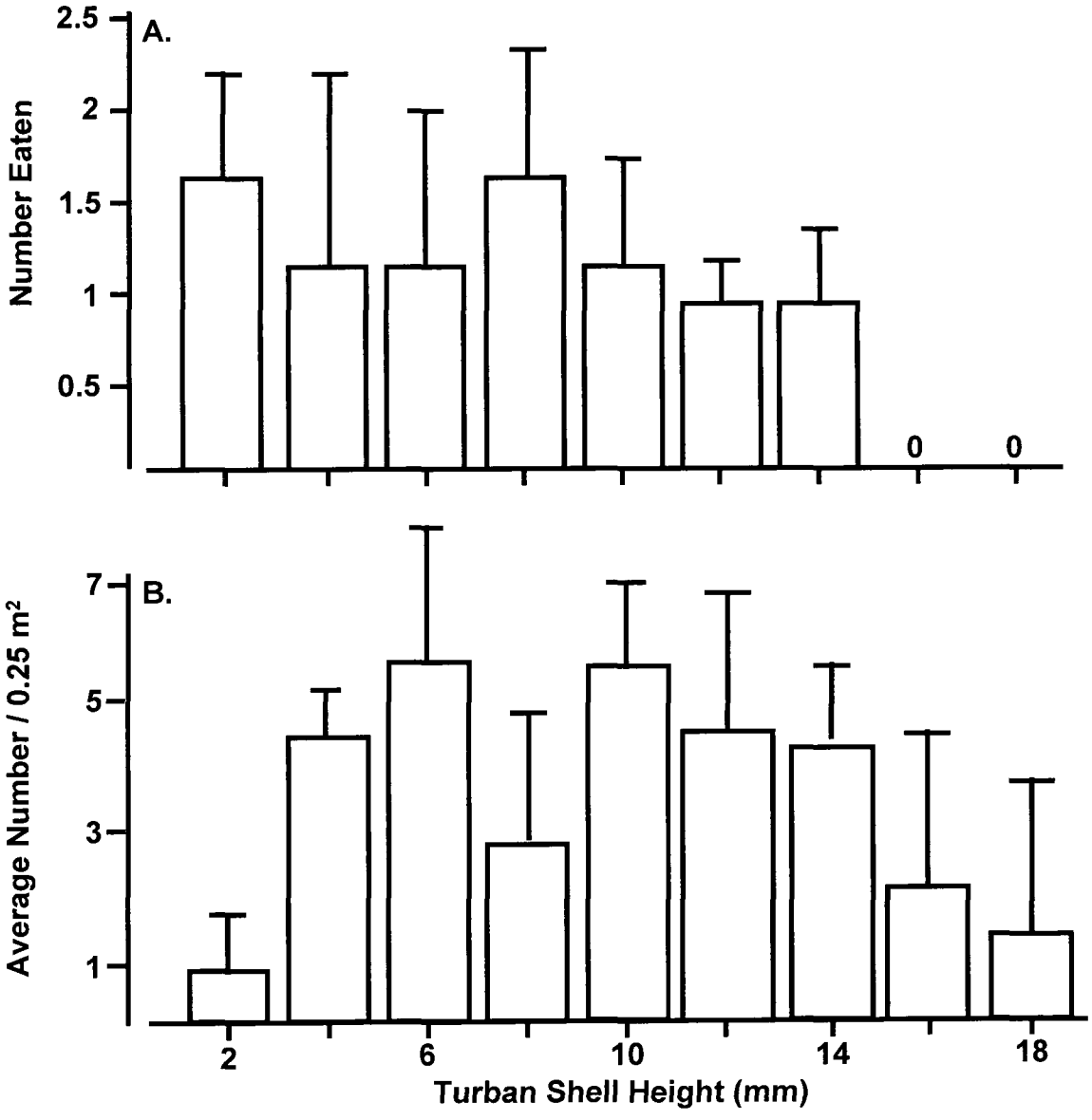

A.
Number Eaten
2.5
2
1.5
1
0.5
0
0
B.
Average Number / 0.25 m²
7
5
3
1
2
6
10
14
18
Turban Shell Height (mm)

Figure 5

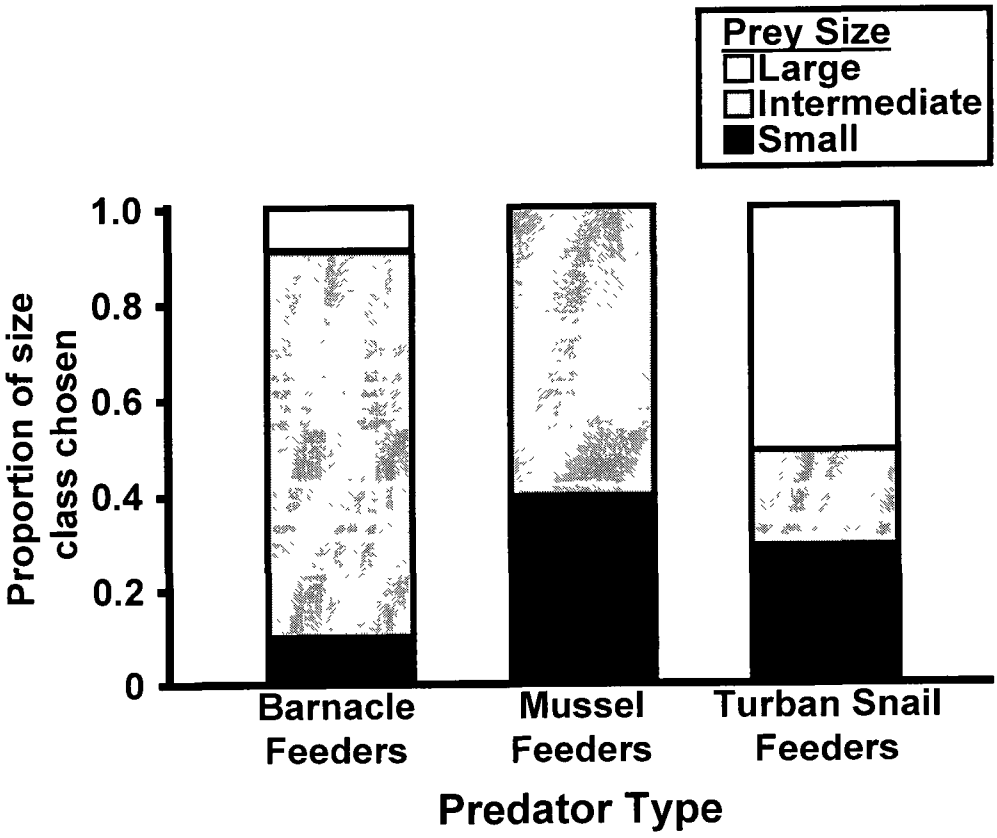

Prey Size
Large
Intermediate
Small
1.0
0.8
0.6
0.4
0.2
0
Proportion of size class chosen
Barnacle Feeders
Mussel Feeders
Turban Snail Feeders
Predator Type

Figure 6

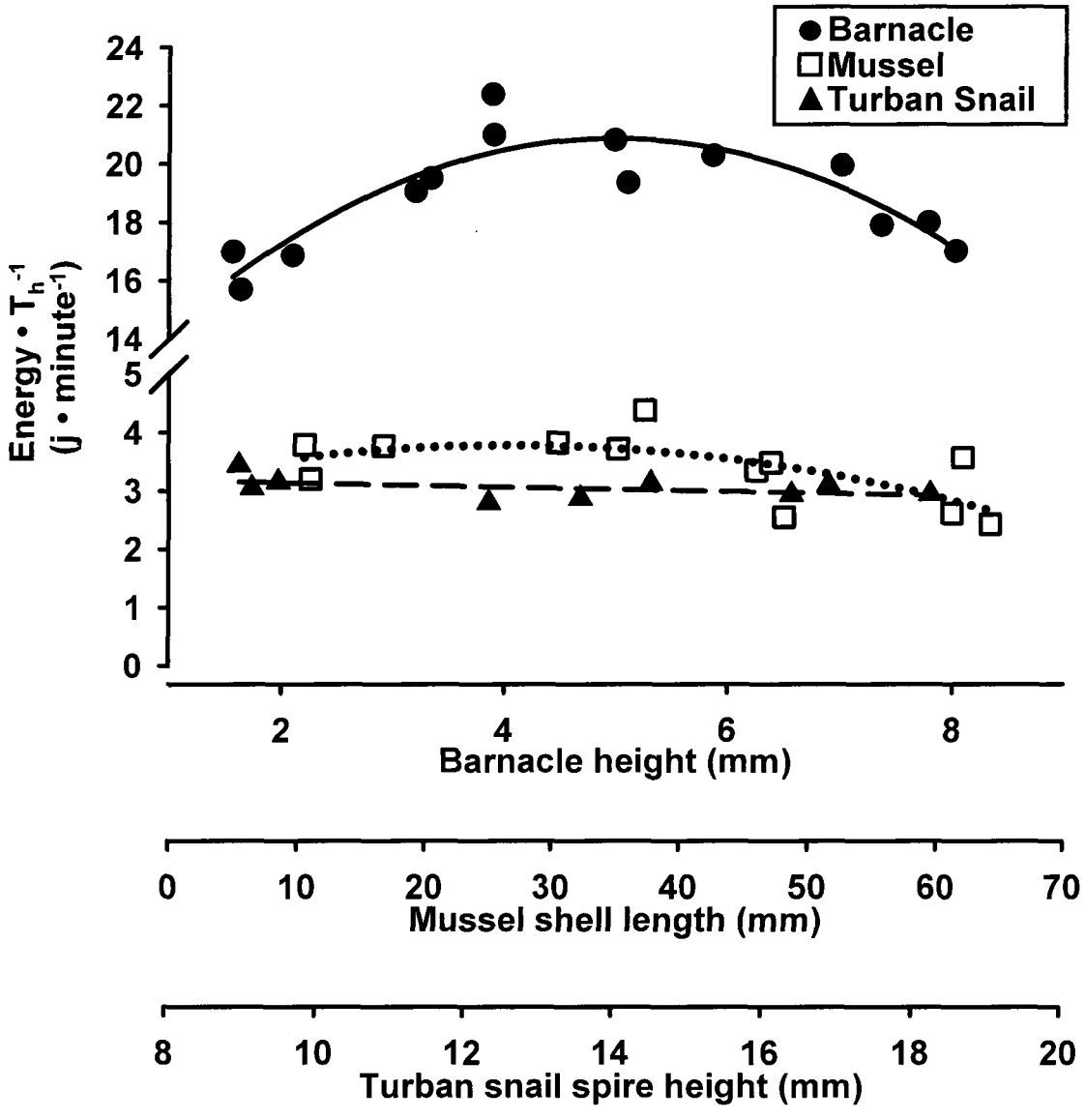

Barnacle
Mussel
Turban Snail
Energy • T_h^{-1} (j • minute^{-1})
24
22
20
18
16
14
5
4
3
2
1
0
2
4
6
8
Barnacle height (mm)
0
10
20
30
40
50
60
70
Mussel shell length (mm)
8
10
12
14
16
18
20
Turban snail spire height (mm)

Figure 7

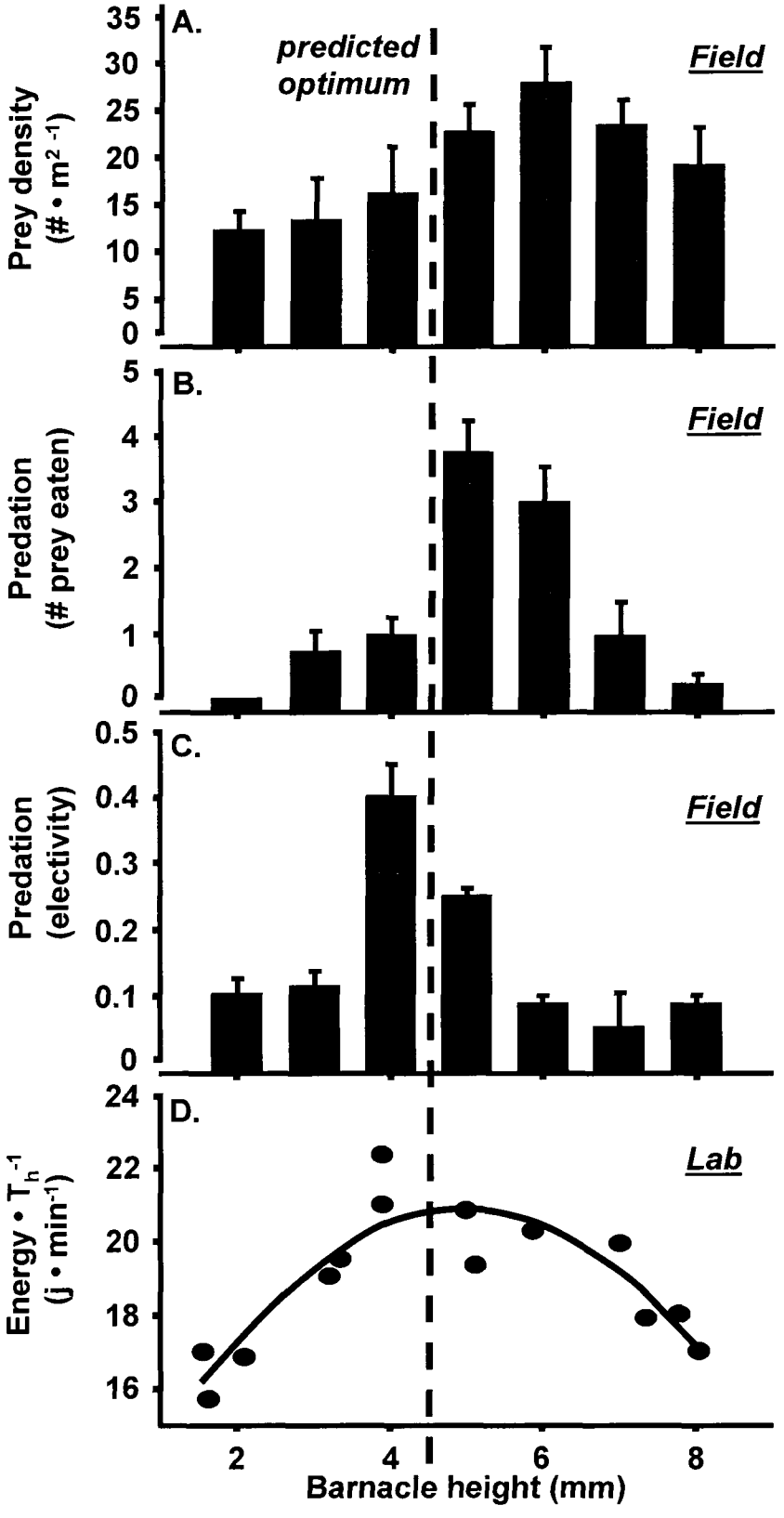

A.
predicted optimum
Field
Prey density (# • m^{-2})
35
30
25
20
15
10
5
0
B.
Field
Predation (# prey eaten)
5
4
3
2
1
0
C.
Field
Predation (electivity)
0.5
0.4
0.3
0.2
0.1
0
D.
Lab
Energy • T_h^{-1} (j • min^{-1})
24
22
20
18
16
2
4
6
8
Barnacle height (mm)

Figure 8

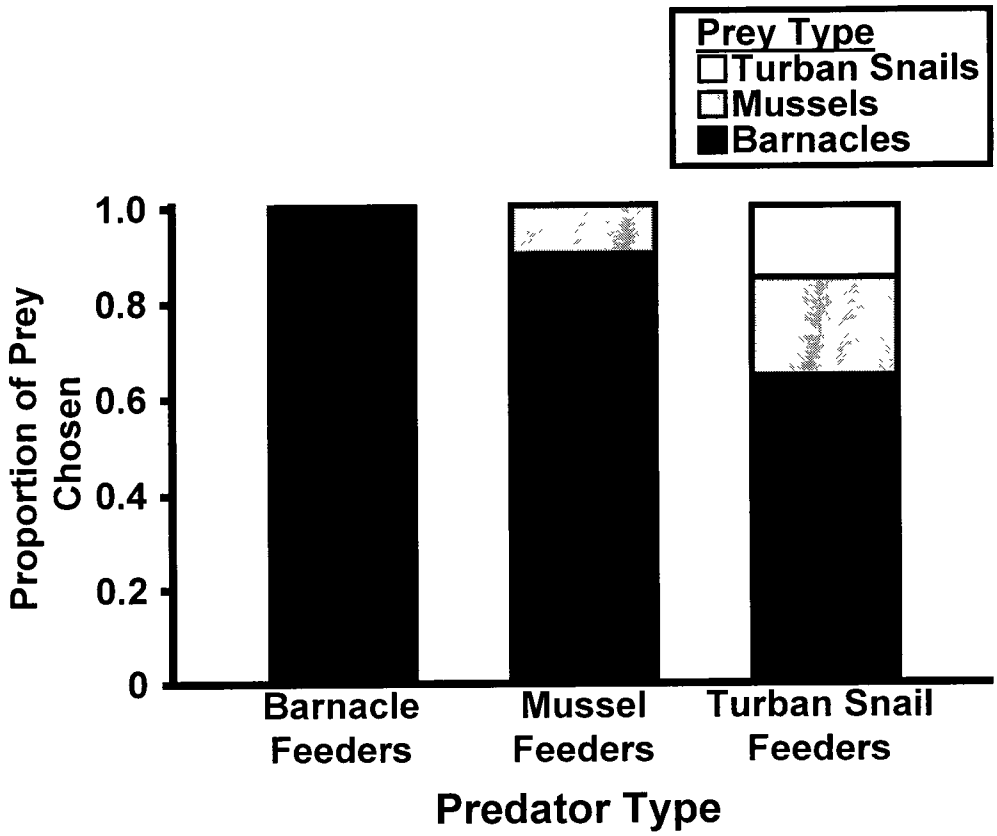

Prey Type
Turban Snails
Mussels
Barnacles
1.0
0.8
0.6
0.4
0.2
0
Proportion of Prey Chosen
Barnacle Feeders
Mussel Feeders
Turban Snail Feeders
Predator Type

Figure 9

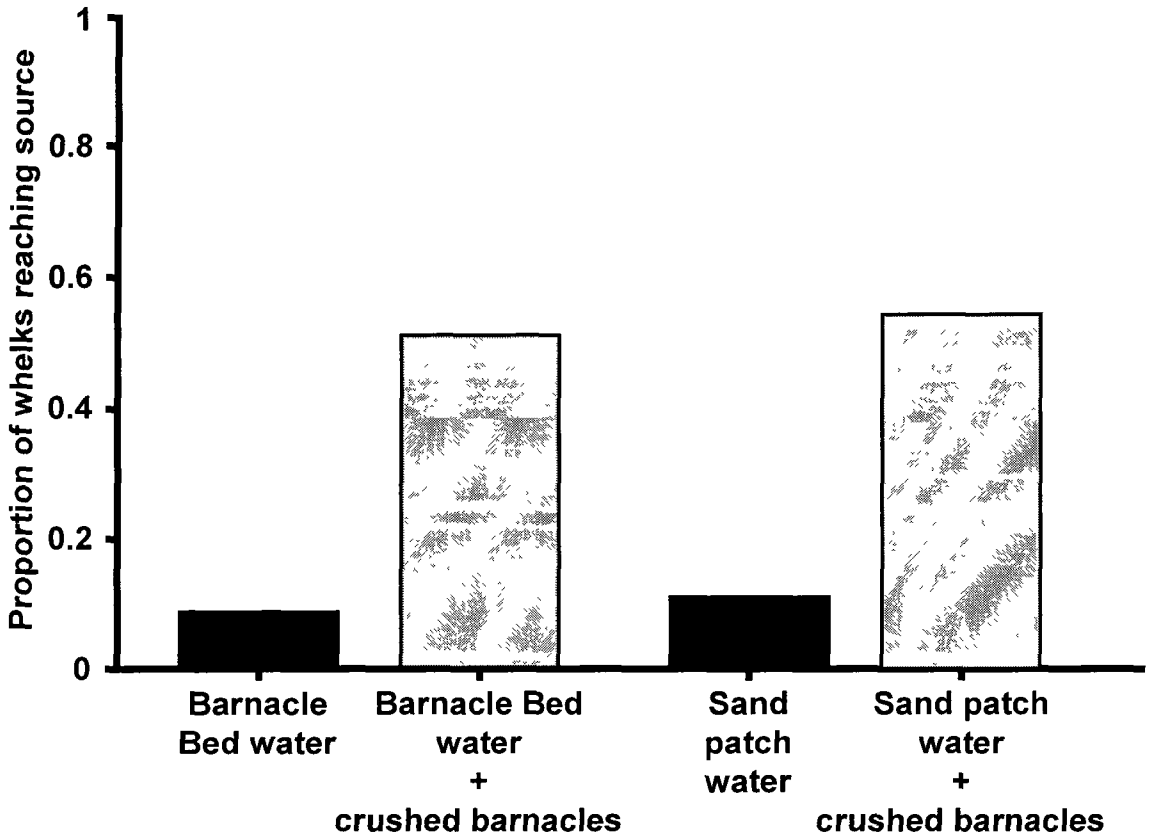

Proportion of whelks reaching source
1
0.8
0.6
0.4
0.2
0
Barnacle Bed water
Barnacle Bed water + crushed barnacles
Sand patch water
Sand patch water + crushed barnacles

Figure 10

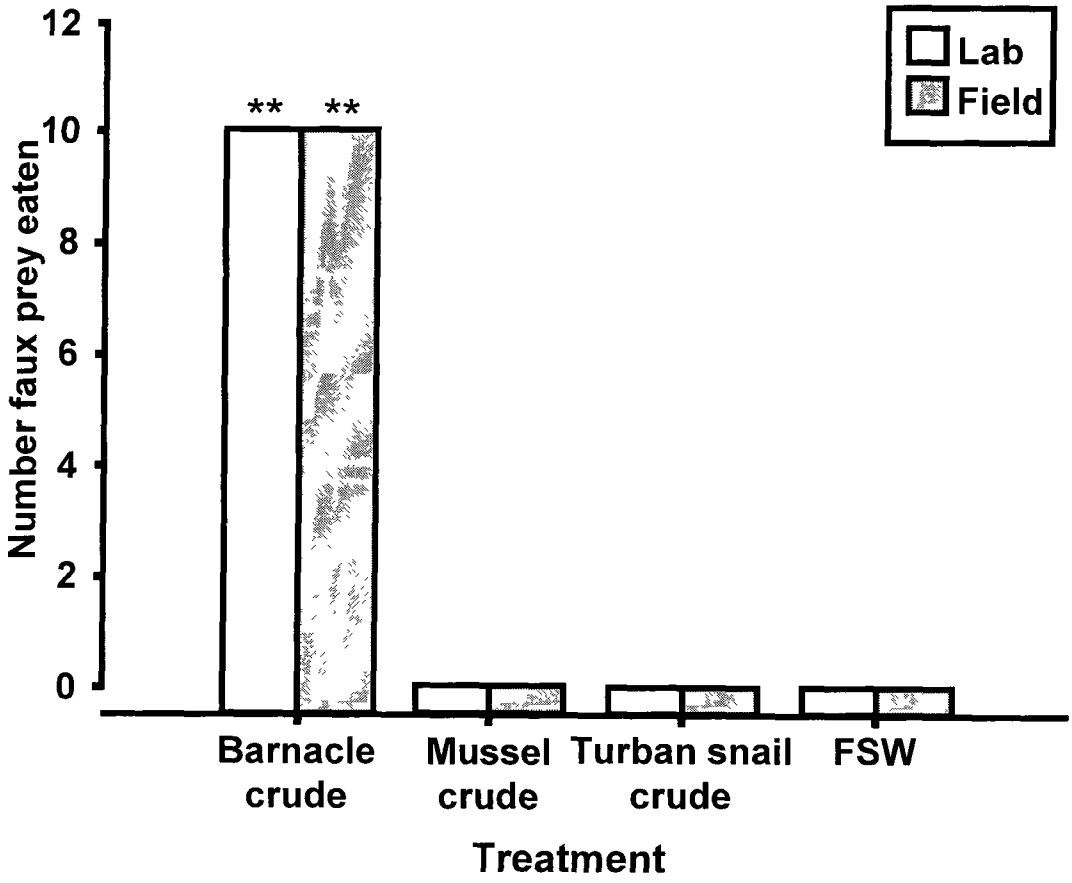

12
10
8
6
4
2
0
Number faux prey eaten
**
**
Lab
Field
Barnacle crude
Mussel crude
Turban snail crude
FSW
Treatment

Figure 11

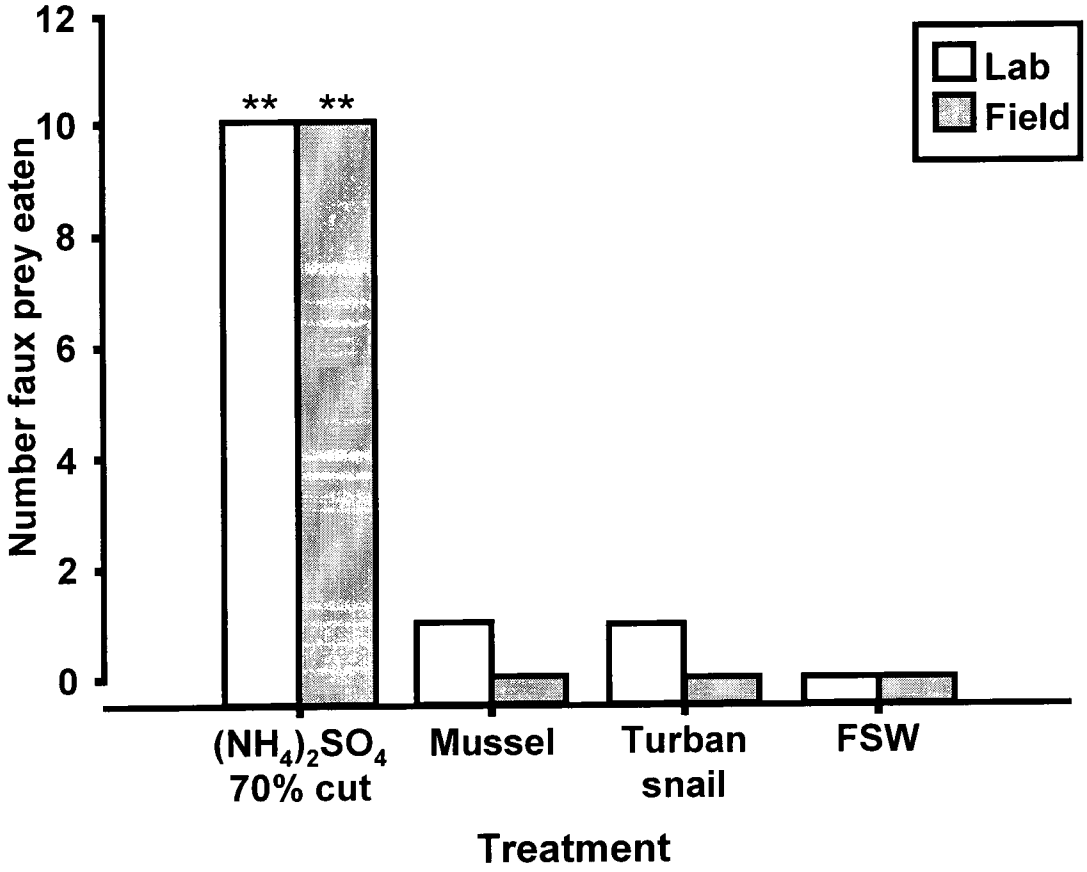

12
10
8
6
4
2
0
Number faux prey eaten
** **
Lab
Field
(NH4)2SO4
70% cut
Mussel
Turban
snail
FSW
Treatment

Figure 12

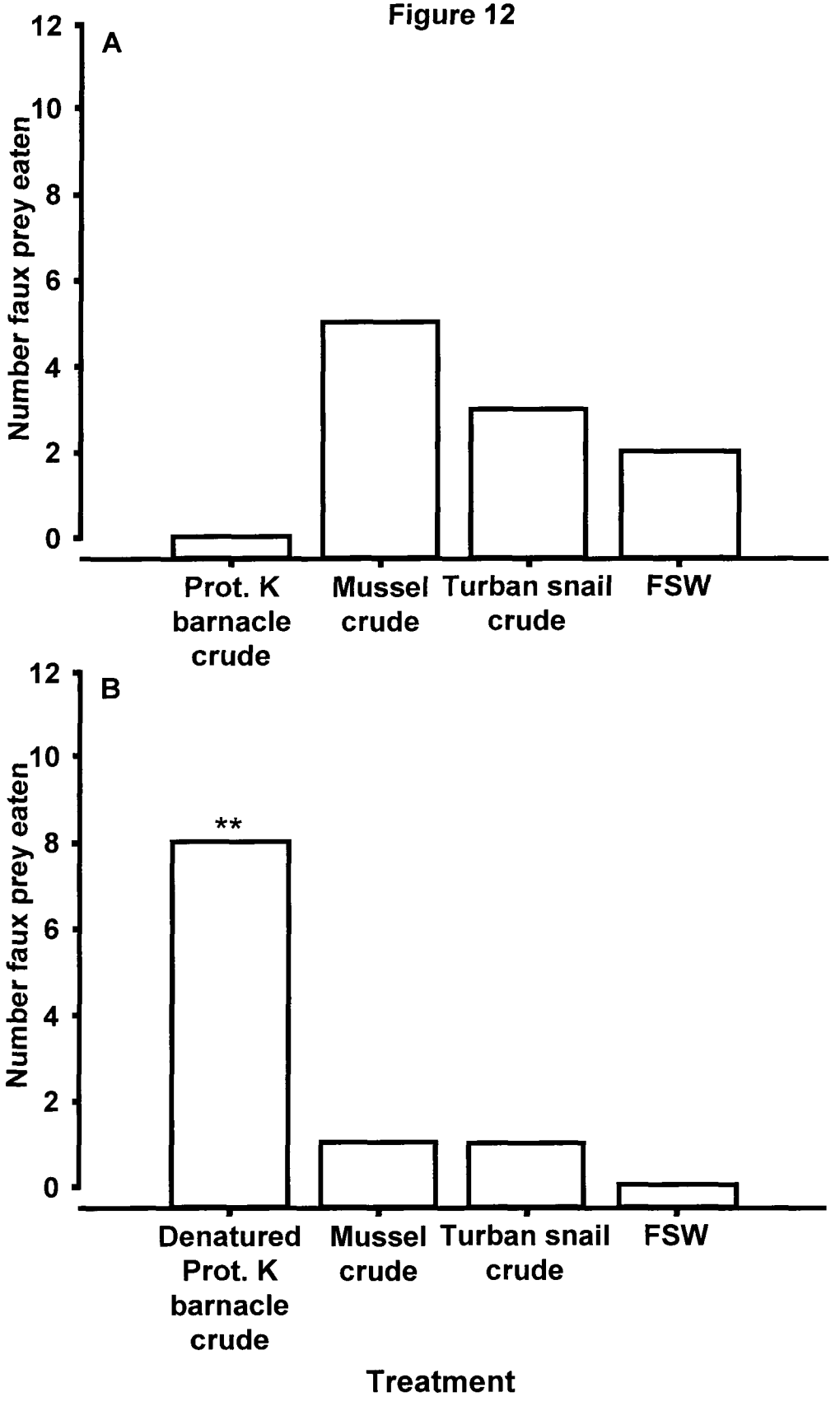

A
12
10
8
6
4
2
0
Number faux prey eaten
Prot. K barnacle crude
Mussel crude
Turban snail crude
FSW
B
12
10
8
6
4
2
0
Number faux prey eaten
**
Denatured Prot. K barnacle crude
Mussel crude
Turban snail crude
FSW
Treatment

Figure Captions

Figure 1-1. The relative abundances of the barnacle *Balanus glandula*, the mussel *Mytilus californianus*, and the black turban snail *Chlorostoma funebralis* at Latigo Canyon (Malibu, CA), Broad Beach (Malibu, CA) and Rancho San Marino (Cambria, CA) recorded from 2006-2008.

Figure 1-2. The number of *Balanus glandula* consumed of a given size class at Broad Beach (Malibu, CA) (A). The average size frequency distribution of 10 randomly selected *B. glandula* sampled every other month at Broad Beach from 2006-2008 (B).

Figure 1-3. The number of *Mytilus californianus* consumed of a given size class at Latigo Canyon (Malibu, CA) (A). The average size frequency distribution of 10 randomly selected *M. californianus* sampled every other month at Latigo Canyon from 2006-2008 (B).

Figure 1-4. The number of *Chlorostoma funebralis* consumed of a given size class at Rancho San Marino (Cambria, CA) (A). The average size frequency distribution of 10 randomly selected *C. funebralis* sampled every other month at Rancho San Marino from 2006-2008 (B).

Figure 1-5. The proportion of prey chosen from each size in laboratory size preference assays for whelks established as barnacle feeders, mussel feeders, or turban snail feeder. Size classes: barnacle: small <1-3mm, intermediate 4-6mm, large 7-10mm; mussel: small 10-20mm, intermediate 30-40mm, large >50mm: turban snail: small 5-10mm, intermediate 11-15mm, large >16mm.

Figure 1-6. The energy gained per unit time handled (in joules • $minute^{-1}$) for whelks feeding on various sizes of *Balanus glandula* (filled circle), *Mytilus californianus* (open square), and *Chlorostoma funebralis* (filled triangle).

Figure 1-7. Predator-prey interaction at Broad Beach. (A) Size-frequency distribution of barnacles in high intertidal (1.3-1.5 m MLLW), sampled bi-monthly and averaged over one year (2007). (B) Prey size selection by whelks. (C) Electivity of whelk predation (Chesson's index). (D) Whelk energy intake (E) per unit handling time (Th), prediciting prey selection by *Acanthinucella spirata.*

Figure 1-8. The proportion of alternate prey chosen during laboratory feeding trials in which equal numbers of each prey type was presented to whelks that were collected from sites with established feeding regimes.

Figure 1-9. The proportion of whelks reaching source water originating from a treatment (barnacle) patch and control (sandy bottom) patch with and without the addition of 15 crushed barnacles. The same letter indicates treatments that are not significantly different (Tukey HSD).

Figure 1-10. The number of faux prey mimics filled with *Balanus glandula* crude protein extract and control solutions eaten by *Acanthinucella spirata* in both laboratory (white bars) and field (grey bars) bioassays. One barnacle of intermediate size (4-6 mm) was found to contain ~ 1.289 mg of total protein. All treatment protein levels were scaled to reflect this. Asterisks indicate a significance level of $p < 0.001$.

Figure 1-11. The number of faux prey mimics filled with the active *Balanus glandula* ammonium sulfate ($(NH_4)_2SO_4$) precipitated pellet and control solutions eaten by *Acanthinucella spirata* in both laboratory (white bars) and field (grey bars) bioassays. One barnacle of intermediate size (4-6 mm) was found to contain ~ 0.541 mg of protein at this solubility level. All treatment and control protein concentrations were scaled to reflect this. Asterisks indicate a significance level of $p < 0.001$.

Figure 1-12. The active protein crude extract was subjected to enzymatic digestion by Proteinase-K resulting in the fragmentation of all the proteins in the sample. This digested sample was made into prey mimics and presented to *Acanthinucella spirata* in laboratory choice feeding assays (A). Additionally, Proteinase-K was inhibited using 5 mM phenylmethylsulfonyl fluoride (PMSF), added to the crude extract, and tested for feeding activity, providing a control for the presence of the enzyme in the feeding gels (B). Asterisks indicate a significance level of $p < 0.001$.

References

Abrams, P.A. 1995. Implications of dynamically variable traits for identifying, classifying, and measuring direct and indirect effects in ecological communities. The American Naturalist 146: 112-134.

Blanchette, C.A., B. Helmuth, and S.D. Gaines. 2007. Spatial patterns of growth in the mussel, *Mytilus californianus*, across a major oceanographic and biogeographic boundary at Point Conception, California, USA. Journal of Experimental Marine Biology and Ecology 340:126-148.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72:248-254.

Bertness, M.D., S.D. Gaines, E.G. Stephens, and P.O. Yund. 1992. Components of recruitment in populations of the acorn barnacle *Semibalanus balanoides* (Linnaeus). Journal of Experimental Marine Biology and Ecology 156:199-215.

Burrows, M.T., and R.N. Hughes. 1990. Variation in growth and consumption among individuals and populations of dogwhelks, *Nucella lapillus*: a link between foraging behaviour and fitness. Journal of Animal Ecology 59:723-742.

Carpenter, S.R., J.F. Kitchell, J.R. Hodgson, P.A. Cochran, J.J. Elser, M.M. Elser, D.M. Lodge, D. Kretchmer, X. He, and C.N. von Ende. 1987. Regulation of lake primary productivity by food web structure. Ecology 68:1863-1876.

Chase, J.M., P.A. Abrams, J.P. Grover, S. Diehl, P. Chesson, R.D. Holt, S.A. Richards, R.M. Nisbet, and T.J. Case. 2002. The interaction between predation and competition: a review and synthesis. Ecology Letter 5:302-315.

Connell, J.H. 1961a. The influence of interspecific competition and other factors on the distribution of the barnacle *Chthamalus stellatus*. Ecology 42:710-724.

Connell, J.H. 1961b. Effects of competition, predation by *Thais lapillus*, and other factors on natural populations of the barnacle *Balanus balanoides*. Ecological Monographs 31:61-104.

Connell, J.H. 1970. A predator-prey system in the marine intertidal region. I. *Balanus glandula* and several predatory species of *Thais*. Ecological Monographs 40:49-78.

Cotton, P.A., S.D. Rundle, and K.A. Smith. 2004. Trait compensation in marine gastropods: shell shape, behavior, and susceptibility to predation. Ecology 85:1581-1584.

Dayton, P.K. 1971. Competition, disturbance, and community organization: the provision and subsequent utilization of space in a rocky intertidal community. Ecological Monographs 41:351-389.

De Moraes, C.M., W.J. Lewis, P.W. Paré, H.T. Alborn, and J.H. Tumlinson. 1998. Herbivore-infested plants selectively attract parasitoids. Nature 393:570-573.

Denny, M.W., L.P. Miller, M.D. Stokes, L.J.H. Hunt, and B.S.T. Helmuth. 2003. Extreme water velocities: topographical amplification of wave-induced flow in the surf zone of rocky shores. Limnology and Oceanography 48:1-8.

Dethier, M.N., E.S. Graham, S. Cohen, and L.M. Tear. 1993. Visual versus random-point percent cover estimations: 'objective' is not always better. Marine Ecological Progress Series 96:93-100.

Dreanno, C., K. Matsumura, N. Dohmae, K. Takio, H. Hirota, R.R. Kirby, and A.S. Clare. An {alpha}2-macroglobulin-like protein is the cue to gregarious settlement of the barnacle *Balanus amphitrite*. Proceeding of the National Academies of Sciences USA 103:14396-14401.

Ferner, M.C., and M.J. Weissburg. 2005. Slow-moving predatory gastropods track prey odors in fast and turbulent flow. The Journal of Experimental Biology 208:809-819.

Ferrer, R.P. and R.K. Zimmer. 2007. The scent of danger: arginine as an olfactory cue of reduced predation risk. The Journal of Experimental Biology 210:1768-1775.

Freeman, A. 2006. Size-dependent trait-mediated indirect interactions among sea urchin herbivores. Behavioral Ecology 17:182-187.

Gaines, S., S. Brown, and J. Roughgarden. 1985. Spatial variation in larval concentrations as a cause of spatial variation in settlement for the barnacle, *Balanus glandula*. Oecologia 67:267-272.

Hughes, R.N. 1979. Optimal diets under the energy maximization premise: the effects of recognition time and learning. The American Naturalist 113:209-221.

Kelly, L.S., and T.W. Snell. 1998. Role of surface glycoproteins in mate-guarding of the marine harpacticoid *Tigriopus japonicus*. Marine Biology 130:605-613.

Koehl, M.A.R., and M.G. Hadfield. 2004. Soluble settlement cue in slowly moving water within coral reefs induces larval adhesion to surfaces. Journal of Marine Systems 49:75-88.

Lively, C.M., and P.T. Raimondi. 1987. Desiccation, predation, and mussel-barnacle interactions in the northern Gulf of California. Oecologia 74:304-309.

Marin, F., and G. Luquet. 2004. Molluscan shell proteins. Comptes Rendus Palevol 3:469-492.

Matsumura, K., M. Nagano, and N. Fusetani. 1998. Purification of a larval settlement-inducing protein complex (SIPC) of the barnacle, *Balanus amphitrite*. The Journal of Experimental Zoology 281:12-20.

Menge, B.A., and J.P. Sutherland. 1976. Species diversity gradients: synthesis of the roles of predation, competition, and temporal heterogeneity. The American Naturalist 110:351-369.

Menge, B.A., and J.P. Sutherland. 1987. Community regulation: variation in disturbance, competition, and predation in relation to environmental stress and recruitment. The American Naturalist 130:730-757.

Murdoch, W.W. 1969. Switching in general predators: experiments on predator specificity and stability of prey populations. Ecological Monographs 39:335-354.

Murdoch, W.W. 1994. Population regulation in theory and practice. Ecology 75:272-287.

Navarrete, S.A., and B.A. Menge. 1996. Keystone predation and interaction strength: interactive effects of predators on their main prey. Ecological Monographs 66:409-429.

Navarrete, S.A., and J.C. Castilla. 2003. Experimental determination of predation intensity in an intertidal predator guild: dominant versus subordinate prey. Oikos 100:251-262.

Nevitt,G.A., R.R. Veit, and P. Kareiva. 1995. Dimethyl sulphide as a foraging cue for Antarctic Procellariiform seabirds. Nature 376:680-682.

Paine, R.T. 1966. Food web complexity and species diversity. The American Naturalist 110:65-75.

Paine, R.T. 1974. Intertidal community structure. Oecologia 15:93-120.

Peacor, S.D., and E.E. Werner. 2000. Predator effects on an assemblage of consumers through induced changes in consumer foraging behavior. Ecology 81:1998-2010.

Peacor, S.D., and E.E. Werner. 2001. The contribution of trait-mediated indirect effects to the net effects of a predator. Proceedings of the National Academies of Sciences USA 98:3904-3908.

Peckarsky, B.L., P.A. Abrams, D.I. Bolnick, L.M. Dill, J.H. Grabowski, B. Luttbeg, J.L. Orrock, S.D. Peacor, E.L. Preisser, O.J. Schmitz, and G.C. Trussell. 2008. Revisiting the classics: considering non-consumptive effects in textbook examples of predator-prey interactions. Ecology 89:2416-2425.

Petraitis, P.S. 1995. The role of growth in maintaining spatial dominance by mussels (*Mytilus edulis*). Ecology 76:1337-1346.

Pohnert, G., M. Steinke, and R. Tollrain. 2007. Chemical cues, defense metabolites and the shaping of pelagic interspecific interactions. TRENDS in Ecology and Evolution 22:198-204.

Post, D.M., M.E. Conners, and D.S. Goldberg. 2000. Prey preferences by a top predator and the stability of linked food chains. Ecology 81:8-14.

Power, M.E., D. Tilman, J.A. Estes, B.A. Menge, W.J. Bond, L.S. Mills, G. Daily, J.C. Castilla, J. Lubchenco, and R.T. Paine. 1996. Challenges in the quest for keystones. Biosciences 46:609-620.

Raffa, K.F. 2001. Mixed messages across multiple trophic levels: the ecology of bark beetle chemical communication systems. Chemoecology 11:49-65.

Raubenheimer, D., and P. Cook. 1990. Effects of exposure to wave action on allocation of resources to shell and meat growth by the subtidal mussel, *Mytilus galloprovincialis.* Journal of Shellfish Research 9:87–93.

Rossini, F.D. in *Experimental Thermochemistry: Measurements of Heats of Reactions* F.D. Rossini Ed. Interscience Publications, Inc., New York 1956.

Schmitz, O.J. 2003. Top predator control of plant biodiversity and productivity in an old-field ecosystem. Ecology Letter 6:156-163.

Schmitz, O.J., V. Krivan, and O. Ovadia. 2004. Trophic cascades: the primacy of trait-mediated indirect interactions. Ecology Letters 7:153-163.

Shabani, S., M. Kamio, and C.D. Derby. 2008. Spiny lobsters detect conspecific blood-borne alarm cues exclusively through olfactory sensilla. The Journal of Experimental Biology 211:2600-2608.

Shanks, A.L., and W.G. Wright. 1986. Adding teeth to wave action: the destructive effects of wave-borne rocks on intertidal organisms. Oecologia 69:420-428.

Smee, D.L., M.C. Ferner, and M.J. Weissburg. 2008. Alteration of sensory abilities regulates the spatial scale of non-lethal predator effects. Oecologia 156:399-409.

Snell, T.W., and M.J. Carmona. 1994. Surface glycoproteins in copepods: potential signals for mate recognition. Hydrobiologia 292/293:255-264.

Steinburg, P.D., R. De Nys, and S. Kjelleberg. 2002. Chemical cues for surface colonization. Journal of Chemical Ecology 28:1935-1951.

Tomaneck, L., and B. Helmuth. 2002. Physiological ecology of rocky intertidal organisms: a synergy of concepts. Integrative and Comparative Biology 42:771-775.

Trussell, G.C., P.J. Ewanchuck, and M.D. Bertness. 2003. Trait-mediated effects in rocky intertidal food chains: predator risk cues alter prey feeding rates. Ecology 84:629-640.

Trussell, G.C., P.J. Ewanchuk, and C.M. Matassa. 2006a. The fear of being eaten reduces energy transfer in a simple food chain. Ecology 87: 2979-2984.

Trussell, G.C., P.J. Ewanchuk, and C.M. Matassa. 2006b. Habitat effects on the relative importance of trait- and density-mediated indirect interactions. Ecology Letters 9:1245-1252.

Trussell, G.C., P.J. Ewanchuk, and C.M. Matassa. 2008. Resource identity modifies the influence of predation risk on ecosystem function. Ecology 89:2798-2807.

Vadas, R.L., M.T. Burrow, and R.N. Hughes. 1993. Foraging strategies of dogwhelks, *Nucella lapillus* (L.): interacting effects of age, diet and chemical cues to the threat of predation. Oecologia 100:439-450.

Weissburg, M.J., and R.K. Zimmer-Faust. 1993. Life and death in moving fluids: hydrodynamic effects on chemosensory-mediated predation. Ecology 74:1428-1443.

Wolfe, G.V. 2000. The chemical defense ecology of marine unicellular plankton: constraints, mechanisms, and impacts. Biological Bulletin 198:225-244.

Zimmer, R.K., J.E. Cummins, and K.A. Browne. 1999. Regulatory effects of environmental chemical signals on search behavior and foraging success. Ecology 80:1432-1466.

Zimmer, R.K., and R.P. Ferrer. 2007. Neuroecology, chemical defense, and the keystone species concept. Biological Bulletin 213:208-225.

Zimmer-Faust, R.K., 1993. ATP: a potent prey attractant evoking carnivory. Limnology and Oceanography 38:1271-1275.

Chapter 2: Barnacle glycoproteins: a keystone molecule that structures natural communities

ABSTRACT

Sensory systems provide critical filters that enable organisms to detect and recognize valuable resources. Trophic cascades, structuring populations and communities, are determined to a large degree by trait-mediated interactions that rely on sensory inputs. Certain molecules serve as chemosensory stimuli and play keystone roles in determining outcomes of predator-prey dynamics at multiple trophic levels. Here, the contributions of surface-adsorbed proteins as signal molecules within wave-swept, rocky intertidal habitats are investigated. Barnacles (*Balanus glandula*) were constrained to produce a high molecular weight, insoluble, glycoprotein complex likely for cuticle/shell biomineralization. This compound was expressed on contactable surfaces and evoked settlement by conspecifics larvae in field assays at biologically relevant concentrations. Thus, glycoproteins operate as a seminal cue for recruitment. Moreover, the same substances triggered barnacle predation by a numerically dominant whelk species (*Acanthinucella spirata*) in lab and field experiments. Such proteins, therefore, simultaneously influence demographic processes that enhance, and diminish, barnacle populations. As dominant competitors for space, the relative balance between barnacle recruitment and predation mortality may have strong, cascading direct and indirect effects

on community dynamics. Hence, the surface-adsorbed glycoprotein plays a keystone role within rocky intertidal habitats.

INTRODUCTION

Many fundamental ecological processes are the result of complex behaviors ultimately driven by sensory inputs. Cellular-level recognition at the sites of receptors (chemo, mechano, and photo) couples the external environment, full of mixed stimuli, with an organism's internal nervous system. Resulting behaviors have wide-ranging individual, population, and community implications. In diverse taxa, ranging from bacteria to humans, olfactory signal production and detection have been identified, and shown, to initiate complex responses (Stern and McClintock, 1998; Bronmark and Hansson, 2000; Waters and Bassler, 2005; Baldwin *et al.*, 2006). Chemically mediated actions, thus, are a key component of intra- and interspecies activities. When examined in a community, or population-level, context they may be the underlying mechanisms in establishment, regulation, and maintenance. Although hypothesized to be of critical importance in both community and population dynamics, largely unknown are the specific identities and functions of multi-trophic species and environmental chemicals signals.

Signal molecule recognition, and the resulting behavioral responses, has been suggested to be an underlying factor of species interactions that initiate trophic cascades. Such compounds have implications for community stability in that they directly influence

predation (Stowe *et al.*, 1987; Zimmer-Faust, 1993; Zimmer *et al.*, 1999; de Boer *et al.*, 2004; Ferner and Weissburg, 2005; Ferrier *et al.*, in prep), mating, mate recognition, and fertilization (Snell and Carmona, 1994, Kelly and Snell, 1998; Riffell *et al.*, 2002; Caskey *et al.*, 2009), settlement and habitat colonization (Raimondi, 1988; Matsumura *et al.*, 1998a; Raffa, 2001; Steinberg *et al.*, 2002; Dreanno *et al.*, 2006; Wheeler *et al.*, 2006; Gerlach *et al.*, 2007), parasitism (De Moraes *et al.*, 1998; Fingerut *et al.*, 2003), and competition (Weir *et al.*, 2004; Bais *et al.*, 2006). Despite the potentially large ecological repercussions from the detection of these signals, few have been explored, or fully characterized, outside the realm of intra-species responses. Largely unexamined are latent multi-species effects and cascading community implications. A notable exception, however, is the pioneering work done in terrestrial habitats on bark beetles (Coleoptera: Scolytidae) and pine trees (Coniferales: Pinaceae) (see Raffa 2001, for review). Research conducted over the last 40 years identified monoterpenes from damaged pines as an attractant for bark beetles both singularly and when co-opted in bark beetle pheromone pathways (Silverstein *et al.*, 1968; Billings, 1985; Erbilgin and Raffa 2000; Seybold *et al.*, 2006). Furthermore, this attractant in its native and modified state also attracts predators of bark beetles. These compounds thus are at the heart of an ecologically important tri-trophic (pine defense-beetle settlement, beetle feeding and mating, and beetles predation) interaction.

Although examples of fully characterized ecological cues are rare, they are not limited to terrestrial habitats. Recent strides have been made in aquatic and marine systems in exploring the multi-trophic role of chemical signals. For example, Zimmer *et*

al. (2007) identified the heterocyclic guanidine alkaloid neurotoxin, tetrodotoxin (TTX), released from the skin of adult newts of the genus *Taricha* to be a multi-trophic signal molecule. TTX was found to play dual ecological roles in which it simultaneously acted to defend adult newts from predation as well as acting as an anti-predator signal for juveniles—often cannibalized by adults in the absences of alternative prey. Furthermore, some higher-order consumers, most notably snakes of the genus *Thamnophis*, exhibit differing degrees of TTX resistance. The bioaccumulation of TTX protects the snakes from raptorial predators thereby limiting their impact in and around riparian stream communities (Brodie and Brodie, 1990; Brodie *et al.*, 2005; Zimmer and Ferrer, 2007). An analog to TTX exists in marine communities– saxitoxin (STX)—produced by some species of puffer fish and toxic dinoflagellates (Shantz *et al.*, 1986). During large algal blooms high concentrations of STX cause mortality in both vertebrate and invertebrate grazer species (White, 1981; Nagai *et al.*, 2000). Evidence from feeding studies and species sampling reveals that some organisms suppress rates of consumption in response to STX, while others, resistant to the neurotoxin, maintain consumption levels and sequester the toxin in their tissues. The effect of STX cascades through the marine environment causing large-scale die-offs and food-chain alterations (Teegarden and Cembella, 1996; Kvitek and Bretz, 2004; Doucette *et al.*, 2006). As these limited examples have shown, identifying the mechanisms by which chemical compounds influence and/or mediate ecological interactions provides valuable insights into the establishment and maintenance of natural communities.

Compounds, such as those briefly described above, with strong, and sometimes opposing, community-level consequences have been termed "keystone molecules" (Zimmer *et al.*, 2007). Much like "keystone species", these molecules exist in low quantity, yet exert strong effects on species interactions at multiple trophic levels. As the potential neurological foundation of behaviors that determine species interrelations, signal molecules operate within an ecological context. Furthermore, the chemistry (smell/taste) of food is a basic property determining palatabilities, eating preferences, and hence, functional-response relationships between predators and prey, irrespective of habitat type. The recognition of prey cues will have important implications for community structure if they induce feeding by ecologically meaningful predators. Mechanisms of signal recognition are highly conserved between diverse taxa (Hildebrand and Shepherd, 1997; Ache and Young, 2005). Processes of recognition in one species, therefore, may apply conceptually, if not explicitly, to another.

The ecological importance of the sensory cues that initiate foraging and feeding are most revealing in systems for which the outcomes of predation set population and distribution limits on lower-tier species. One of the best examples is found in the rocky wave-swept intertidal where, for the better part of 50 years, studies have been conducted on the effects of predation on species distributions. Here, we explore a classic predator-prey system known to structure intertidal communities and examine the role of a surface-bound protein in mediating settlement and mortality of a competitively dominant prey species. Whelks, specifically *Acanthinucella spirata* (Neogastropoda, Muricidae), are primary predators of barnacles on the temperate wave-swept shores of central and

southern California. Predatory activities of whelks ease competitive pressure among species in the upper intertidal by opening up space (a limiting resource of the intertidal) (Connell, 1970; Weiters and Navarrete, 1998). With a preference hierarchy which places barnacles at the top, *A. spirata* are amazingly adept at finding live adult barnacles of an ideal size that maximizes net energy intake (Ferrier *et al.*, in prep). In examining whelk-barnacle interactions, the active molecule used for barnacle recognition is identified, and furthermore the role of this in the settlement of conspecific larvae is determined. With this barnacle protein eliciting dual, yet opposing, demographic processes that are partly responsible for structuring the upper intertidal community this signal is an archetypal "keystone molecule".

MATERIALS AND METHODS

Measuring whelk response to isolated contact cues using a feeding bioassay

Foraging and feeding may be mediated by cues recognized only when contacted. Our goal, therefore, was to determine if signals, secreted and bound to the surface of barnacles, initiate feeding once touched. Laboratory feeding trials were conducted at the University of California, Los Angeles (UCLA). Adult whelks (17-21 mm, shell height) of the species *Acanthinucella spirata* were collected at Broad Beach, Malibu CA and held at UCLA in sea water aquaria (18 °C) for a one week acclimation period prior to the initiation of each experiment. Broad Beach was targeted for collection as a previous study identified this site to have a population of *A. spirata* that feeds exclusively on

barnacle prey (Ferrier *et al.*, in prep). During captivity whelks were kept on a 12 hour light:dark cycle and were not fed.

The behavioral responses of whelks to isolated barnacle compounds were measured at the end of the 1 week acclimation period. In blind feeding trials, whelks were provided an array of treatment and control solutions embedded in artificial prey. Faux prey molds were made by collecting whole adult barnacles (4-6 mm test height) from Broad Beach and dissecting out the soft tissues of the animals. The empty barnacle tests were next scrubbed with pipe cleaners in ddH_2O before being bathed, and stirred, in a 5% HCl solution for 15 minutes. Each barnacle test was then rinsed again with ddH_2O and any remaining tissue was taken off before each was placed in a 130°C oven and baked over night. Finally, burned material was carefully removed, and the resulting calcium carbonate served as a clean foundation to be filled and coated (< 0.5 mm) with impregnated carboxymethyl cellulose gel thereby creating prey mimics.

An accurate examination of sensory signal roles requires experiments to be dynamically scaled especially with respect to the concentrations used. The quantity of protein in each prey mimic, therefore, was adjusted to represent ecologically-relevant amounts at every purification step. This started by determining whole animal protein concentrations of intermediate sized barnacles from crude extractions of a known number of individuals utilizing the protein-dye binding method (Bradford, 1976). Crude protein extractions were repeated ten times each year (2006-2009) and protein content averaged so extract concentrations could be diluted to ecologically-realistic levels. These yearly averaged extractions served as our reference point for the total protein value of a

barnacle; proteins separated out in subsequent purifications steps were referenced to this starting level (as a percentage of the total) and concentrations scaled appropriately.

The artificial prey gel used to make the mimics was produced by mixing treatment (barnacle extracts) or control (mussel (*Mytilus californianus*) extracts, turban snail (*Chlorostoma funebralis*) extracts, or filtered sea water) solutions with carboxymethyl cellulose sodium salt (MP Biomedicals) at 4°C. Carboxymethyl cellulose (herein referred to as CMC) was the matrix of choice as gel pore diameter is smaller than compounds ostensibly acting as prey cues preventing the release of any signal which may act as a waterborne attractant (Zimmer-Faust, 1993). Furthermore, CMC polymerizes on ice thereby preventing protein denaturation. Faux prey were made by mixing 1 ml of ecologically scaled test extract, control extract, or filtered sea water (FSW) with 0.117 g of CMC . This amount of CMC represented the optimal weight for polymerization of 1 ml of solution. Furthermore, 1 ml was the smallest volume for which gel production could be done successfully. The volume of an intermediate barnacle was found to be ~0.333 ml so 1 ml of faux prey gel was used to make 3 artificial barnacles (concentrations were scaled to fit this). Once thoroughly mixed, barnacles were filled completely with the gel and a thin coat was smeared along the outer surface before they were placed on ice to harden for 5 minutes. Barnacles mimics (n=12; 3 barnacles per treatment) were than randomly arrayed in a circle in each flow-through test tank (10 x 10 x 3 cm). Fresh sea water was continuously plumbed in from above with excess exiting each tank as overflow. A single whelk was placed in the center of each tank and acclimated to the conditions for 10 minutes before the 50 minute observation period.

Whelks were used in only one trial, and the response to each contact signal was monitored by recording each faux barnacle visited and fully consumed. The resulting choices were compared with maximum likelihood tests.

Measuring whelk response to isolated contact cues under natural field conditions

It is often difficult to recreate field conditions in the laboratory, and so results from controlled feeding trials may not reflect the behavior of animals in their native environment. Our goal, thus, was to determine if whelks respond and select prey in the same manner under natural environmental conditions as was observed in the laboratory. The behavioral response of whelks to contact cues was tested at Broad Beach using five experimental plots (10 x 10 cm) in which all but 12 randomly assigned barnacles were removed. These remaining 12 barnacles had the live animals pulled out and the shells and surrounding rock scrubbed and cleaned with a 5% HCl solution. The plots were left to the intertidal for 2 weeks and any newly settled species removed every three days before trials commenced. Feeding trials were run such that each barnacle in a plot was randomly assigned a treatment using a random number generator. Faux prey gels were prepared (as described above) on site, and the individual barnacle shells filled, 20 minutes prior to complete plot submersion. *A. spirata* extensively forages within barnacles beds when they are fully submerged (author's personal observation), therefore, when the water completely covered the test plot 10 whelks were placed randomly around the periphery and permitted to forage as normal. As is the nature of working with this sort of system, many whelks simply moved away from the plots, however, those that

entered were tracked by snorkeling and the barnacles visited and eaten were recorded over 1 hour. As with the laboratory experiments, choices were compared using maximum likelihood analysis.

Measuring the response of cyprid larvae to isolated contact cues

For over 50 years signals originating in adult barnacles have been assumed to be the mediating factor driving cyprid settlement and the gregariousness of adult barnacles (Crisp and Meadows, 1963; Larman and Gabbot, 1975; Raimondi, 1988; Clare *et al.*, 1995; Matsumura *et al.*, 1998a,b; Dreanno *et al.*, 2006). Despite several attempts a single definitive settlement signal was not fully sequenced until 2006 when one was found for *Balanus amphitrite* (the settlement-inducing protein complex (SIPC)) (Dreanno *et al.*, 2006). Here we seek to add to this by identifying the settlement signal of the gregarious barnacle, *Balanus glandula*. In this study, settlement plates (5 x 5 cm) were cut from the rock that *B. glandula* cyprids naturally settle on at Broad Beach, brought to UCLA, and cleaned. Prior to sample addition the plates were autoclaved and heat-dried to assist in the binding of protein test and control solutions. Population surveys conducted at the Broad Beach site found that 5 x 5 cm plots in a barnacle bed had, on average, 30 live barnacles attached. Samples were prepared so that the protein concentrations bound to each plate would approximate this number of barnacles. For example, an average barnacle contains ~1.289 mg of total protein of which 10.5% may be expressed on the surface (assuming a barnacle is a cone minus the top and only the surface may be contacted; in the case of the barnacle the surface down to 0.1 mm represents 10.5 % of

the total volume) leaving 0.1353 mg available for contact on the whole barnacle. Furthermore, we assume the bottom of the barnacle, and its surface, can not be contacted which further reduced the total by ~35% leaving 0.088 mg of total protein available for contact for each of the 30 barnacles represented on each plate. Each purification step was scaled to its appropriate representative level compared to the total. To bind proteins, 1 ml of sample was applied to a dried-out plate. The plate was then tightly wrapped in plastic and soaked for 10 hours at 4 °C. Following the soaking, the protein concentrations of the residual solutions were measured and the total amount of proteins bound to each plate estimated. As 100% of the sample rarely binds to a plate, the contact cues readily available in these experiments, and thus the number of cyprids responding, likely represents a conservative estimate.

The attraction of cyprid larvae to extracted and plate-bound barnacle solutions was measured by deploying settlement plates in the barnacle zone at Broad Beach during the peak settlement season in Los Angeles County (May-June) from 2007-2009. One month prior to the trials, all surround barnacles were removed from the area that plates were to be deployed and settlement was monitored until experiments ended. During the settlement period plates bound with treatment and control solutions (mussel extracts, turban snail extracts, and FSW) were deployed in a randomized linear array (each plate separated by 10 cm) as the tide was coming in. Plates remained attached to the settlement plot for one complete tidal cycle before they were retrieved, brought back to UCLA, and the number of newly settled cyprids counted using a dissecting microscope. To insure settled cyprids were indeed *Balanus glandula* plates were periodically placed in

flowing sea water tanks and newly metamorphed barnacles grown and monitored until they could be positively identified to genus. The number of newly settled cyprids was compared between treatments and controls using ANOVA with sample type as the main effect.

Preparation and isolation of the active barnacle factor

An initial experiment was performed to determine if naturally expressed barnacle proteins induced a response in foraging *Acanthinucella spirata* and/or cyprid larvae of *Balanus glandula*. A barnacle crude protein extract was prepared following the methods of Matsumura *et al.* (1998c). Whole, live, adult barnacles (~100 g) were collected from Broad Beach and brought to UCLA where they were fast-frozen at -80 °C. Frozen barnacles were next crushed and homogenized in a 1.5 volume of 50 mM tris-HCL (w:v), pH 7.5, and stirred for 120 minutes at 4 °C. The resulting slurry was rough-filtered through gauze and centrifuged at 40,000g for 30 minutes. After centrifugation the supernatant was filtered using Whatman no. 3 filter paper, and the resulting solution served as our initial crude extract. Control solutions—crude extractions of the mussel *Mytilus californianus*, and the turban snail *Chlorostoma funebralis*—were prepared in exactly the same way. Protein concentrations were determined using the Bradford method (Bradford, 1976) with bovine serum albumin as the standard, and all samples not immediately used were stored at -80 °C. The activity of the barnacle crude extract was assayed alongside control crude extractions in laboratory and field feeding and settlement trials during the peak predatory (April-November) and settlement (May-June) seasons

from 2007-2009. Following positive feeding and settlement responses to the barnacle crude extract in relation to the controls the extract was next fractionated and purified as follows:

1. Ammonium sulfate ($(NH_4)_2SO_4$) precipitation

$(NH_4)_2SO_4$ was added to the crude extract in a series saltation cuts starting with 35% (0.21 g/ml), next going to 70% (0.42 g/ml), and finally to full saturation (0.6 g/ml). Each cut was gently stirred for 20 minutes, or until all $(NH_4)_2SO_4$ was fully dissolved, and the solution centrifuged at 40,000g for 15 minutes. The resulting supernatant was separated, and the pellet re-suspended in 50 mM tris-HCL, pH7.5. All solutions were dialyzed against 50 mM tris-HCl to remove residual $(NH_4)_2SO_4$ (and for long-term storage at -80 °C) and then against FSW prior to use. The proportion of protein from the crude extract distributed in each supernatant and re-suspended was pellet determined using the Bradford assay. Each supernatant and resuspended pellet from every $(NH_4)_2SO_4$ cut was bioassayed for activity in laboratory and field feeding and settlement trials against controls to determine at what $(NH_4)_2SO_4$ concentration the active molecule precipitated and, hence, its relative solubility.

2. Size exclusion chromatography

The bioactive fraction from the ammonium sulfate precipitation was further purified, and the molecular weight range determined, using a HiPrep 16/60 high resolution S-200 sephacryl size exclusion column (SEC) (GE Healthcare) set up on a fast performance liquid chromatography (FPLC) pump system (BioRad BioLogic

Chromatography system) eluting at 0.8 ml/min in 50 mM tris-HCl, pH 7.5. The column was calibrated using a molecular-grade standard solution of: horse spleen Ferretin (M_r=770,000), sweet potato β-amylase (M_r=224,316), human hemoglobin A (M_r=64,667), bovine carbonic anhydrase (M_r=28,980), and horse heart cytochrome C (M_r=12,318), and the effect of molecular weight on column retention time was determined by a model 1 regression. The approximate molecular weights of the unknown compounds eluting from the column could be determined using the resulting regression equation. Fractions (1ml each) were pooled around elution peaks and the total protein concentration of each was determined by the Bradford method. Pooled SEC fractions were concentrated using ultracentrifuge filters with molecular weight cut offs (MWCO) appropriate to the size range of the compounds in use. The distribution of proteins in each elution peak from the active ammonium sulfate cut (% of the active cut) was determined and samples scaled to an appropriate protein concentration in relation to what would be expressed in that molecular weight range in an average adult barnacle. SEC peaks were bioassayed in laboratory and field feeding and settlement trials first against controls and then against each other.

3. Lentil-lectin affinity chromatography

The active molecular weight fractions were applied to an affinity column hand-packed with lentil-lectin sepharose (LCA) beads (75 ml of beads; GE Healthcare), and equilibrated with 0.5 M NaCl, 25 mM tris-HCl, pH 7.5. Following sample application, the column was washed using an FPLC (BioRad BioLogic Chromatography system) at a rate of 0.8 ml/min with 100 ml of the same buffer and eluted compounds pooled around

the void peak. Compounds bound to the LCA beads were eluted using 150 ml of 0.2 M methyl α,D-mannopyranoside (MMP) in the same buffer (Matsumura, 1998a). Fractions (1 ml each) coming off the column following the addition of MMP were pooled around the predominant peak. LCA fractions were dialyzed against 50 mM tris-HCl and were concentrated using ultracentrifuge filters (MWCO = 10,000 Da, Millipore). Prior to use, samples were dialyzed against filtered sea water and the distribution of the active SEC proteins between void and LCA peaks determined so samples could be diluted to ecological concentrations. LCA peaks were bioassayed in laboratory and field feeding and settlement trials first against controls and then against each other.

4. Active signal purification and distribution within adult barnacles

Purification was monitored using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (Laemmli,1970). 40 μL of a 0.25 mg/ml protein solution was mixed with 10 μL of 5x loading buffer and heat denatured for 5 minutes at 100°C. Samples were loaded on a 7.5% acrylamide (acrylamide:BIS = 29:1) slab gel (65 mm x 90 mm x 1 mm) and electrophoresed at 80 v. Proteins were stained with coomasie brilliant blue, and the molecular weight of each protein was estimated using a model 1 regression of the migration of Precision Plus Protein Standards (Bio-Rad) through the gel.

Following the identification of the active signal, its distribution within a barnacle was determined from barnacle tissue dissections. Whole barnacles, barnacle cirri, animals without shells, cuticle ,and scrapings from live and empty (dead) shells collected from the field were isolated under a dissecting microscope and kept on ice. Purification

of the active compound from each dissected tissue and scraping followed the above established protocols, and following lentil-lectin affinity chromatography 40 μL of 0.12 mg/ml was mixed with 10 μL of 5x loading buffer and heat denatured for 3 minutes at 100 °C. The sample was applied to a 10% SDS-PAGE acrylamide (acrylamide:BIS = 29:1) slab gel (65 mm x 90 mm x 1 mm) under reducing conditions in the presence of β-mercaptoethanol. A shorter denaturing time and the use of 10% acrylamide gels were used so a higher resolution between protein bands could be obtained. The molecular weight of each protein band from each tissue was compared to determine were the active signal would be found in an adult barnacle.

RESULTS

Whelk response to barnacle protein factors

An initial experiment was performed to determine if the predatory whelk, *Acanthinucella spirata*, recognizes and responds to protein signals originating in its preferred prey- the barnacle *Balanus glandula*. Whelk behavior was monitored in artificial prey feeding assays in both the laboratory and the field in which *A. spirata* was provided an equal amount of treatment (extracted barnacle proteins) and control (extracted mussel proteins, extracted turban snail proteins, or FSW) prey mimics and the type and number of each consumed was recorded. In blind laboratory feeding trials repeated throughout the primary predatory season from 2006-2009 *A. spirata* preferentially consumed, in its entirety, faux prey imbedded with the crude protein extract

of *B. glandula* (maximum likelihood test: G^2= 57.76, D.F.= 3, $p < 0.0001$, n=59; Fig 1a). This choice remained consistent and significant in feeding trials conducted under natural field conditions (G^2= 27.73, D.F.= 3, $p < 0.0001$, n=12; Fig. 1a). Together, these results establish that a natural feeding cue is present in adult barnacles to which predatory *A. spirata* strongly respond.

Cyprid response to barnacle protein factors

Previous work has shown that proteins extracted from a related barnacle species, *B. amphitrite*, induces settlement of conspecific cyprid larvae once contacted (Matsumura *et al.*, 1998a; Dreanno *et al.*, 2006). Here settlement assays were done to determine if the cyprid larvae of *B. glandula* is induced to settle when extracted conspecific proteins are detected. Cyprid response was quantified using settlement plates bound with treatment and controls solutions (solutions were the same as for the whelk feeding trials) deployed in the barnacle zone at Broad Beach (Malibu, CA) during the peak settlement season. Binding efficiency of the barnacle crude extract to settlement plates was on average 71%. The amount of protein available on a given plate, thus, represents a conservative estimate of the amount of protein expressed on the surface and the number of cyprids settled is likely an underestimate. As with whelks, cyprid larvae of *B. glandula* responded strongly and settled in significantly greater numbers on barnacle crude extract-bound plates than on controls (one-way ANOVA: $F_{3,79}$= 32.59, $p < 0.0001$, n = 20/treatement; Fig 1b). Subsequent identification following the juvenile growing period indicated all settled barnacles to be *Balanus glandula*. Furthermore, cyprid rearing to juvenile barnacles was

done periodically with each active purification step and all barnacles were positively identified as *B. glandula*.

Isolation of whelk feeding cue via bioassay-guided fractionation

The feeding signal of *Acanthinucella spirata* was isolated from a bulk protein extraction of *Balanus glandula* by bioassay-guided fractionation using a series of increasing ammonium sulfate ($(NH_4)_2SO_4$) cuts. A 35 % saturation cut (0.21 g/ml $(NH_4)_2SO_4$) was initially preformed and the precipitate separated from the supernatant using ultracentrifugation. This cut was replicated ten times and the amount of protein retained in the supernatant was determined to be 47.5 % of the total protein found in a barnacle. Feeding trials were done on both the 35 % supernatant and pellet, and all activity was retained in the supernatant (maximum likelihood test: G^2= 19.69, D.F.= 3, p = 0.0002; n=12). The active 35 % supernatant was further separated with $(NH_4)_2SO_4$ to a 70 % saturation level (0.42 g/ml $(NH_4)_2SO_4$), and the resulting precipitate was pelleted. This step was again repeated ten times and the quantity of protein retained in this supernatant was found to be 4.4 %, whereas that of the pellet was 42.66% of the total. Both the pellet and the supernatant were assayed individually; no significant activity was found for the supernatant, but nearly all whelks fed when the 70% pellet was contacted (maximum likelihood test: G^2= 19.69, D.F.= 3, p = 0.0002; n=12; Fig. 2a). Finally, the non-active 70% supernatant was brought to 100% $(NH_4)_2SO_4$ saturation (0.6 g/ml $(NH_4)_2SO_4$) which precipitated out all remaining proteins. The resulting supernatant retained <0.01% of the total protein, and the pellet contained the remaining 4.4% of the

total barnacle proteins. Both pellet and supernatant were assayed and no significant feeding activity was observed in either sample. All feeding activity in our laboratory trials was retained in the 36-70% $(NH_4)_2SO_4$ saturation level. When this sample was tested under natural field conditions the foraging and feeding patterns were consistent and nearly identical. Most whelks, when contacting the 70% cut, ceased foraging and began to consume the prey mimic indicating this sample contained the active feeding cue (maximum likelihood test: $G^2 = 27.73$, D.F.= 3, $p < 0.0001$; n=10; Fig. 2a).

Isolation of cyprid settlement cue via bioassay-guided fractionation

As was done for the whelk feeding cue, the cyprid settlement factor was partially isolated from adult barnacle crude protein extract with bioassay-guided fractionation using increasing saturation states of $(NH_4)_2SO_4$. Settlement plates were deployed with each $(NH_4)_2SO_4$ cut (35, 70, 100 % pellets) bound to them at ecologically-relevant levels and the number of cyprids settled compared to control solutions. Binding of proteins to settlement plates ranged from ~90 % for the 35 % $(NH_4)_2SO_4$ cut to ~94 % for the 70% cut and ~62% for 100% $(NH_4)_2SO_4$ cuts. Settlement in response to the 35 % cut was not significantly different from that of the controls, indicating the active compound did not pellet out at that saturation level. In response to the 70 % cut, however, significantly more cyprids settled than did on the control plates (one-way ANOVA: $F_{3,79} = 28.06$, $p < 0.0001$, n = 20/treatmtent; Fig. 2b). Finally, no significant activity was found in response to the 100 % pellet when compared to controls further confirming that the active settlement compound is found in the 35-70 % $(NH_4)_2SO_4$ cut as it had been for *A. spirata*.

Molecular weight identity of the feeding and settlement cue

The feeding and settlement cue was further isolated from a bulk preparation of the active ammonium sulfate cut. Solutions of the resuspended 70 % pellet were loaded onto a HiPrep 16/60 sephacryl S-200 high resolution gel exclusion chromatography (GEC) column eluting at 0.8 ml/min with 50 mM tris-HCl, pH 7.5. One major and three minor peaks were found at an absorbance of 280 nm (Fig. 3a). Fractions were pooled around each peak before being concentrated to protein values representing the proportion of each weight class in an average adult barnacle (e.g. the major peak represents 38.5 % of total barnacle proteins). Feeding trials were preformed in the lab and the field; all activity for both experiments was contained in the major peak (corresponding molecular weight class: 527-170 kDa) (whelk feeding maximum likelihood test lab: G^2= 18.18, D.F.= 3, p = 0.0004, n=13; maximum likelihood test field: G^2= 23.8, D.F.= 3, p < 0.0001, n=11; Fig 3b). No significant difference was found in feeding response when the three other molecular weight size classes were tested against the controls.

Binding efficiency of the GEC peak fractions to the plates proved to be close to that of the 70% cut and ranged from 92-96 %. Additionally, cyprid response was found to be similar to that of whelks. Following deployment of all molecular weight fractions a significantly greater number of whelks settled on the high molecular weight group than did on the any of the other fractions (Fig 3c). When the active molecular weight peak was tested solely against controls the activity was retained with significantly more cyprids settling on plates bound with the peak fractions (Fig 3c; one-way ANOVA:

$F_{3,59}$= 4.78, p = 0.005, n = 15/treatment). When the other three peaks were tested against controls no significant response was found. The combined results of the feeding and settlement assays reveal the active molecule for both *Acanthinucella spirata* and *Balanus glandula* cyprid larvae is of high molecular weight.

Identification of the natural feeding and settlement cue

The final purification of the active feeding and settlement signal was conducted with lentil-lectin affinity chromatography of a bulk preparation of the high molecular weight GEC isolate. One major peak was identified prior to the addition of methyl α,D-mannopyranoside (MMP) corresponding to high molecular weight void proteins. Following the addition of MMP a second peak was identified that was composed of glycoproteins (fig 4a). Both peaks were assayed for whelk feeding activity in the lab and field as well as for settlement response. No preferential feeding was observed with the void fractions, and although settlement was observed in response to the void peak it was significantly less than that of the glycoprotein peak (fig 4b,c). The proteins contained in the glycopeak were found to represent 12.6 % of the total protein concentration of an adult barnacle (e.g. an average adult barnacle contains 0.16 mg of glycoprotein) and all trails were scaled to represent this value. The active peak was assayed against controls in laboratory feeding trials and whelks preferentially responded to, and consumed in its entirety, prey mimics imbedded with the isolated glycoprotein (whelk feeding maximum likelihood test lab: G^2= 23.8, D.F.= 3, p < 0.0001, n=11), this pattern was retained in field feeding trials (whelk feeding maximum likelihood test field: G^2= 19.69, D.F.= 3,

p = 0.0002, n=12; Fig 4b).

The active cue found in the glycoprotein peak was further assayed against controls in settlement trials, and plates typically bound 93 % of the protein found in these fractions. Glycoproteins induced significantly more cyprids to settle than any of the controls (one-way ANOVA: $F_{3,87}$= 3.64, p = 0.016, n = 22/treatment; Fig 4c). The feeding and settlement signal, a glycoprotein of high molecular weight and low solubility, was found to be the same for both *Acanthinucella spirata* and *Balanus glandula* cyprid larvae.

The activity of the purified glycoprotein was checked against that of the crude barnacle protein extract, the *Chlorostoma funebralis* extract control, and filtered sea water. Feeding response was found to be comparable between the crude protein extract and the isolated glycoprotein, both of which were significantly greater than either control despite the difference in overall protein concentration (whelk feeding maximum likelihood test lab: G^2= 13.37, D.F.= 3, p = 0.004, n=22; whelk feeding maximum likelihood test field: G^2= 9.9, D.F.= 3, p = 0.02, n=22; Fig 5a). In much the same manner settlement response to both the isolated glycoprotein and crude extract were significantly different from controls but not from each other (one-way ANOVA: $F_{3,39}$= 16.56, p < 0.0001; Tukey-Kramer (HSD); n = 5/treatment; Fig 5b).

SDS-PAGE analysis of the active glycoprotein peak was performed on a 7.5 % reducing acrylamide slab gel in the presence of β-mercaptanoethanol. Coomasie staining identified four major bands with molecular weights estimated to be 199, 98, 88, and 76 kDa (fig. 6). These results, when examined alongside the active gel exclusion column

high molecular weight peak, suggest that the active barnacle compound is a protein complex. Furthermore, examining the SDS-PAGE results of the barnacle tissue dissections, which were subjected to less heat denaturing, produced a solitary band of approximately 390 kDa which likely represents the intact protein complex (fig. 7b).

The active feeding and settlement compound was originally purified from whole barnacle extracts. Although this technique enabled us to find the active compound it limited our ability to determine where the signal would be and thus whether it would be available on contactable surfaces. Following LCA purification, the active glycoprotein was identified in all tissues examined (fig. 7a and b). The active complex was found in high quantity in the feeding legs (cirri) and the scrapings of the shell of live animals, however two of four subunits (88 and 76 kDa subunits) were found in limited quantities. This difference in subunit concentration found in the dissections is the result of reduced denaturation of the intact complex (390 kDa band) at the top of the gel, but this was anticipated and provided information on the complex as a whole in all of the dissected tissue. Furthermore, the signal was not present in the empty (deceased barnacle) shells collected from the field implying that the means by which whelks are able to sense live versus dead barnacles is the detection of the this signal on the outer surfaces of live prey. These results are consistent with the findings that the active feeding and settlement cue is of low solubility and thus requires contact in order to initiate a response.

DISCUSSION

The identity of a multifunctional intertidal signal

A 390 kDa protein complex of low solubility originating in adult barnacles of the species *Balanus glandula* is the natural settlement signal for conspecific larvae, as well as the primary feeding and foraging cue of a predominant predator of this barnacle, the whelk *Acanthinucella spirata*. With the active protein binding to lentil-lectin sepharose it was determined to be a glycoprotein. Furthermore, the distribution of this signal in barnacle tissue showed that the complex is present on the outer, contactable, surfaces of adult barnacles. As a requisite component of biomineralization, glycoproteins are expressed at high concentrations in barnacles and are thus available at detectable levels on the outer surfaces, and surrounding substrate, of *B. glandula* (Weiner *et al.*, 1984; Marin and Luquet, 2004). When explored in feeding and settlement trials, this signal was very active (50-65% response) at concentrations naturally found in adult barnacles. Furthermore, whelk and cyprid responses were not context dependent— similar results were found under controlled laboratory settings and natural field conditions. The low solubility of this glycoprotein requires direct contact for detection by an organism. Search behavior exhibited by whelks—periodic sampling of the substrate with tentacles—corroborates this scenerio. However, recognition appears to be independent of prey shape and conspecific presence as whelks consumed gels placed on variety of forms (including the legs of natural predator, *Cancer spp.*) and cyprids settled on bare rock.

The response of both whelks and cyprid larvae, thus, is dependent on chemo- rather than mechano-reception.

The discovery that our active intertidal signal is of low solubility represents a shift from what has become a common marine paradigm—that marine signal molecules are, typically, waterborne and therefore operate from a distance as attractors and/or deterrents (Duval *et al.*, 1994; Trussel *et al.*, 2003; Dalziel and Boulding 2005). Evidently this idea is not as broadly applicable in marine environments as assumed previously. Although waterborne signals are likely to be active in subtidal and open-ocean environments, the turbulence associated with the wave-swept rocky intertidal, where water velocities can exceed 25 $m \cdot s^{-1}$ (Denny *et al.*, 2003), dilutes the concentration of most soluble cues to unrecognizable levels within a few millimeters of the source (Smee *et al.*, 2008). In a previous experiment, we tested the effects of waterborne signals on whelk foraging (Ferrier *et al.*, in prep). Field trials, in which water from an actively feeding barnacle bed was pumped through a series of flow-through chambers, showed that no more whelks were able to track to the source than in the control experiment (water from a barren sand bed). These results, when combined with the molecular identity of the active feeding and settlement cue, highlight the importance of surface-bound signals in the rocky intertidal. At sites that are exposed to the brunt of turbulent ocean conditions, contact cues may be the most parsimonious solution for signal detection. Furthermore, as glycoproteins are typically long lived, surface-bound, and produced *en masse* by most shelled animals, this class of protein may prove to be a critical signaling component within the intertidal community.

Glycoproteins as signaling molecules

Glycoproteins represent an important class of bio-molecule. For many invertebrates they are obligatory to biomineralization as a primary component of the organic matrix that serves as the template for shell and cuticle (Weiner *et al.*, 1984; Albeck *et al.*, 1996; Marin and Luquet, 2004). The requisite nature of glycoproteins for shelled animals, therefore, makes them viable targets for exploitation. In fact, strong evidence has linked cuticular glycoproteins to mate recognition and guarding in copepods (Snell and Carmona, 1994; Kelly and Snell, 1998) implicating this class of protein in critical, species-level processes. Additionally, glycoproteins were found to control the settlement of cyprid larvae of the barnacle *Balanus amphitrite* (Matsumura *et al.*, 1998a,b; Dreanno *et al.*, 2006, 2007). As barnacles are compulsory internal fertilizers, requiring genetically distinct individuals to settle within penis length, glycoproteins would again play a critical role in reproduction and population dynamics. Furthermore, because glycosylation of proteins are tightly coupled to overall environmental conditions (Goochee and Monica, 1990), glycoproteins may serve as reliable indicators for cyprids of a habitat that supports an adult population.

Signals that are reliable indicators/attractors for one species may present a chemosensory opportunity to another. This phenomenon was observed in plant-herbivore-predator insect food webs where chemical signals released from injured plants attract more herbivores, but also attract the predators and/or parasites of those insects (Dicke *et al.*, 1990; Turlings *et al.*, 1990). Predators also have evolved pheromone mimics to attract their primary prey; one of the best examples is bolas spiders

(*Mastophora spp.*) which attract male moths by mimicking the female pheromone (Eberhard, 1977; Haynes *et al.*, 2002). As the recognition of glycoproteins is conserved between the cyprid larvae of two distinct barnacle species and indicates the presence of adult barnacle populations, this signal could be co-opted by foraging whelks for much the same reason, albeit for very different outcomes. As with the tri-trophic insect web and pheromone mimicry, the outcomes of signal recognition are very different depending on which species is examined, and ultimately will influence community dynamics. Whereas small chemical compounds are ubiquitous as signal molecules in multi-trophic terrestrial interactions, large, long-lived, proteins may prove to be commonly recognized and exploited in the viscous and turbulent intertidal habitat.

Barnacle glycoproteins: intertidal keystone molecules

Glycoproteins produced by *Balanus glandula* play a keystone role within the upper intertidal of rocky wave-swept shores. This glycoprotein drives dual, yet opposing, demographic processes with community-wide ecological consequences. As a foundation species, *B. glandula* out-competes other species, including smaller acorn barnacles (*Chthamalus fissus*), thereby forming large monoculture barnacle beds of low relative diversity (Connell, 1961a; Dayton, 1971; Menge, 2000). By inducing the settlement of this barnacle, glycoproteins are the sensory signal of competitive dominance in the upper intertidal. However, this also cues *Acanthinucella spirata* to feed on live adults within a barnacle bed. Glycoprotein recognition by predatory whelks, thus, plays an additional role which balances barnacle populations. Furthermore, strong secondary effects are

associated with the whelk predation in that feeding indirectly facilitates an increase in diversity by releasing a limited resource, space, to competitive subordinates (Connell, 1961b; Dayton, 1971; Navarrete and Menge, 1996; Raimondi *et al.*, 2000; Navarrete and Castilla, 2003). Thus, a conceptual model emerges for the sensory basis of predator-prey interactions on wave-swept shores in which barnacles are physiologically constrained to express this glycoprotein and do so in small quantities (<0.015 mg/cm^2 surface expressed). The recognition of this signal is, however, conserved between at least two separate species. Effects converge on the rocky wave-swept intertidal that both directly and indirectly influence the distribution and abundances of species. Because small amounts of surface-bound signal appear to have far reaching ecological consequences, barnacle glycoproteins are here considered "keystone molecules".

The activity of surface-bound glycoproteins in the rocky wave-swept intertidal provides critical insight into what may prove to be an important class of signaling molecules in turbulent environments. Long-lived, surface-adsorbed, signals likely act as reliable cues in habitats in which water velocity dilutes soluble signal concentrations to below detectable thresholds. As behavior is almost always stimulus-driven, the underlying cue initiating it is of critical importance to fully understanding the neuroecological foundations of species interactions. Recent strides have been made in both terrestrial and aquatic environments. Examples of the multi-trophic effect of a single compound include: dimethylsulfonipropionate (DMSP), and its metabolites (dimethyl sulfide and acrylate), which influence several pelagic trophic levels and convey feeding and defense information (Nevitt *et al.*, 1995; Zimmer and Butman, 2000; Pohnert

et al., 2007), guanidine alkaloids which manipulates food webs in riparian forests (TTX) and marine (STX) habitats (Kvitek *et al.*, 1991; Teegarden and Cembella, 1996; Zimmer *et al.*, 2006; Ferrer and Zimmer, 2007a,b), and monoterpenes which drive plant-herbivore-predator interactions in coniferous forests (Billings, 1985; Seybold *et al.*, 2006) Future work, driven by collaboration between ecologists, physiologist, and molecular biologists, will ultimately see the discovery of numerous compounds that underlie vital ecological interactions. No doubt, it is an exciting time for chemical ecology.

Figure 1

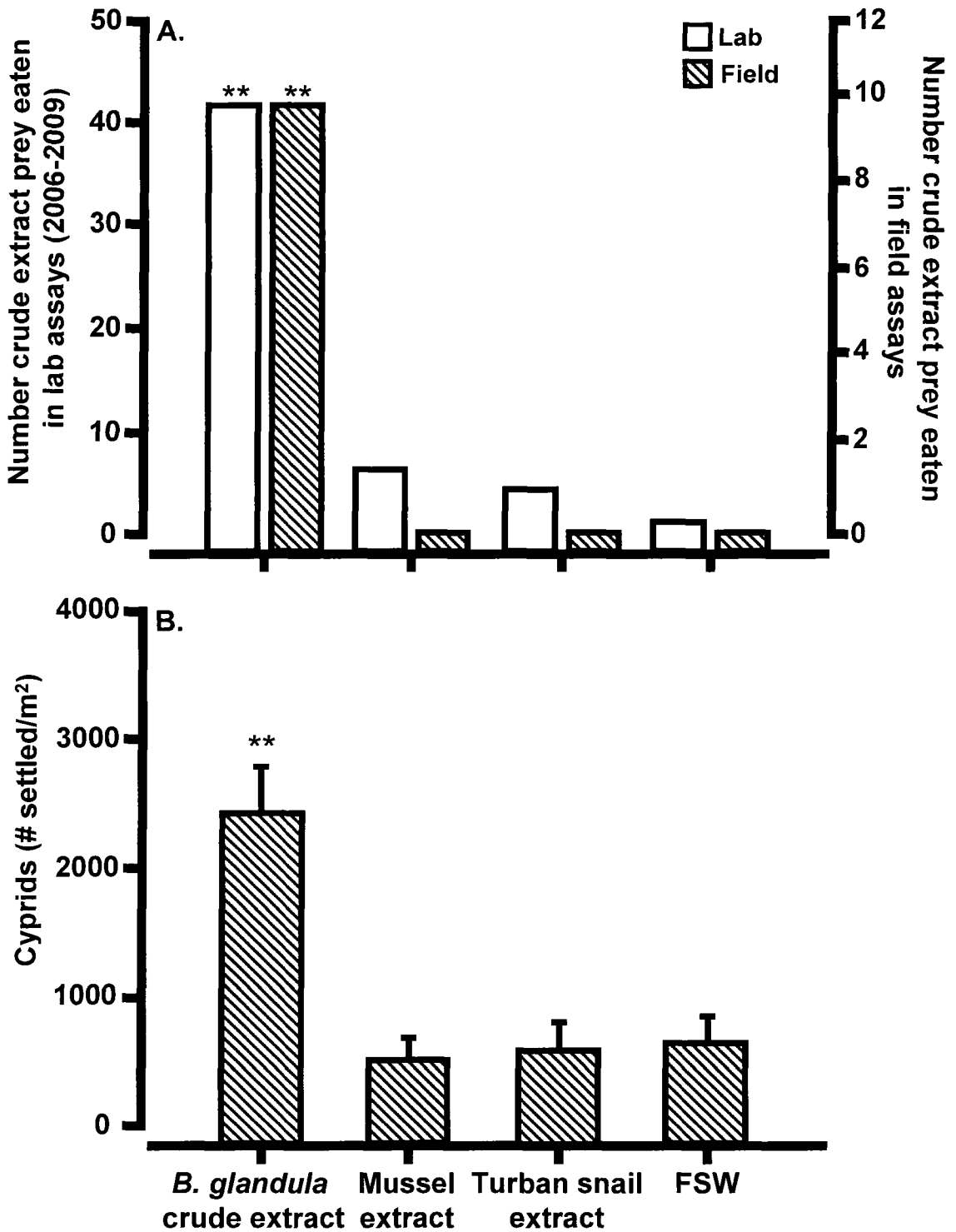

A.
Lab
Field
Number crude extract prey eaten in lab assays (2006-2009)
Number crude extract prey eaten in field assays
50
40
30
20
10
0
12
10
8
6
4
2
0
**
**
B.
Cyprids (# settled/m²)
4000
3000
2000
1000
0
**
B. glandula crude extract
Mussel extract
Turban snail extract
FSW

Figure 2

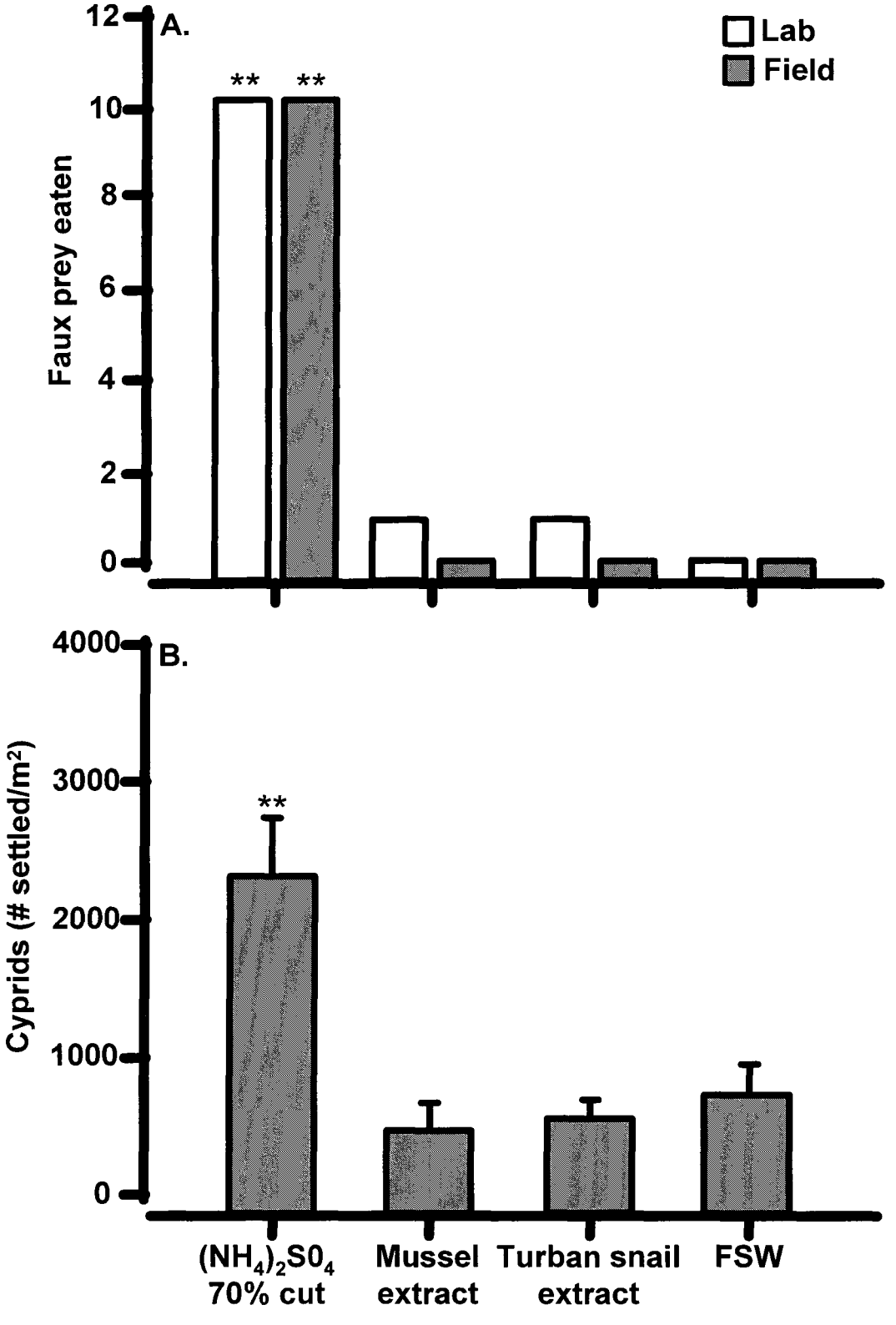

A.
Lab
Field
**
**
12
10
8
6
4
2
0
Faux prey eaten
B.
**
4000
3000
2000
1000
0
Cyprids (# settled/m²)
(NH4)2S04 70% cut
Mussel extract
Turban snail extract
FSW

Figure 3

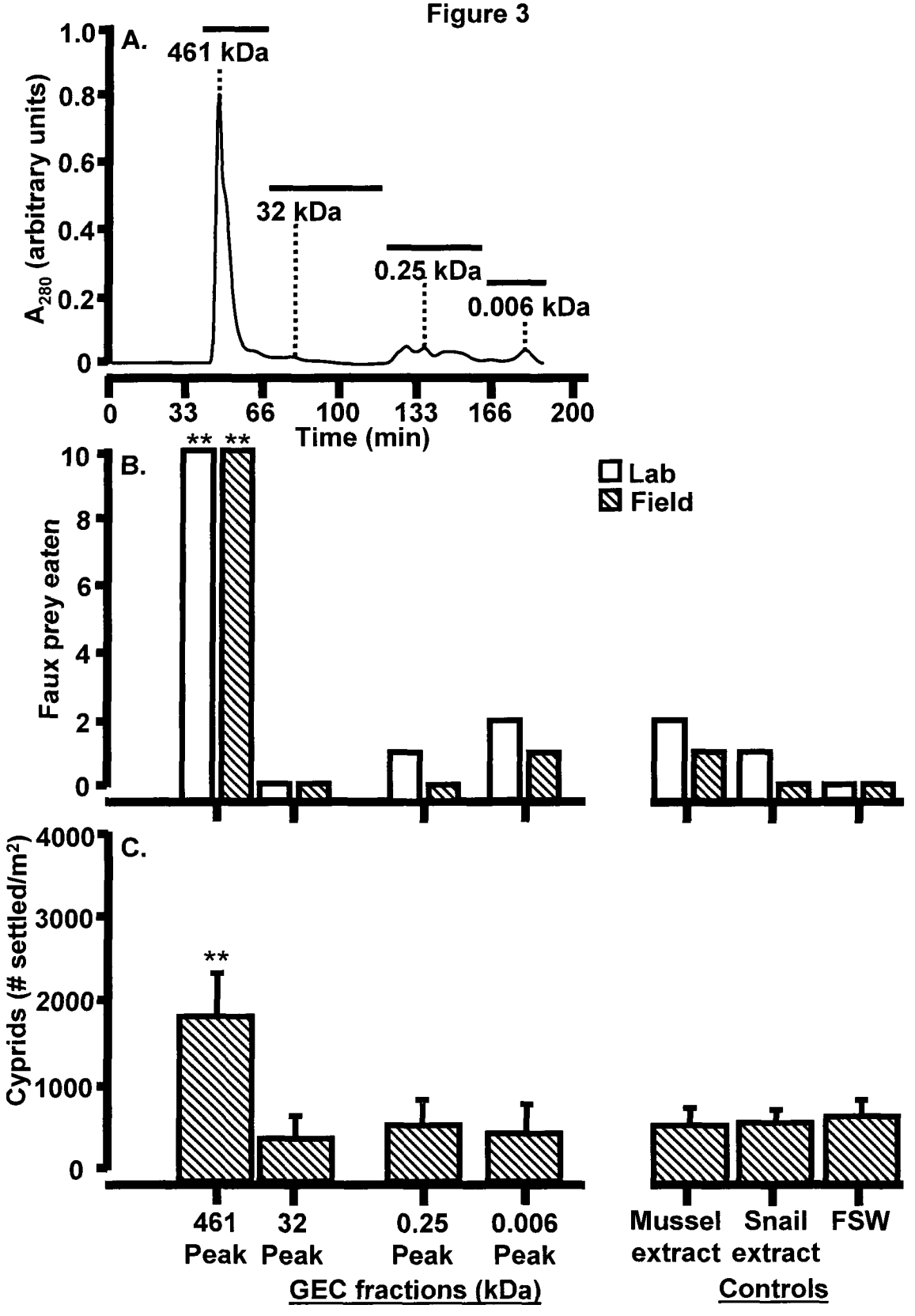

A.
461 kDa
32 kDa
0.25 kDa
0.006 kDa
A_{280} (arbitrary units)
1.0
0.8
0.6
0.4
0.2
0
0
33
66
100
133
166
200
Time (min)
B.
** **
Lab
Field
Faux prey eaten
10
8
6
4
2
0
C.
**
Cyprids (# settled/m²)
4000
3000
2000
1000
0
461 Peak
32 Peak
0.25 Peak
0.006 Peak
GEC fractions (kDa)
Mussel extract
Snail extract
FSW
Controls

Figure 4

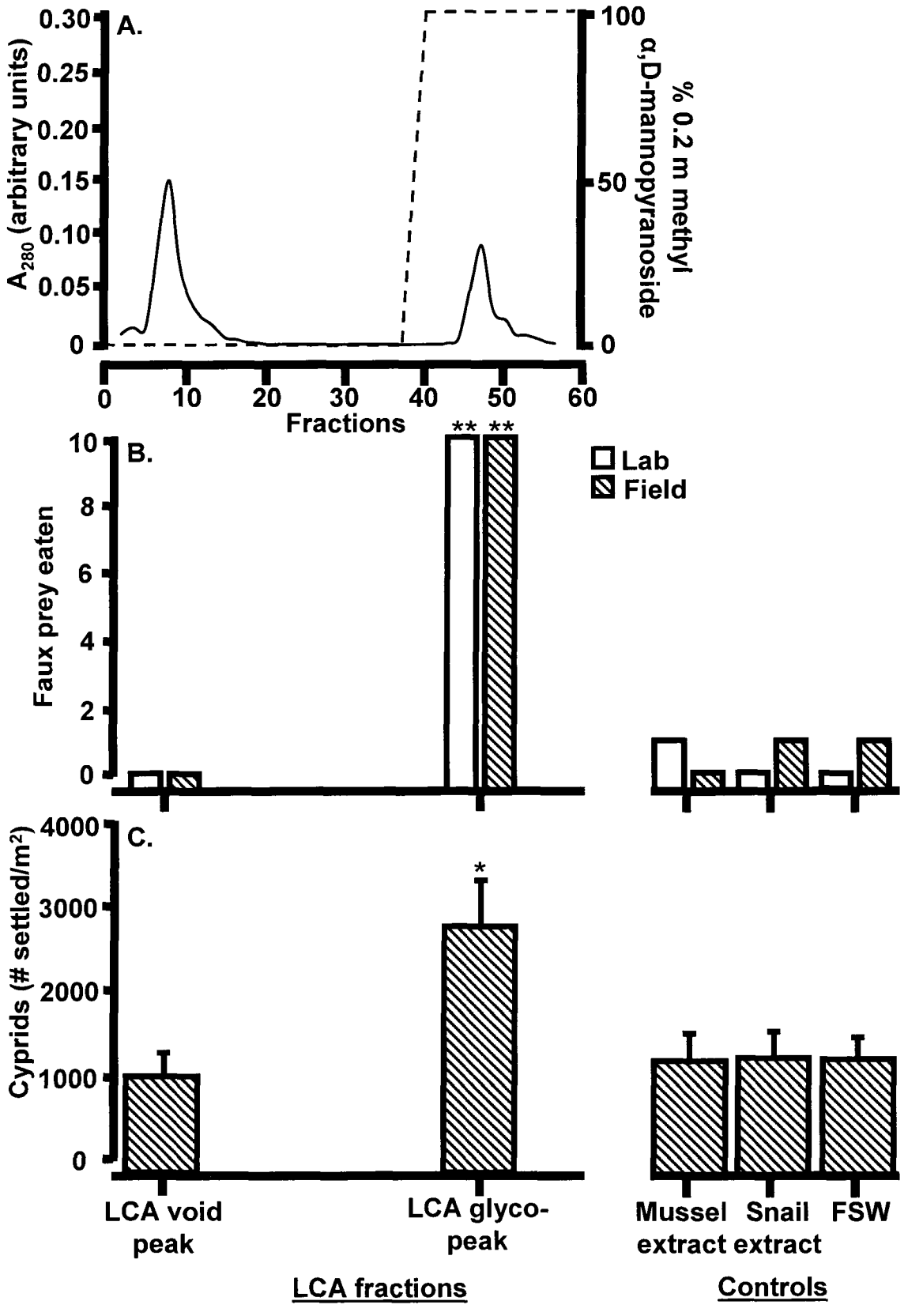

A.
0.30
0.25
0.20
0.15
0.10
0.05
0
A_{280} (arbitrary units)
100
50
0
% 0.2 m methyl α,D-mannopyranoside
0
10
20
30
40
50
60
Fractions
B.
10
8
6
4
2
0
Faux prey eaten
** **
Lab
Field
C.
4000
3000
2000
1000
0
Cyprids (# settled/m²)
*
LCA void peak
LCA glyco-peak
LCA fractions
Mussel extract
Snail extract
FSW
Controls

Figure 5

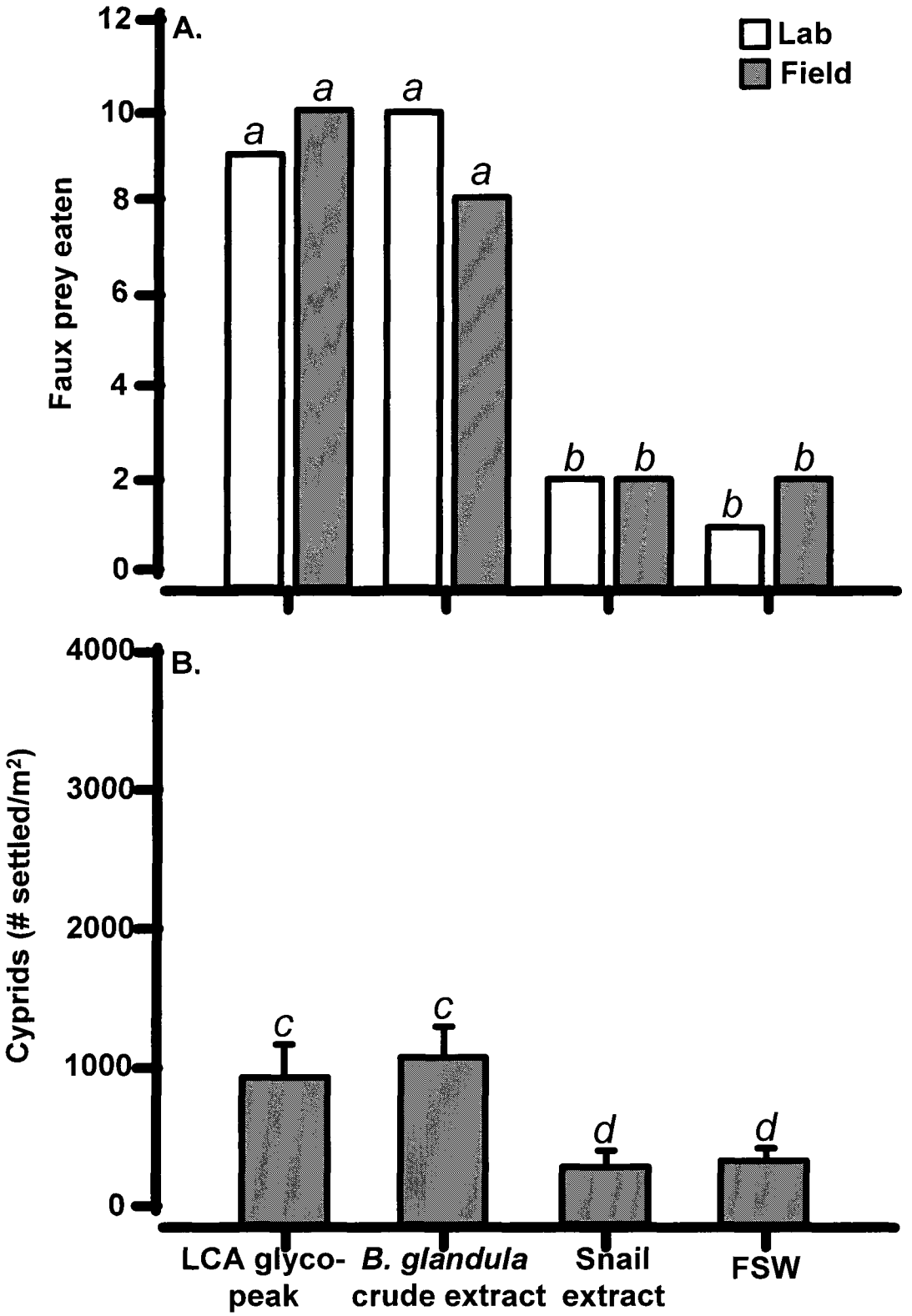

A.
Lab
Field
Faux prey eaten
12
10
8
6
4
2
0
a
a
a
a
b
b
b
b
B.
Cyprids (# settled/m²)
4000
3000
2000
1000
0
c
c
d
d
LCA glyco-peak
B. glandula crude extract
Snail extract
FSW

Figure 6

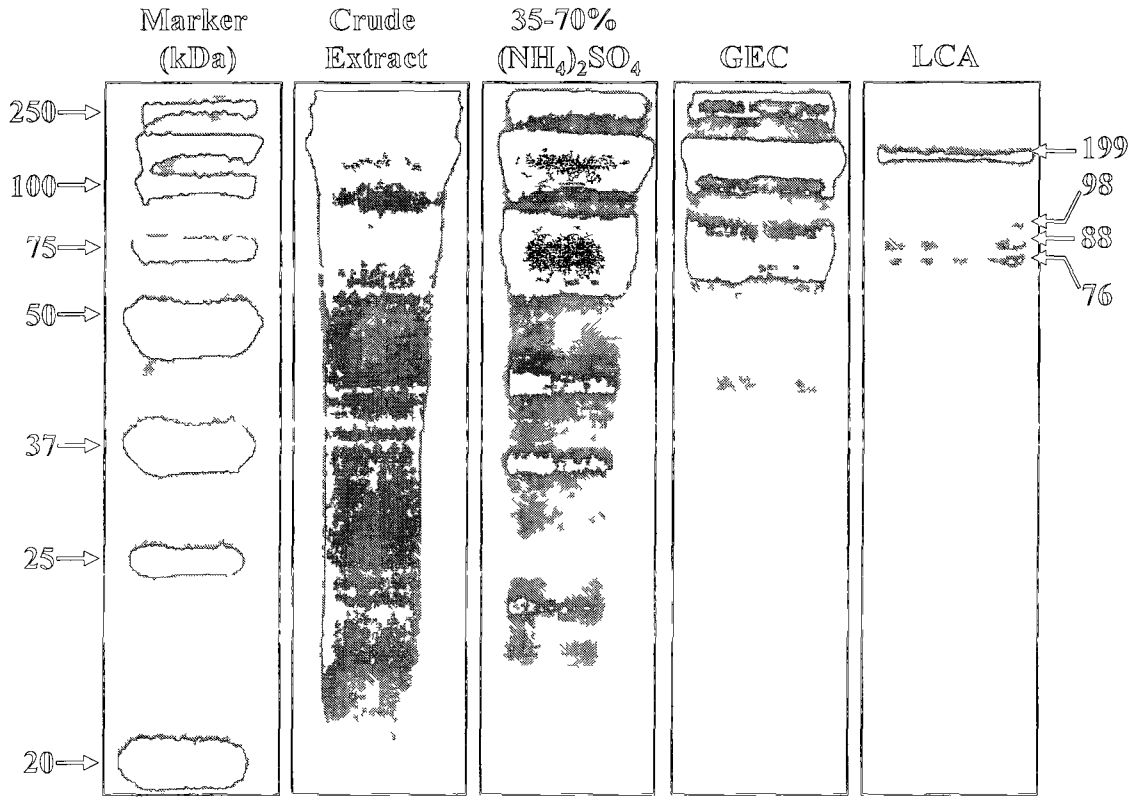
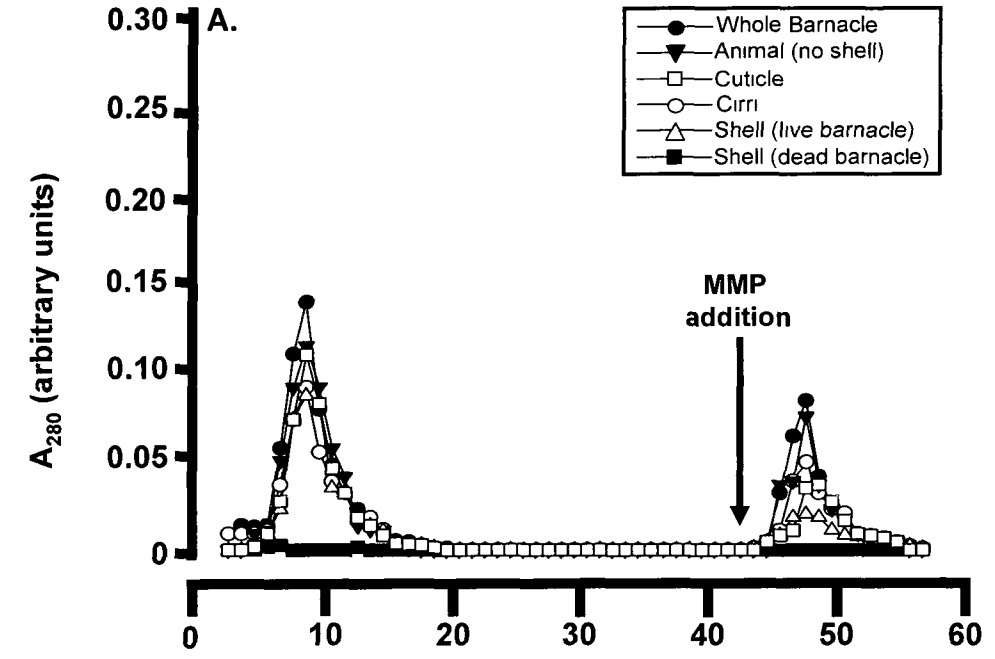

Marker (kDa)
Crude Extract
35-70% $(NH_4)_2SO_4$
GEC
LCA
250
100
75
50
37
25
20
199
98
88
76

Figure 7


A.
0.30
0.25
0.20
0.15
0.10
0.05
0
A_{280} (arbitrary units)
Whole Barnacle
Animal (no shell)
Cuticle
Cirri
Shell (live barnacle)
Shell (dead barnacle)
MMP addition
0
10
20
30
40
50
60

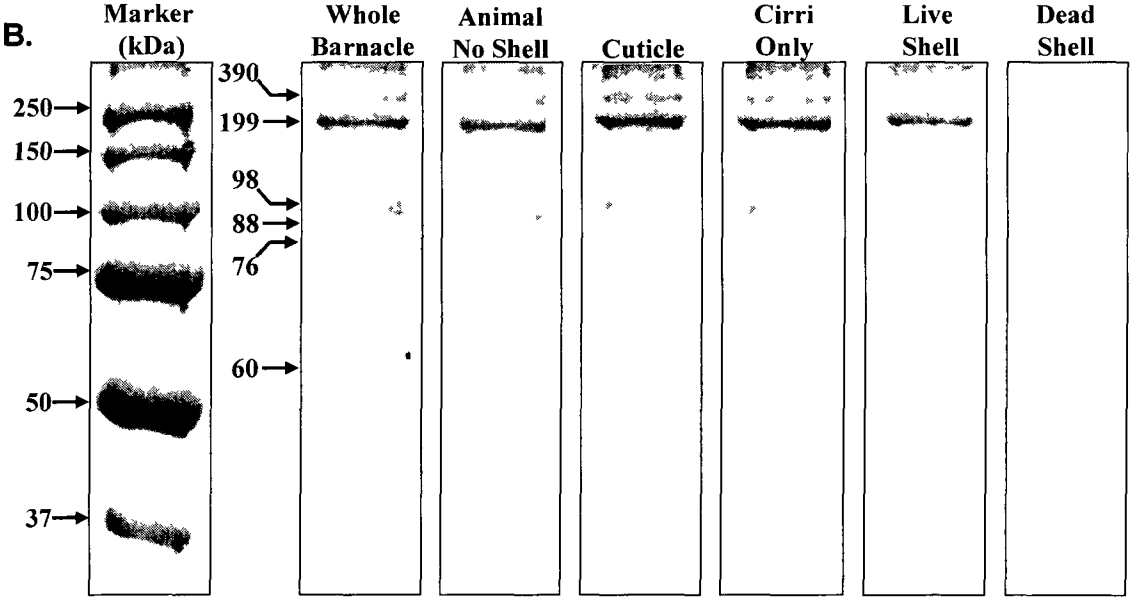

B.
Marker (kDa)
Whole Barnacle
Animal No Shell
Cuticle
Cirri Only
Live Shell
Dead Shell
250
150
100
75
50
37
390
199
98
88
76
60

Figure Captions

Figure 2-1. Whelk feeding assays using *Balanus glandula* crude protein extract and controls in the laboratory from 2006-2009 (N=59) and in the field (n=12) (A). *B. glandula* cyprid larvae settlement on crude extract or control solution-bound plates (n=20) (B). A total protein concentration of an intermediate-sized barnacle was determined to be 1.289 mg. Experiments were scaled to this concentration. Asterisks denotes a significance level $p < 0.005$.

Figure 2-2. Whelk feeding assays using the 70% ammonium sulfate ($(NH_4)_2SO_4$) precipitate of *Balanus glandula* and control solutions in laboratory and field feeding trials (A). *B. glandula* cyprid larvae settlement on 70% $(NH_4)_2SO_4$ cut or control solution-bound plates (B). The 70% cut contained approximately 42% of the total proteins contained in an intermediate-sized barnacle. Feeding and settlement occurred on samples scaled to this level. Asterisk denotes a significance level $p < 0.005$.

Figure 2-3. The molecular weight fractionation of proteins found in the 70% $(NH_4)_2SO_4$) cut as run through a size exclusion column (SEC) eluting at 0.8 ml/min. Bars over peaks indicate fractions pooled together (A). Whelk feeding responses to each of the pooled eluted peaks and the response of the active peak when assayed against control solutions (B). Cyprid settlement on plates bound with each of the pooled eluted peaks and the response of the active peak when assayed against control solutions (C). The active peak (~461 kDa) was found to represent ~38.7% of the total barnacle protein. Feeding and settlement were both induced upon contacting samples scaled to this. Asterisk denotes a significance level $p < 0.005$.

Figure 2-4. The separation of the 461 kDa peak from size exclusion chromatography into component void and glycoprotein fractions before and after the addition of 0.2 M methyl α, D-mannopyranoside (MMP), respectively (A). Whelk feeding response to the void and glycoprotein peaks and the response of the active peak when assayed against control solutions (B). Cyprid settlement on plates bound with the void and glycoprotein peaks and settlement response of the active glycoprotein when assayed against control solutions (C). The active glycoprotein was found to represent 12.6% of the total barnacle protein concentration (0.16 mg/barnacle). Feeding and settlement were both induced upon contacting samples scaled to this. Asterisk denotes a significance level $p < 0.005$.

Figure 2-5. The active glycoprotein was assayed against the crude extract and control solutions in both whelk feeding (A), and cyprid settlement (B). The glycoproteins sample contained a protein concentration of 12.6% that of the crude and turban snail extracts. Bars with the same letter indicate responses that were not significantly different.

Figure 2-6. SDS-PAGE gel, run under reducing conditions in the presence of β- mercaptanoethanol, of each purification step showing the isolation of the final glycoprotein complex (here broken into its component subunits) from all other crude extract component proteins.

Figure 2-7. Lentil-lectin (LCA) affinity chromatography elution profile showing the presence of a glycoprotein peak following the addition of 0.2 M methyl α, D-mannopyranoside (MMP) in the whole barnacle extract (filled circle), the animal without the shell (filled triangle), isolated cuticle (open square), cirri (open circle), scrapings of the outer surface of live shells (open triangles), and scrapings of the outer surface of dead barnacle shells (filled square) (A). SDS-PAGE gel of the eluted glycoproteins from the LCA column runs of each of the dissected tissues (B). The glycoprotein complex and its component subunits are present in all tissues tested, but do not show up in the dead shell scrapings.

References:

Ache, B.W., and J.M. Young. 2005. Olfaction: diverse species, conserved principles. Neuron 48:417-430.

Albeck, S., S. Weinerm, and L. Addadi. 1996. Polysaccharides of intracrystalline glycoproteins modulate calcite crystal growth in vitro. Chemistry a European Journal 2:278-284.

Bais, H. P., T.L. Weir, L.G. Perry, S. Gilroy, and J.M. Vivanco. 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. Annual Review of Plant Biology 57:233-266.

Baldwin, I.T., R. Halitschke, A. Paschold, C.C. von Dahl, and C.A. Preston. 2010. Volatile signaling in plant-plant interactions: "talking trees" in the genomics era. Science 311:812-815.

Billings, R. F. 1985. Southern pine bark beetles and associated insects. Journal of Applied Entomology 99:483-491.

Bradford, M. 1976. A Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72:248-254.

Bronmark, C., and L. Hansson. 2000. Chemical communication in aquatic systems: an introduction. Oikos 88:103-109.

Brodie, E.D. III, and E.D. Brodie jr. 1990. Tetrodotoxin resistance in garter snakes: an evolutionary response of predators to dangerous prey. Evolution 44:651-659.

Brodie, E.D. III, C.R. Feldman, C.T. Hanifin, J.E. Motychak, D.G. Mulcahy, B.L. Williams, and E.D. Brodie jr. 2005. Parallel arms races between garter snakes and newts involving tetrodotoxin as the phenotypic interface of coevolution. Journal of Chemical Ecology 31:343-356.

Caskey, J. L., K.H. Hasenstein, and R.T. Bauer. 2009. Studies on contact sex pheromones of the caridean shrimp. Invertebrate Reproduction and Development 53:92-103.

Clare, A.S., R.F. Thomas, and D. Rittschof. 1995. Evidence for the involvement of cyclic amp in the pheromonal modulation of barnacle settlement. The Journal of Experimental Biology 198:655-664.

Connell, J.H. 1961(a). The influence of interspecific competition and other factors on the distribution of the barnacle *Chthamalus stellatus*. Ecology 42:710-723.

Connell, J.H. 1961(b). Effects of competition, predation by *Thais lapillus*, and other factors on natural populations of the barnacle Balanus balanoides. Ecological Monographs 31:61-104.

Connell, J. H. 1970. A predator-prey system in the marine intertidal region. I. *Balanus glandula* and several predatory species of *Thais*. Ecological Monographs 40:49-78.

Crisp, D.J., and P.S. Meadows. 1963. The chemical basis of gregariousness in Cirripedes. The Proceedings of the Royal Society London B 156:500-520

Dalziel, B., and E.G. Boulding. 2005. Water-borne cues from a shell-crushing predator induce a more massive shell in experimental populations of an intertidal snail. Journal of Experimental Marine Biology and Ecology 317:25-35.

Dayton, P.K. 1971. Competition, disturbance, and community organization: the provision and subsequent utilization of space in a rocky intertidal community. Ecological Monographs 41:351-389.

DeBoer, J.G., M.A. Postumus, and M. Dicke. 2004. Identification of volatiles that are used in discrimination between plants infested with prey or non-prey herbivores by a predatory mite. Journal of Chemical Ecology 30:2215-2230.

DeMoraes, C., W. Lewis, P. Pare, H. Alborn, and J.H. Tumlinson. 1998. Herbivore-infested plants selectively attract parasitoids. Nature, 393:570-573.

Denny, M.W., L.P. Miller, M.D. Stokes, L.J.H. Hunt, and B.S.T. Helmuth. 2003. Extreme water velocities: topographic amplification of wave-induced flow in the surf zone of rocky shores. Limnology and Oceanography, 48:1-8.

Dicke, M., M.W. Sabelis, J. Takabayashi, J. Bruin, and M.A. Posthumus. 1990. Plant strategies of manipulating predator- prey interactions through allelochemicals: prospects for application in pest control. Journal of Chemical Ecology 16:3091-3118.

Doucette, G. J., A.D. Cembella, J.L. Martin, J. Michaud, T.V.N. Cole, and R.M. Rolland. 2006. Paralytic shellfish poisoning (PSP) toxins in North Atlantic right whales *Eubalaena glacialis* and their zooplankton prey in the Bay of Fundy, Canada. Marine Ecological Progress Series 306:303–313.

Dreanno, C., K. Matsumura, N. Dohmae, K. Takio, H. Hirota, R.R. Kirby, and A.S. Clare. 2006. An alpha2-macroglobulin-like protein is the cue to gregarious settlement of the barnacle *Balanus amphitrite*. Proceedings of the National Academy of Sciences of the United States of America 103:14396-401.

Dreanno, C., R.R. Kirby, and A.S. Clare. 2007. Involvement of the barnacle settlement-inducing protein complex (SIPC) in species recognition at settlement. Journal of Experimental Marine Biology and Ecology 351:276-282

Duval, M.A., A.M. Calzetta, and D. Rittschof. 1994. Behavioral response of *Littoraria irrorata* (Say) to water-borne odors. Journal of Chemical Ecology 20:3321-3334.

Eberhard, W.G. 1977. Aggressive chemical mimicry by a bolas spider. Science, 198:1173-1175.

Erbilgin, N., and K.F. Raffa. 2000. Opposing effects of host monoterpenes on responses by two sympatric species of bark beetles to their aggregation pheromones. Journal of Chemical Ecology 26:2527-2548.

Ferner, M. C., & Weissburg, M. J. 2005. Slow-moving predatory gastropods track prey odors in fast and turbulent flow. The Journal of Experimental Biology 208:809-19.

Ferrer, R.P., and R.K. Zimmer. 2007(a). Chemosensory reception, behavioral expression, and ecological interactions at multiple trophic levels. The Journal of Experimental Biology 210:1776-1785.

Ferrer, R.P., and R.K. Zimmer. 2007(b). The scent of danger: arginine as an olfactory cue of reduced predation risk. Journal of Experimental Biology 210:1768-1775.

Fingerut, J., C.A. Zimmer, and R.K. Zimmer. 2003. Patterns and Processes of Larval Emergence in an Estuarine Parasite System. Biological Bulletin 205:110 -120.

Gerlach, G., J. Atema, M.J. Kingsford, K.P. Black, and V. Miller-Sims. 2007. Smelling home can prevent dispersal of reef fish larvae. Proceedings of the National Academy of Sciences of the United States of America 104:858-863.

Gillespie, A.S., and E. Elliott. (2005). Comparative advantages of imidazole-sodium dodecyl sulfate-zinc reverse staining in polyacrylamide gels. Analytical biochemistry 345:158-60.

Goochee, C.F., and T. Monica. 1990. Environmental effects on protein glycosylation. Nature Biotechnology 8:412-427.

Haynes, K.F., C. Gemeno, K.V. Yeargan, J.G. Millar, and K.M. Johnson. 2002. Aggressive chemical mimicry of moth pheromones by a bolas spider: how does this specialist predator attract more than one species of prey? Chemoecology 12:99-105.

Hildebrand, J.G., and G.M. Shepherd. 1997. Mechanisms of olfactory discrimination: converging evidence for common principles across phyla. Annual Review of Neuroscience 20:595-631.

Howe, N.R., and Y.M. Sheikh. 1975. Anthopleurine: a sea anemone alarm pheromone. Science 189:386-388.

Kelly, L.S., and T. Snell. 1998. Role of surface glycoproteins in mate-guarding of the marine harpacticoid *Tigriopus japonicus*. Marine Biology 130:605-612.

Kvitek, R.G., A.R. DeGange, and M. K. Beitler. 1991. Paralytic shellfish poisoning toxins mediate feeding behavior of sea otters. Limnology and Oceanography 36:393–404.

Kvitek, R.G., and C. Bretz. 2004. Harmful algal bloom toxins protect bivalve populations from sea otter predation. Marine Ecological Progress Series 271:233-234.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227:680-685.

Larman, V.N., and P.A. Gabbot. 1975. Settlement of cyprid larvae of *Balanus balanoides* and *Elminius modestus* induced by extracts of adult barnacles and other marine animals. Journal of the Marine Biological Association of the UK 55:183-190.

Marin, F., and G. Luquet. 2004. Molluscan shell proteins. Comptes Rendus Palevol 3: 469-492.

Matsumura, K., M. Nagano, and N. Fusetani. 1998 (a). Purification of a Larval Settlement-Inducing Protein Complex (SIPC) of the Barnacle, *Balanus amphitrite*. Journal of Experimental Zoology 281:12-20.

Matsumura, K., M. Nagano, Y. Kato-Yoshinaga, M. Yamazaki, A.S Clare, and N. Fusetani. 1998(b). Immunological studies on the settlment-inducing protein complex (SIPC) of the barnacle Balanus amphitrite and its possible involvement in larva-larva interactions. Proceedings of the Royal Society of London 265:1825-1830.

Matsumura, K., S. Mori, M. Nagano, and N. Fusetani. 1998(c). Lentil-lectin inhibits adult extract-induced settlement of the barnacle, *Balanus amphitrite*. The Journal of Experimental Zoology 280:213-219.

Menge, B.A. 2000. Top-down and bottom-up community regulation in marine rocky intertidal habitats. Journal of Experimental Marine Biology and Ecology 250:257-289.

Nagai, K., Y. Matsuyama, T. Uchida, S. Akamatsu, and T. Honjo. 2000. Effect of a natural population of the harmful dinoflagellate *Heterocapsa circularisquama* on the survival of the pearl oyster *Pinctada fucata*. Fisheries Science 66: 995–997.

Navarrete, S.A., and B.A. Menge. 1996. Keystone predation and interaction strength: interactive effects of predators on their main prey. Ecological Monographs 66:409-429.

Navarrete, S.A., and J.C. Castilla. 2003. Experimental determination of predation intensity in an intertidal predator guild: dominant versus subordinate prey. Oikos 100:251-262.

Nevitt, G.A., R.R. Veit, and P. Kareiva. 1995. Dimethyl sulfide as a foraging cue for Antarctic Procellariiform seabirds. Nature 376:680– 682.

Pohnert, G. 2005. Diatom/copepod interactions in plankton: the indirect chemical defense of unicellular algae. ChemBioChem 6:946-959.

Raffa, K.F. 2001. Mixed messages across multiple trophic levels: the ecology of bark beetle chemical communication systems. Chemoecology 11:49-65.

Raimondi, P.T. 1988. Settlement cues and determination of the vertical limit of an intertidal barnacle. Ecology 69(2):400-407.

Raimondi, P.T, S.E. Forde, L.F. Delph, and C.M. Lively. 2000. Processes structuring communities: evidence for trait-mediated indirect effects through induced polymorphisms. Oikos 91:353-361.

Riffell, J.A., P.J. Krug, and R.K. Zimmer. 2002. Fertilization in the sea: the chemical identity of an abalone sperm attractant. The Journal of Experimental Biology 205:1439-1450.

Schantz, E.J. 1986. Chemistry of saxitoxin and related toxins. Annals of the New York Academy of Sciences 479:15-23.

Seybold, S. J., D.P. Huber, J.C. Lee, and A.D. Graves. 2006. Pine monoterpenes and pine bark beetles: a marriage of convenience for defense and chemical communication. Phytochemistry Reviews 5:143-178.

Silverstein, R.M., R.G. Brownlee, T.E. Bellas, D.L. Wood, and L.E. Browne. 1968. Brevicomin: principle sex attractant in the frass of the female western pine beetle. Science 159: 889-891.

Smee, D.L., M.C. Ferner, and M.J. Weissburg. 2008. Alteration of sensory abilities regulates the spatial scale of nonlethal predator effects. Oecologia 156:399-409.

Snell, T., and M.J. Carmona. 1994. Surface glycoproteins in copepods: potential signals for mate recognition. Hydrobiologia 292/293: 255-264.

Steinberg, P. D., R.D. Nys, and S. Kjelleberg. 2002. Chemical cues for surface colonization. Journal of Chemical Ecology 28(10):1935-1951.

Stern, K., and M.K. Mcclintock. 1998. Regulation of ovulation by human pheromones. Nature 392:177-179.

Stowe, M.K., J.H. Tumlinson, and R.R. Heath. 1987. Chemical mimicry: bolas spiders emit components of moth prey species sex pheromones. Science 236:964–967

Teegarden, G.J., and A.D. Cembella. 1996. Grazing of toxic dinoflagellates, *Alexandrium spp.*, by adult copepods of coastal Maine: implications for the fate of paralytic shellfish toxins in marine food webs. Journal of Experimental Marine Biology and Ecology 196:145–176.

Trussell, G.C., P.J. Ewanchuk, and M.D. Bertness. 2003. Trait-mediated effects in rocky intertidal food chains: predator risk cues alter prey feeding rates. Ecology 84:629-640.

Turlings, T.C.J., J.H. Tumlinson, and W.J. Lewis. 1990. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. Science 250:1251-1253.

Waters, C.M., and B.L. Bassler. 2005. Quorum Sensing: Cell-to-Cell Communication in Bacteria. Annual Review of Cell and Developmental Biology 21:319-346.

Weir, T.L., S. Park, and J.M. Vivanco. 2004. Biochemical and physiological mechanisms mediated by allelochemicals. Current Opinion in Plant Biology 7:472-479.

Wieters, E.A., and S.A. Navarrete. 1998. Spatial variability in prey preferences of the intertidal whelks Nucella canaliculata and Nucella emarginata. Journal of Experimental Marine Biology and Ecology 222:133-148.

Wheeler, G. L., K. Tait, A. Taylor, C. Brownlee, and I. Joint. 2006. Acyl-homoserine lactones modulate the settlement rate of zoospores of the marine alga *Ulva intestinalis* via a novel chemokinetic mechanism. Plant, Cell, and Environment 29:608-618.

Weiner, S., W. Traub, and S.B. Parker. 1984. Macromolecules in mollusc shells and their functions in biomineralization [and discussion]. Philosophical transactions of the Royal Society London B 304:425-434.

White, A.W. 1981. Sensitivity of marine fishes to toxins from the red-tide dinoflagellate *Gonyaulax excavata* and implications for fish kills. Marine Biology 65:255-260.

Zimmer, R. K., Commins, J. E., & Browne, K. A. 1999. Regulatory effects of environmental chemical signals on search behavior and foraging success. Ecology, 80:1432-1446.

Zimmer, R.K., and C.A. Butman. 2000. Chemical signaling processes in the marine environment. Biological Bulletin 198:168-187.

Zimmer, R.K., D.W. Schar, R.P. Ferrer, P.J. Krug, L.B. Kats, and W.C. Michel. 2006. The scent of danger: tetrodotoxin (ttx) as an olfactory cue of predation risk. Ecological Monographs 76:585-600.

Zimmer, R.K., and R.P. Ferrer. 2007. Neuroecology, Chemical Defense, and the Keystone Species Concept. Biological Bulletin 213:208-225.

Zimmer-Faust, R.K. (1993). ATP: A potent prey attractant evoking carnivory. Limnology and Oceanography 38:1271-1275.

Chapter 3: Barnacle MULTIFUNCin- a multifunctional barnacle protein at the core of community dynamics

ABSTRACT

Interactions between species often have an underlying sensory basis. Many of these interactions induce trophic cascades that, in part, structure communities. Thus, the chemical signals driving the interactions are of significant ecological importance. Here, we identify a glycoprotein signal—a secreted multifunctional protein factor (MULTIFUNCin)—which is the putative settlement cue for cyprid larvae of the barnacle *Balanus glandula*. Furthermore, MULTIFUNCin is recognized as the feeding stimulant for a numerically dominant barnacle predator, the whelk *Acanthinucella spirata*. Utilizing electrospray ionization coupled with gas-phase electrophoretic mobility molecular analysis (ESI-GEMMA), the glycoprotein was identified as a 390 kDa complex. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and ESI-GEMMA, run under denaturing conditions, revealed this signal to be a protein dimer composed of two identical 199 kDa proteins. From feeding and settlement assays, it was determined that all the activity of the complex could be found in the 199 kDa monomeric subunit. Subsequent amino acid translation from a sequenced 5.1 kb mRNA transcript found the active glycoprotein to be composed of 1550 amino acid residues (mw = 171 kDa). Conserved domains were discovered between MULTIFUNCin and members of the α_2-macroglobulin (A2M) protein superfamily; however, missing were

certain protein motifs characteristic of members of this family. MULTIFUNCin is, therefore, a novel protein that likely evolved from an A2M ancestral gene into a signal with cellular, individual, population, and community level functionality.

INTRODUCTION

Natural communities form as a result of numerous species interacting in space and over time. These processes fundamentally result from complex behaviors that are driven by central nervous system responses to internal and external stimuli. Under natural conditions, organism- and environmentally derived chemical signals saturate the surroundings; nevertheless, many species have evolved modes of detection that identify only those cues which impart useful information (e.g. mate, danger, food, and/or shelter identifiers), suggesting strong selection pressures for chemical receptors (Berenbaum, 2002). There are numerous examples of such bioactive compounds, and many of them induce behavioral and/or morphological responses in receiver species (see reviews by Vos *et al.*, 2005, and Paul *et al.*, 2007). Despite the importance of sensory signals to biology, physiology, ecology and evolution, many bioactive molecules have been only partially characterized. Thus, although the intra- and interspecies responses of many of these signals is irrefutable, only full characterization would reveal the signal origin, natural concentrations and persistence, likely receptors, genetic underpinning, and potential ecological roles. Possibly of greater significance, such insights into how species respond to, and interact with, naturally produced and occurring chemical signals will

undoubtedly shed light on the selective forces which drive ecology and evolution (Zimmer and Butman, 2000).

As chemical cues and signals are diverse and abundant in many natural systems, the task of parsing out, identifying, and characterizing those critical to individual-, population-, and community-wide processes is, at best, daunting. That said, significant strides have been made in terrestrial, aquatic, and marine systems. For example, numerous fully characterized pheromones, and their biochemical pathways, have been identified for moth reproduction (see Tillman *et al.*, 1999 and references therein). Moreover, Heptadecene-1 is an aggregating compound (for mating and possibly anti-predation) of the lake zooplankter *Polyphemus pediculus* (Wendal and Jüttner, 1997; Burks and Lodge, 2002). In marine systems, histamine from the alga *Delisea pulchra* is a settlement cue for the sea urchin, *Holopneustes purpurascens* (Swanson *et al.*, 2004). A near universal theme in the fully characterized signals to date is that they facilitate conspecific processes important to the population dynamics only of the single species in question. Biologically this makes intuitive sense, but few investigations have moved beyond intraspecies responses to look at a broader role for these identified signals.

The emission of signal molecules into the surrounding environment can elicit a multitude of responses from individuals of the same species. These very signals, however, might be recognized by other species with positive, negative, and/or neutral consequences. Such cues can serve as the sensory signals that underpin species interactions, and thus, are of critical importance to understanding organismal behavior, food web complexity, and, ultimately, community dynamics. Identifying community-

driving chemical cues is challenging as they often represent a small proportion of the signal molecules within a system. There are, nevertheless, some very notable cases where signal molecules have been fully identified. Arguably, the most prominent examples are those found in plant-herbivore-predator interactions involving the emission of plant volatile organic compounds (VOCs). These signals, emitted during, and after, grazing, attract both general and specific predators, as well as triggering neighboring plants to begin producing defense compounds that deter potential future attack (see Raffa 2001 for bark beetle review, De Boer *et al.*, 2004; and Baldwin *et al.*, 2006). VOCs, therefore, have strong primary and secondary roles which influence multiple trophic levels and can manipulate community dynamics.

Signals that are available in limited quantity but have strong structural consequences through behavioral, morphological, and/or physiological modifications have been termed "keystone molecules" (Zimmer and Ferrer, 2007). Only a few such molecules have been identified, many of which are defense compounds (e.g. tetrodotoxin and saxitoxin) co-opted as alarm cues and/or incorporated through digestion (Teegarden and Cembella, 1996; Kvitek and Bretz, 2004; Zimmer and Ferrer, 2007). Structural compounds—those that are requisite to some aspect of organismal physiology—may represent an alternate class of multifunctional signals. As many organisms are physiologically constrained to produce such compounds, they may lend themselves to easy exploitation by con- or heterospecifics. Despite underlying selection pressure that likely acts on chemical receptors, there have been few studies exploring the potential role of obligatory structural elements as signal molecules. Furthermore, proteins—ubiquitous

bioactive molecules—have been examined in a very limited capacity as signal molecules in natural systems. This work highlights the potential for structural compounds to act as signals that influence cellular, individual, population, and community-level processes by fully identifying, characterizing, and sequencing a barnacle protein signal that strongly influences the community dynamics of wave-swept rocky shores along the Pacific Northeast of North America.

Previous studies conducted in our labs focused on interactions between the barnacle *Balanus glandula* and a primary barnacle predator, the whelk *Acanthinucella spirata*. Using field and laboratory settlement and feeding assays, a naturally expressed glycoprotein from adult *B. glandula* that induced settlement of conspecific larvae was isolated. This same glycoprotein triggered whelks to begin feeding upon contacting the signal (Ferrier *et al.*, in prep.). This study was the first to show that a molecule can be both a potent settlement inducer as well as a strong signal for the primary predator of that species. At the heart of barnacle population dynamics, this glycoprotein drives dual, yet opposing, demographic processes that both directly and indirectly influence the distribution and abundance of many organisms in the upper intertidal of rocky wave-swept shores.

Here, the glycoprotein signal was identified as a protein complex, and the activity of the constituent subunits, and cleavage products, determined in settlement and feeding assays. Utilizing mass spectrometry and ion mobility spectrometry, we identified the native weight of the intact complex as well as the molecular weights of each subunit. Finally, using rapid amplification of cDNA ends (RACE) we sequenced the mRNA

transcript which codes for the active protein identifying a keystone molecule that strongly influences community dynamics on rocky shores.

MATERIALS AND METHODS

Bioassays of active barnacle factor

The target glycoprotein active in both cyprid settlement and barnacle predation was isolated from live adult *Balanus glandula* according methods described in chapter 2. The active signal was previously estimated to be a protein complex of ca. 400 kDa which breaks down into four components under reducing conditions (199, 98, 88, and 76 kDa; migration estimated on 7.5% polyacrylamide gels). As the activity of the intact complex may be found in only one of the protein components each was isolated and tested in feeding assays in which each was embedded, at ecologically realistic concentrations, in carboxymethyl cellulose creating prey mimics. Following positive feeding responses proteins were tested under natural field conditions at Broad Beach (Malibu , CA). The number of prey mimics eaten by the predatory whelk, *Acanthinucella spirata*, was compared between treatment and control (mussel extracts, turban snail extracts, and filtered sea water) mimics in maximum likelihood tests. In addition, the effect of each protein component on *B. glandula* cyprid settlement was assayed by binding each to settlement plates (cut from the rock cyprids naturally settle on) at ecologically scaled levels (dissertation chapter 2). These plates were then deployed in the barnacle zone at Broad Beach for one complete tidal cycle during the primary settlement period (May-

June) of 2009. The resulting number of settled cyprids were counted and compared to control treatments (mussel extracts, turban snail extracts, and FSW) of the same protein concentration in a one-way ANOVA.

Subunit isolation and purification

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on the purified active glycoprotein using a 7.5% acrylamide preparative slab gel (acrylamide:bis = 29:1; 16 x 22 cm) under reducing conditions in the presence of β-mercaptoethanol to dissociate the complex. Isolated, non-fixed, protein components were visualized using imidazole-SDS-zinc heptahydrate reverse staining before each was excised using a sterile scalpel. Following excision, gel fragments were de-stained using SDS-PAGE Laemmli buffer. Protein bands were dissociated from the polyacrylamide by placing gel fragments in 10 cm of Spectrum Spectra/Por™ regenerated cellulose membrane tubing (Spectrum) (10,000 Da molecular weight cutoff) filled with 8ml of Laemmli buffer. Each tube was sealed and placed in a western blotting rig modified for electroelution (cooled with ice) and subjected to an electric current inducing each protein to migrate out of the gel and into solution. Each isolated protein was then dialyzed against 50 mM tris-HCl before being concentrated to biologically-relevant levels. Prior to bioassays, samples were dialyzed against filtered sea water.

Molecular weight determination of glycoprotein complex and component subunits

The native weight of the active complex was measured using electrospray ionization coupled to a gas-phase electrophoretic mobility molecular analyzer (ESI-GEMMA, TSI Inc. Minneapolis, MN). As a soft ionization technique, ESI allows for mass measurements of weakly bound non-covalent protein complexes (Kaddis *et al.*, 2007). Coupled with GEMMA the weight of the large complex was determined without the difficulties of multiply charged molecules which are commonly produced by the ESI process (Kaddis and Loo, 2007; Kaddis *et al.*, 2007). In addition, the molecular weight of each active component (isolated with SDS-PAGE as described above) was determined using matrix-assisted laser desorption ionization time of flight (MALDI-ToF, Voyager DE-STR: Applied Biosystems, San Jose, CA) mass spectrometry and SDS-PAGE migration distance.

Amino acid sequencing

The active glycoprotein complex was purified from live adult barnacles before being chemically digested using cyanogen bromide (CNBr). As a targeted digester CNBr hydrolyzes peptide bonds at the C-terminus of methionine residues generating long peptide chains. CNBr digested proteins were electrophoretically separated on Tricine gels. Each peptide fragment was then excised before being in-gel digested with trypsin. Following this second digestion, samples were applied to a reverse-phase high performance liquid chromatography (HPLC) system run online with a tandem quadrupole-time-of-flight mass spectrometer (LC/Q-ToF) (Sciex QSTAR XL; Applied

Biosystems). This technique separates the peptide fragments by mass before each is then analyzed using tandem mass spectrometry (MS/MS). Sequence and database searches were done using the algorithm MASCOT (Matrix Science, London, UK). Amino acid composition of each fragment was determined from the resulting spectra creating a partial peptide fingerprint of the active compound. These sequenced peptides were the basis from which degenerate primers were designed for use in the isolation and amplification of mRNA transcripts from adult *B. glandula.*

mRNA isolation

The cirri of 10 *Balanus glandula* barnacles were dissected on site from collected adults at Broad Beach (Malibu, CA). The cirri were chosen as the target tissue for mRNA isolation as it was previously determined that the glycoprotein was expressed in large quantity there, and that the suggested mode of application to the outer surfaces was through these appendages (Ferrier *et al.*, in prep). To prevent ribonuclease activity from breaking down mRNA transcripts, dissected cirri were immediately submerged in RNAlater RNA stabilization buffer (Qiagen) and transported to UCLA on ice. Stabilized barnacle cirri were ground into a powder under liquid nitrogen, and the total RNA was extracted using RNeasy total RNA isolation kit (Qiagen). As the focus of our work was to obtain the sequence of the active signal we isolated mRNA from the total RNA population using a NucleoTrap poly (A) RNA purification kit (Clontech). Isolated mRNA was kept at -80 °C until use in first strand cDNA synthesis.

Amplification of Balanus glandula *cDNA and amino acid composition*

Isolated mRNA of *B. glandula* was reverse transcribed into first strand cDNA with a SMARTer IIA rapid amplification of cDNA ends (RACE) kit (Clontech) to create a total cDNA template ready for amplification. A partial sequence of the active *B. glandula* cDNA was amplified by PCR using degenerate oligonucleotide primers designed from peptide fingerprints obtained in our MS/MS sequencing. The resulting partial cDNA fragment was sequenced providing the 5' untranslated region (UTR) and first 800 base pairs (bp). From this sequence, gene-specific primers were designed and used in conjunction with RACE-PCR to amplify the entire open reading frame (ORF) of the active signal. The full length ORF was sequenced in both 5' and 3' directions by automated direct sequencing. As this technique typically yields 700-1500 reliable base pair reads, new gene-specific primers were produced as needed, and sequence gaps filled, until the entire transcript was decoded.

Sequence analysis

Database searches on DNA and amino acid sequence homologies were carried out using the web servers of the National Center for Biotechnology Information (NCBI) and their Basic Local Alignment Search Tool (BLAST) for species comparisons, protein matches, and conserved domain identification. The Expert Protein Analysis System (ExPASy) of the Swiss Institute of Bioinformatics (SIB) was used for protein sequence translation and database searches. Sequence alignments to *Balanus amphitrite* SIPC (Dreanno *et al.*, 2006) and other previously described proteins in the macroglobulin

families were done using Clustal-W (European Bioinformatics Institute). Relatedness to conserved protein domains and phylogenetic tree construction was done using Molecular Evolution Genetic Analysis (MEGA 5.0) (Kumar *et al.*, 2008). Location of potential signal peptide cleavage sites were done using SignalP (v3.1) (Center for Biological Sequence Analysis).

RESULTS

Molecular weight of the native and denatured complex

The active glycoprotein complex extracted from *Balanus glandula* was subjected to SDS-PAGE electrophoresis under denaturing conditions causing the complex to dissociate into four prominent protein components (Fig. 1). Each protein band was individually isolated and electroeluted from the polyacrylamide gel for further mass identification. The molecular weight of the native complex was determined using ESI-GEMMA. Based on the electrophoretic mobility diameter (EMD) of the protein complex, and using the relationship between EMD and molecular weight (Kaddis *et al.*, 2007), the intact complex was shown to have an approximate mass of 390 kDa (Fig. 2).

To obtain more accurate masses of each protein band, a combination of mass spectrometry (MALDI-ToF) and molecular weight estimations from protein mobility in SDS-PAGE gels was done. The MALDI-ToF spectrum of the heaviest of the components found a dominant peak at 199 kDa (Fig. 3a). The next protein band was analyzed and this was found to have a molecular weight of approximately 98 kDa. The

size of both 199 and 98 kDa proteins were confirmed by measuring the mobility of each on 7.5% polyacrylamide gels. As the SDS-PAGE gels provided accurate measurements of two of the four proteins bands, the two smaller component masses were estimated using only their mobility, and were found to be 88 kDa and 76 kDa respectively (Fig. 1).

Subunit activity in cyprid settlement and whelk feeding

Each denatured glycoprotein component was individually isolated and electroeluted from the SDS-PAGE gels to be used in settlement and feeding assays. Cyprid settlement response was measured by deploying settlement plates, bound with each protein, in the barnacle zone for one complete tidal cycle during the primary settlement season (May-June) of 2009. Previous studies identified the intact glycoprotein complex to represent 12.6% (0.16 mg/barnacle) of the total protein content of an adult *B. glandula* (dissertation chapter 2). Isolated protein bands were dynamically scaled to represent this concentration in both settlement (as proportion expressed) and feeding bioassays (as total protein in a barnacle mimic). Plates bound on average 93% of the isolated 199 kDa protein and these induced significantly more cyprids to settle than did plates bound with control solutions (one-way ANOVA: $F_{3,31}= 7.56$, $p < 0.005$; Fig. 4a). When the 199 kDa subunit was embedded in prey mimics and presented to foraging *Acanthinucella spirata* along side controls whelks preferentially consumed the faux prey laced with the subunit ($G^2= 11.05$, D.F.= 3, $p = 0.011$, n=16; Fig. 4e). In addition to the dominant 199 MALDI-ToF peak, a protein of approximately 100 kDa appears in the spectrum as well. It is likely that this peak represents a cleavage product

of the intact 199 kDa subunit. Furthermore, a protein of 98 kDa was identified in SDS-PAGE gels, further indicating this as a cleavage product. This protein fragment was thus tested in settlement and feeding assays to determine if only a portion of the full signal is required to induce settlement and feeding responses. Significantly more cyprids settled in response to this cleaved portion of the protein than did to controls (one-way ANOVA: $F_{3,31}$= 8.04, $p < 0.005$; Fig. 4b). Likewise, this protein fragment was preferentially chosen over controls in laboratory feeding assays (G^2= 13.75, D.F.= 3, p = 0.0033, n=14; Fig. 4f).

The role of the 88 kDa protein in both *B. glandula* cyprid settlement and whelk feeding was also tested. Plates were bound with the isolated subunit, and on average 90% of the proteins were adsorbed. Despite being tested during the same settlement period as the 199 kDa subunit and the 98 kDa cleavage product, the 88 kDa protein did not induce any more cyprids to settle than did the controls (Fig. 4c). Furthermore, this subunit failed to induce any preferential feeding in whelks (Fig. 4g), and in a total of 43 bioassays only 9 whelks fed at all. Due to the lack of activity observed in laboratory feeding trials this protein was not assayed in field feeding trails.

Finally, the activity of the 76 kDa protein was assayed for settlement and feeding. Plates typically bound 94% of the protein, but again failed to induce any more settlement than did plates bound with control solutions (Fig. 4d). When this subunit was presented to foraging whelks, however, there was some observed feeding in response to contact, but this preference was not found to be significant (G^2= 4.79, D.F.= 3, p = 0.188; Fig. 4h).

Although some whelk feeding activity may be associated with contacting this portion of the glycoprotein complex the response of whelks was not as great as it was for the 199 kDa band. Paired with the settlement data, this strongly indicates that the activity of the intact complex originates in the 199 kDa subunit (or a portion therein).

Finally, the activity of the 199 kDa protein was compared to that of the active *B. glandula* crude extract and the purified glycoprotein complex in both settlement and feeding assays. Despite a near 10-fold difference in protein concentrations between the crude, the native glycoprotein, and the 199 kDa subunit all remained active inducing significantly more cyprids to settle than on the FSW control, but no significant difference between each other (one-way ANOVA: $F_{3,31}$= 3.37, p = 0.0325; Fig. 5a). As was seen for settling cyprids, the 199 kDa protein, intact glycoprotein complex, and crude barnacle extraction all induced preferential feeding over the FSW control in both laboratory and field feeding trails, and again there was no preference over any one of these treatments over another indicating the active predation compound is present at recognizable levels in each purification provided (lab feeding: G^2= 15.51, D.F.= 3, p = 0.001; field feeding: G^2 =9.90, D.F.= 3, p = 0.019; Fig 5b).

Peptide Fingerprint, RACE-PCR, and the sequence of the active glycoprotein

Internal peptide sequences were obtained from chemical and enzymatic digests of the active 199 kDa subunit and active 98 kDa cleaved protein. The resulting peptide fragments were separated using HPLC and sequenced online with a quadrupole time-of-flight mass analyzer (LC-MS/MS). Eight peptides were unambiguously sequenced from

multiple locations within the active protein sequence (Table 1). From these sequenced fragments degenerate primers were developed to be used in rapid amplification of cDNA ends (RACE). Initial RACE-PCR amplification with degenerate primers yielded numerous non-specific binding events with first strand cDNA. One peptide sequence (ETFLTLVQTDK), however, provided both a 3' and a 5' degenerate primer that yielded discrete DNA products. The PCR product was processed using automated sequencing which yielded a sequence of the 36 base pairs coding for the 5' untranslated region (UTR) (including the start codon (ATG)) and an 800 bp length of the open reading frame (ORF) from which gene-specific primers were produced. RACE-PCR was repeated using gene-specific primers in both the 5' and 3' direction, and a full length cDNA of 5,089 base pairs was obtained including the 5' UTR (36 bp), the ORF (4,704 bp), and the 3' UTR (385bp) . The full-length ORF encoded 1567 amino acids residues producing a protein with a molecular mass of 172.5 kDa. Each of the internal N- and C-terminal sequences obtained in tandem mass spectrometry peptide fingerprinting were found in the translated protein confirming we had obtained the correct mRNA transcript. Searching the sequence in the SignalP database identified a 17-residue signal peptide at the N-terminus of the glycoprotein indicating this cue is secreted. As the signal peptide is cleaved following site secretion the unmodified mass of the active protein is 170.7 kDa.

Sequence homologies and protein family associations

Comparison of the multifunctional protein factor (MULTIFUNCin) to other sequences in the GenBank database using the NCBI BLASTp algorithm found this

protein most closely resembles the settlement inducing protein complex (SIPC) of *Balanus amphitrite* (Dreanno *et al.*, 2007; GenBank accession # AAR33079) with a score (S) = 2355 bits (E = 0) and an overall sequence homology to SIPC of 73%. Alignment of sequences also found considerable overlap with the Thiolester-containing protein 7 (TEP 7) of the wasp *Nasonia vitripennis* (S = 667 bits, E = 0, 30% homologous) and of the bee *Apis mellifera* (S = 640, E = 0, 29% homologous). A QTD motif (142-144) and an FxVxxYxLPxFE region (228-239) were found in the MULTIFUNCin sequence which is characteristic of different members of the TEP family. Although both of these TEP proteins gave a significant alignment score, the highly conserved GCGEQ motif characteristic of most TEP proteins is not found in MULTIFUNCin. In addition to the TEP 7 protein alignment, numerous α_2-macroglobulin (A2M) proteins significantly matched this signal including those the horseshoe crab *Limulus spp.* (S = 666, E = 0, 30% homologous), the ticks *Ixodes ricinus* and *Ornithodoros moubata* (S = 657, E = 0, 29% homologous; S = 642, E = 0, 29% homologous, respectively), the prawn *Macrobrachium rosenbergii* (S = 572, E = $1e^{-160}$, 29% homologous), the crab *Eriocheir sinensis* (S = 559, E = $1e^{-156}$, 28% homologous), and the shrimps *Fenneropenaeus chinensis*, *Marsupenaeus japonicus*, and *Litopenaeus vannamei* (S = 555, E = $2e^{-155}$, 32% homologous; S = 554, E = $3e^{-155}$, 27 % homologous; S = 551, E = $3e^{-154}$, 28% homologous, respectively) (Fig. 6).

With a threshold expected value (E value) set to 0.01, MULTIFUNCin was found to share conserved domains with the A2M protein superfamily (including the thiolester bond forming region (TEP)) and the complement factors C3-5. The conserved domains

between MULTIFUNCin and members of the A2M protein family of various crustaceans and arthropods suggest that this signal may have evolved from an ancestral crustacean A2M gene arising either through duplication or mutation (Fig. 6). However, the conservation observed between *B. amphitrite* SIPC and the *B. glandula* MULTIFUNCin may suggest a new class of secreted protein, similar to A2M proteins, but with a physiological and ecological functionality that was previously unknown.

DISSCUSSION

The identity of an ecologically important signal molecule

Here a protein complex was identified and the mRNA transcript sequenced for a molecule that induces settlement of, and predation on, the barnacle *Balanus glandula*. ESI-GEMMA revealed the native complex to have a mass of 390 kDa. As the mRNA transcript was found to encode an unmodified protein of approximately 171 kDa, this data implies the active complex is a dimer of two repeating 199 kDa proteins (the post-translationally modified 171 kDa protein identified in sequencing). This result is further corroborated by the ESI-GEMMA spectrum of the denatured complex and the SDS-PAGE gels which identified a prominent peak and protein band, respectively, at approximately 199 kDa. An additional protein band was identified in the denatured complex of 98 kDa. Initially this was thought to be a unique subunit of the active complex and so was tested in feeding and settlement assays along with other isolated fragments. However, the subsequent mRNA transcript was found to code for a protein of

greater mass. This result conflicts with the previous notion of the 98 kDa protein as a unique subunit. The most likely explanation is, thus, that the 199 kDa subunit is cleaved during denaturation into two 98 kDa fragments of near-identical mass that migrate through the gels at the same rate and would thus be indistinguishable. A similar phenomenon would occur in the MALDI-ToF in that if the protein cleaves into fragments of near-equal mass they would be represented by a single m/z peak (the slight difference in mass can be explained by the cleaving of sugar chains). The notion that the 98 kDa band is a cleavage product is further supported by the fact that sequenced peptides from the 98 kDa protein fragment were identified in both the N- and C-terminus of the intact protein (Table 1). Moreover, the 98 kDa subunit was found to be as active in feeding and settlement as the 199 kDa subunit suggesting that after cleavage the active portion of the protein remains intact.

Previous studies have shown that the glycoprotein is present in feeding cirri, cuticle, gut, and on the outer surface of the shell (dissertation chapter 2). Furthermore, a 17-residue signal peptide was identified at the N-terminus, indicating secretion of this protein. These results strongly suggest that the protein is cuticle-derived and deposited on the outer surfaces of barnacles. In holding tanks, barnacles were routinely observed cleaning the shell using a "sweeping" action of their cirri which may bind the signal to the substrate. Although the active glycoprotein identified here shares a high degree of homology with the SIPC of *B. amphitrite*, it also has more functions than SIPC. Specifically, this study identified cellular (cuticle), population (settlement), and community-level (predation) functionality associated with it. As this protein is likely

physiologically requisite to cuticle formation, induces cyprid settlement and whelk predation, is secreted and bound to outer surfaces, and thus is multifunctional, the signal identified here has been termed the multifunctional protein factor (MULTIFUNCin).

An adult *Balanus glandula* expressed on average 0.16 mg of the active glycoprotein signal (Ferrier *et al.*, in prep). Prey mimics thus contained ecologically scaled concentrations of this molecule which was eaten upon contact by the predatory whelk *Acanthinucella spirata* in both laboratory and field feeding assays. This study showed that a biologically relevant concentration of 0.0132 mg/cm^2 was effective at inducing *B. glandula* settlement under natural field conditions. Remarkably, this concentration is similar to the effective concentration (0.011mg/cm^2) that induces settlement of *B. amphitrite* cyprid larvae in laboratory settlement trials (Matsumura *et al.* 1998).

Both *B. glandula* MULTIFUNCin and *B. amphitrite* SIPC share conserved domains with the α_2-macroglobulin (A2M) protein superfamily, the thiolester-containing protein (TEP) family embedded within the A2M family, as well as to the complement factors C3-5 of a variety of species (Fig. 6). With an approximate homology of 73%, SIPC and MULTIFUNCin are likely the same class of signal molecules with minor species-specific differences at the amino acid residue level. MULTIFUNCin and SIPC contain a QTD motif (142-144) and an FxVxxYxLPxFE region (228-239) both of which are structurally important to the TEP family (Dodds, 2002), but MULTIFUNCin lacks the STQDT motif found in the SIPC. In addition, both signals lack the γ-glutamyl thioester signature sequence (GCGEQ) of the TEP family which is necessary for native

conformation and activity of many A2M proteins (Armstrong and Quigley, 1999; Dodds, 2002; Blandin and Levashina, 2004; Dreanno *et al.*, 2006). Although MULTIFUNCin contains sequence motifs found in the conserved domain of the A2M superfamily, the lack of the thioester bond sequence and the STQDT motif suggests that this signal belongs to a different class of protein. A 17 amino acid residue was found for MULTIFUNCin indicating secretion of the expressed protein. Furthermore, MULTIFUNCin is found at ecologically relevant concentrations on the outer surfaces of barnacles and may belong to a class of cuticular proteins that are constantly produced as the organism undergoes biomineralization. MULTIFUNCin shares significant homology with A2M, and thus, the signal likely evolved from an ancestral arthropod A2M gene, an idea supported by phylogenetic analysis (Fig. 6). MULTIFUNCin, and likely SIPC, may be the first described members of a group of multifunctional cuticular glycoproteins that play roles in biochemical, individual, population-, and community-level processes.

The ecological role of the secreted multifunctional protein factor

Chemical signals are a powerful force in population level processes. In terrestrial, aquatic, and marine habitats, species-specific mating, settlement, and feeding cues influence the population dynamics of the species. A primary focus of chemical ecology has been the identification of conspecific signaling molecules and how they facilitate/mediate population dynamics. Although effects of conspecific cues on population dynamics are well recognized, rarely considered is the possibility of a cue being hijacked by other species. Such multi-species cue recognition has important

implications for community ecology, and may serve as the underlying chemosensory signals that force species interactions.

In this study, we identified one such molecule—MULTIFUNCin. As the putative settlement cue for the cyprid larvae of *B. glandula*, the detection of this protein signal results in a rapid increase in barnacle population size during the settlement season and, ultimately, is responsible for observed gregariousness. Barnacles are dominant space occupiers and competitors in the upper intertidal of many temperate rocky intertidal habitats (Connell, 1961). MULTIFUNCin may be the underlying sensory cue initiating competitive interactions that shape these communities. The signal is also recognized by a primary predator of *B. glandula*—*Acanthinucella spirata*—which when contacted induces feeding. The recognition of MULTIFUNCin, therefore, acts as the sensory basis for a critical interaction which drives opposing demographic processes—simultaneously increasing barnacle populations through settlement and decreasing populations through predator-induced mortality. Indirectly, this signal is at the heart of a trophic cascade resulting from competitive exclusion by barnacles, which pulls the community towards monospecific assemblages, and the release of space through selective feeding by whelks. This freeing of space facilitates the settlement of competitive subordinates, which pulls the community towards greater diversity (Murdoch, 1969; Dayton, 1971). Much like keystone species, which have a disproportionate effect on their communities relative to their biomass (Paine, 1966), the MULTIFUNCin protein has a disproportionate effect on the intertidal community relative to its concentration. Due to these effects, MULTIFUNCin can be considered a keystone molecule (Zimmer and Ferrer, 2007).

In terms of chemical cues, proteins have not been widely considered as important signaling molecules, however, there have been numerous suggested roles for small peptides in the settlement of abalone (Robert, 2001), oysters (Zimmer-Faust and Tamburri, 1994), and nudibranchs (Lambert *et al.*, 1997). Large macromolecules, of low relative solubility and of a structural nature, may prove to be reliable signals in turbulent environments. Many organisms produce glycoproteins for cuticle and shell biomineralization, many of which are requisite and long-lived (Marin and Luquet, 2004). These signals may prove to be reliable intra- and interspecies cues that indicate food, mates, habitat, and defense. This study found a glycoprotein—remarkably similar in amino acid composition between two different species—induces settlement. These results indicate that a high level of conservation may exist in Balanomorph barnacle glycoprotein signals. Further inquiry into the expression of this protein in other species may reveal a common production/recognition paradigm that is responsible (both directly and indirectly) for barnacle-whelk interactions and underlie intertidal community dynamics. This new class of α_2-macroglobulin-like glycoproteins, comprised of MULTIFUNCin and SIPC, thus represents the first discovery of a large protein complex significantly contributing to community dynamics.

Table 1: Peptide fragment sequences obtained from LC-MS/MS fingerprinting used for degenerate primer design. Location and peptide fragment origin are giving.

Sequence	Origin	Fragment Location
ACLSLFNLAGPNR	98 kDa	N-terminal
ETFLTLVQTDK	199, 98 kDa	N-terminal
ESGEVISDSR	98 kDa	Internal
AAPNSVCGISAVDK	199, 98 kDa	Internal
TSITTFKPFFTEVSLPYSLK	98 kDa	Internal
GGEAALTAFVM	199 kDa	Internal
FSSTLEVPDPAFSLGVAAK	98 kDa	C-terminal
VHDFYRPEER	199 kDa	C-terminal

Figure 1

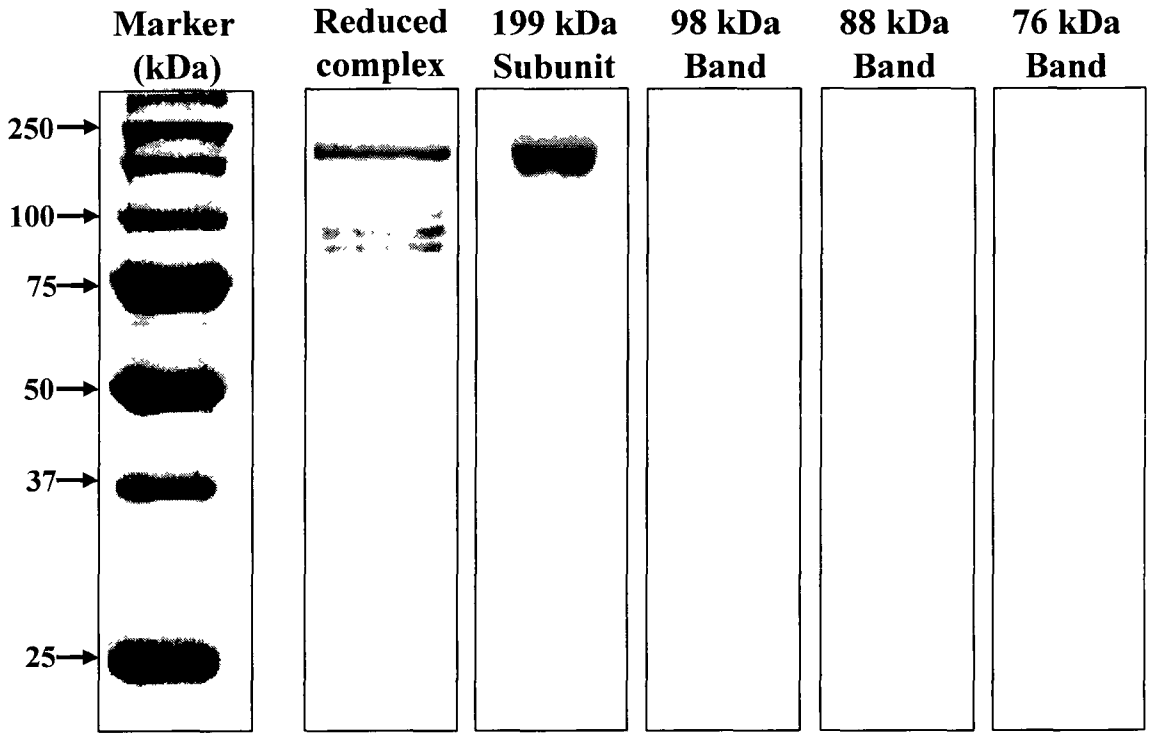

Marker (kDa)
Reduced complex
199 kDa Subunit
98 kDa Band
88 kDa Band
76 kDa Band
250
100
75
50
37
25

Figure 2

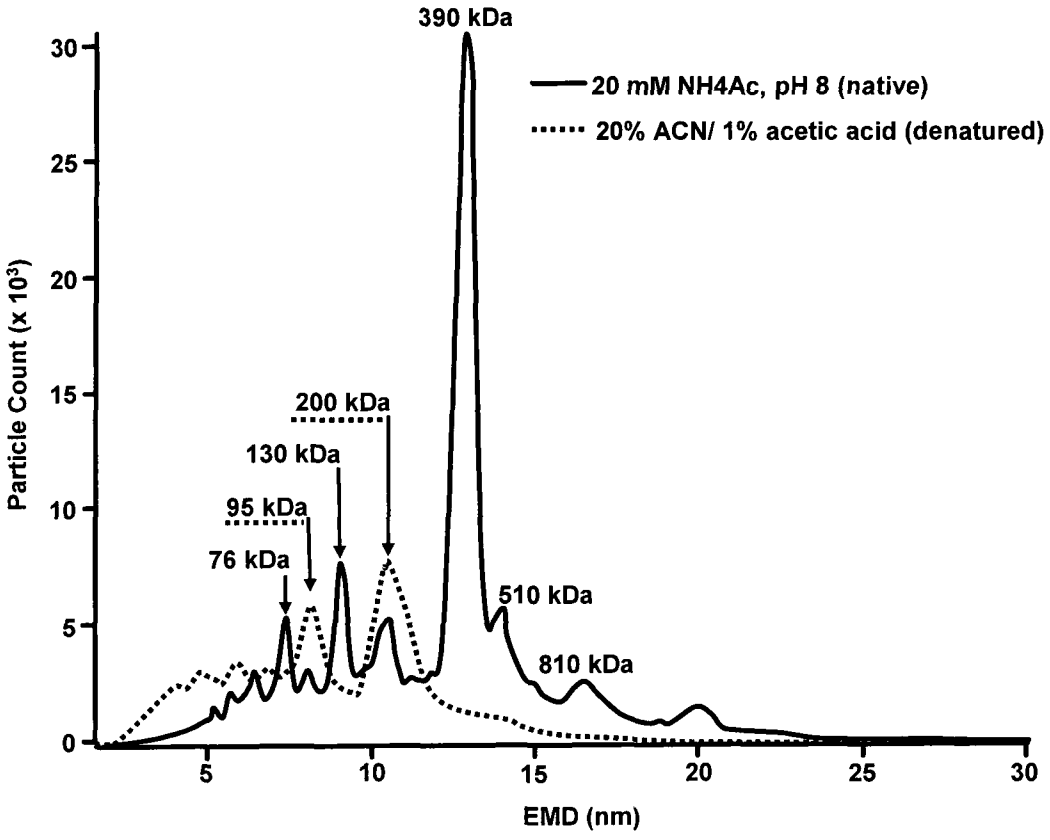

390 kDa
20 mM NH4Ac, pH 8 (native)
20% ACN/ 1% acetic acid (denatured)
200 kDa
130 kDa
95 kDa
76 kDa
510 kDa
810 kDa
Particle Count (x 10³)
EMD (nm)
0
5
10
15
20
25
30

Figure 3

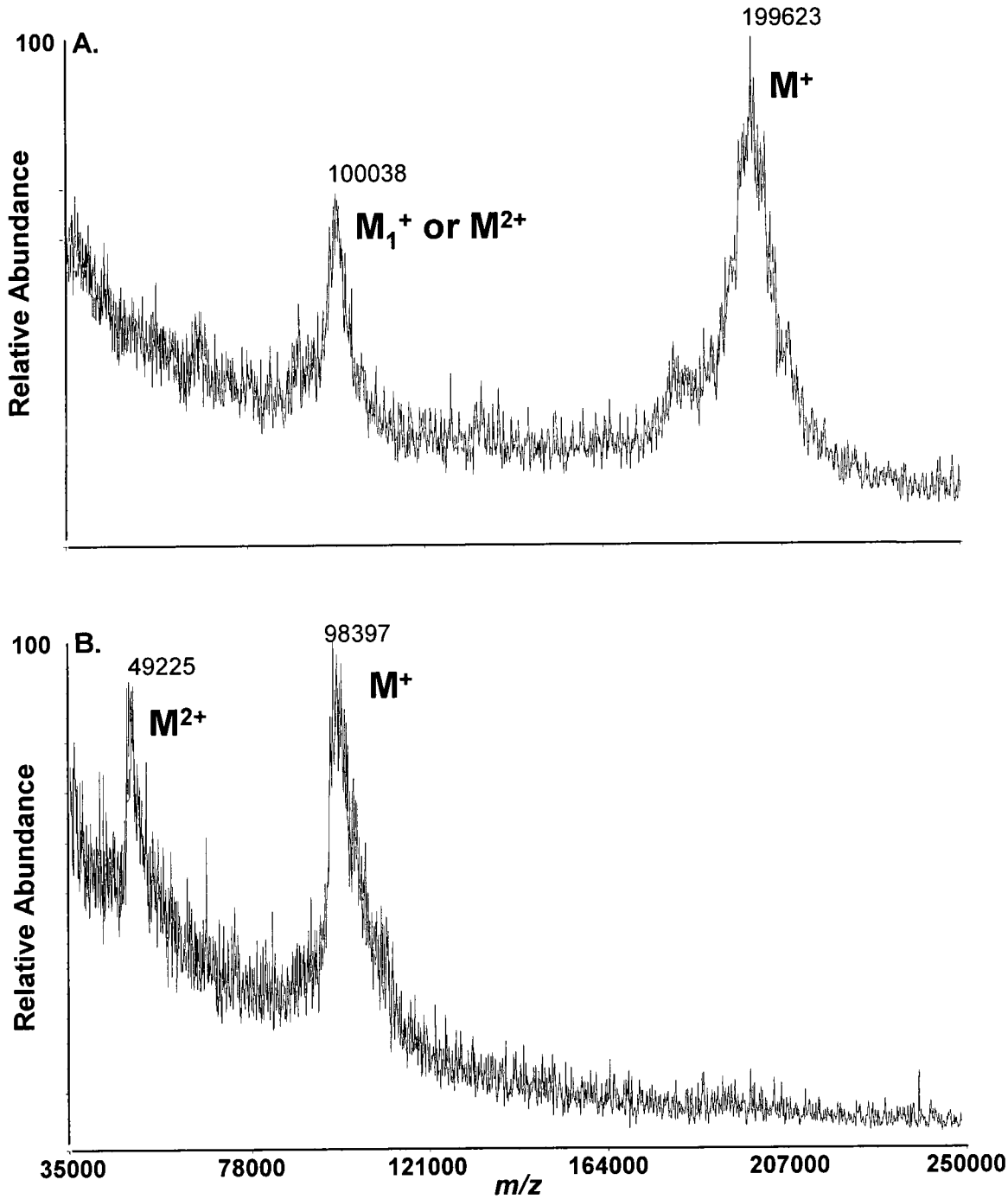

100
A.
199623
M+
100038
M1+ or M2+
Relative Abundance
100
B.
98397
49225
M+
M2+
Relative Abundance
35000
78000
121000
164000
207000
250000
m/z

Figure 4

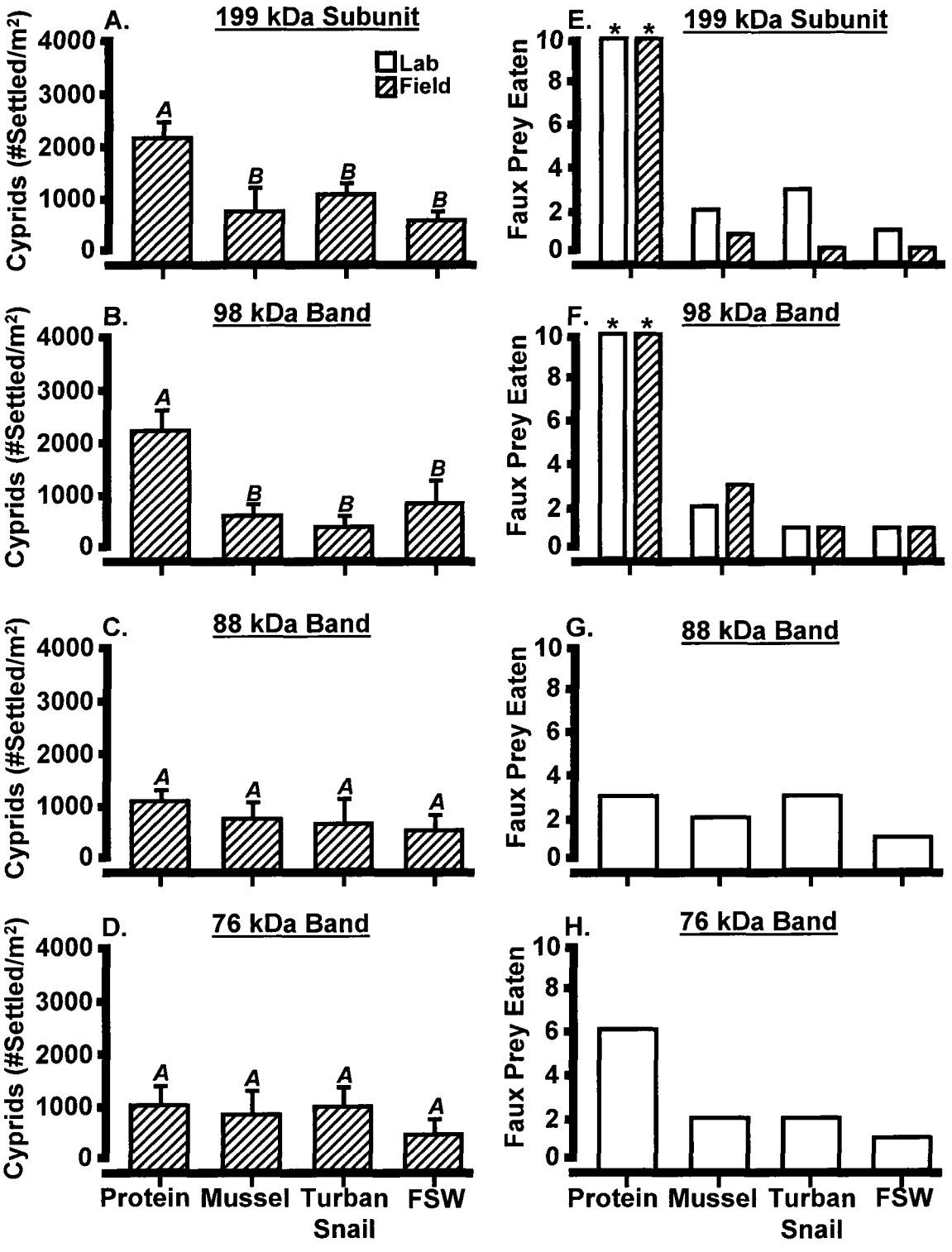

A.
199 kDa Subunit
Lab
Field
Cyprids (#Settled/m²)
4000
3000
2000
1000
0
A
B
B
B
E.
199 kDa Subunit
Faux Prey Eaten
10
8
6
4
2
0
*
*
B.
98 kDa Band
A
B
B
B
F.
98 kDa Band
C.
88 kDa Band
A
A
A
A
G.
88 kDa Band
D.
76 kDa Band
A
A
A
A
H.
76 kDa Band
Protein
Mussel
Turban Snail
FSW

Figure 5

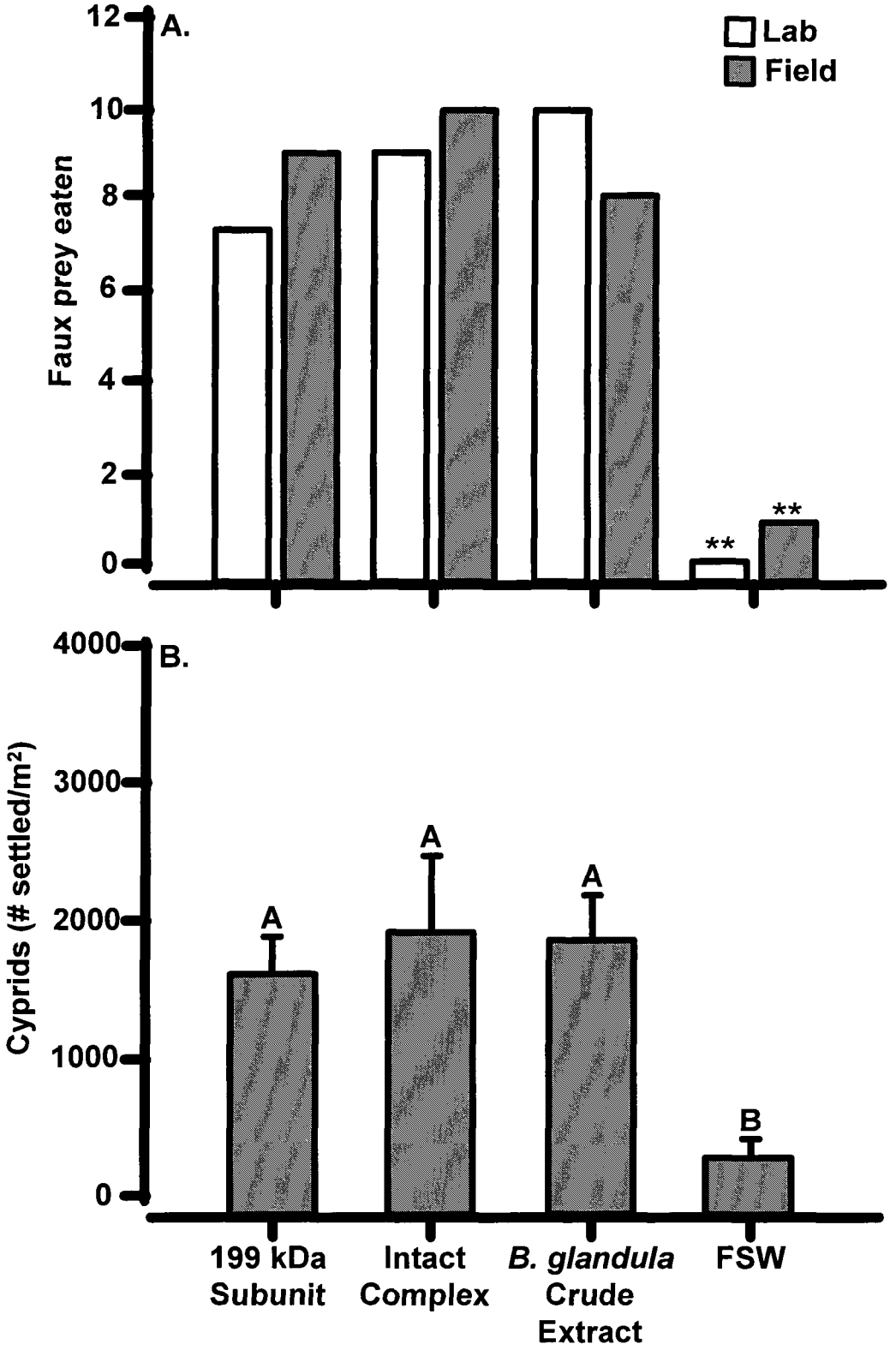

A.
Lab
Field
Faux prey eaten
12
10
8
6
4
2
0
**
**
B.
Cyprids (# settled/m²)
4000
3000
2000
1000
0
A
A
A
B
199 kDa Subunit
Intact Complex
B. glandula Crude Extract
FSW

Figure 6

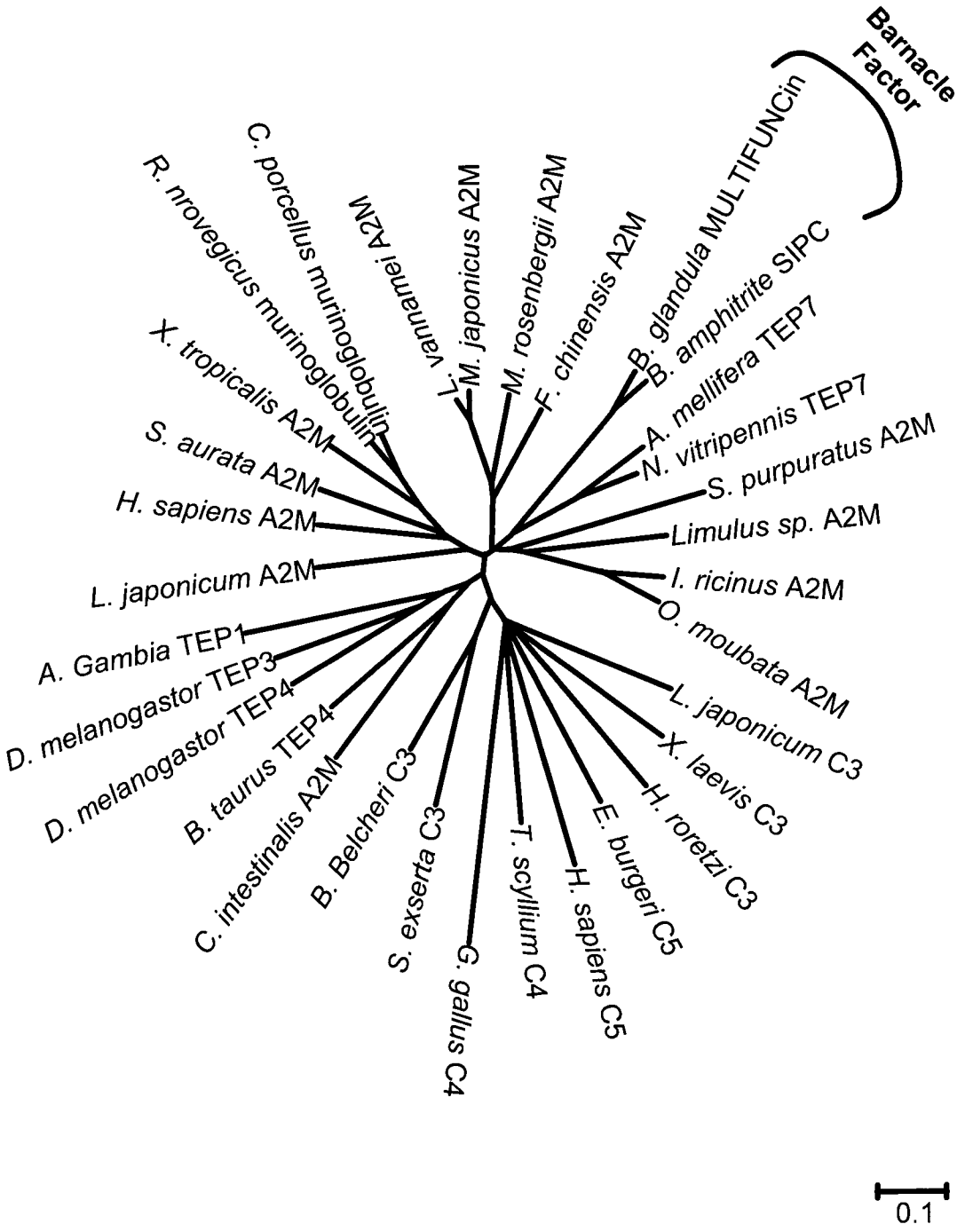

Barnacle Factor
B. glandula MULTIFUNCin
B. amphitrite SIPC
A. mellifera TEP7
N. vitripennis TEP7
S. purpuratus A2M
Limulus sp. A2M
I. ricinus A2M
O. moubata A2M
L. japonicum C3
X. laevis C3
H. roretzi C3
E. burgeri C5
H. sapiens C5
T. scyllium C4
G. gallus C4
S. exserta C3
B. Belcheri C3
C. intestinalis A2M
B. taurus TEP4
D. melanogastor TEP4
D. melanogastor TEP3
A. Gambia TEP1
L. japonicum A2M
H. sapiens A2M
S. aurata A2M
X. tropicalis A2M
R. nrovegicus murinoglobulin
C. porcellus murinoglobulin
vannamei A2M
L.
M. japonicus A2M
M. rosenbergii A2M
F. chinensis A2M
0.1

Figure Captions

Figure 3-1. SDS-PAGE gel run under denaturing conditions in the presence of β-mercaptanoethanol showing the dissociated glycoprotein complex and each of the isolated subunits that were tested in feeding and settlement assays. Subunits were isolated from excised gel fragments by electroelution using a modified western blotting apparatus.

Figure 3-2. Electrospray ionization gas-phase electrophoretic mobility molecular analysis (ESI-GEMMA) of the intact glycoprotein complex. Particle counts of the native protein identified a dominant peak corresponding to a complex with a mass of 390 kDa. A dominant peak was found in the ESI-GEMMA spectrum of the denatured complex of ca. 199 kDa suggesting the complex is likely a dimer composed of two subunits.

Figure 3-3. Matrix-assisted laser desorption ionization time of flight (MALDI-ToF) spectra of the two active bands observed in the SDS-PAGE gels. The spectra of the first active band indicated a protein subunit with a mass of 199 kDa (A). The spectra of the next active band indicated a mass of 98 kDa (B). Subsequent sequence analysis revealed the 98 kDa component likely represents a cleavage product of the 199 kDa subunit.

Figure 3-4. Settlement and feeding responses of *Balanus glandula* cyprid larvae and *Acanthinucella spirata*, respectively, to each isolated subunit. Cyprids settled in significantly greater numbers in response to the 199 kDa subunit and the 98 kDa cleavage product than to controls (A and B), but did not settle any more when the 88 and 76 kDa subunits were contacted than on controls (C and D). Bars sharing the same letter are not significantly different ($p \geq 0.05$). Like cyprids, whelks preferentially consumed prey mimics embedded with the 199 kDa subunit and the 98 kDa fragment (E and F) over controls, but did not feed significantly more upon contacting the 88 and 76 kDa subunits than on controls (G and H). Asterisks denote highly significant preferences ($p < 0.005$).

Figure 3-5. The active 199 kDa subunit was assayed against the intact complex, the crude barnacle extract and the control filtered sea water (FSW) in both whelk feeding (A), and cyprid settlement (B). The subunit and the intact complex contained a protein concentration of 12.6% that of the crude. Bars with the same letter indicate responses that were not significantly different; asterisks denote highly significant preferences ($p < 0.005$).

Figure 3-6. The inferred evolutionary history of MULTIFUNCin and members of the α2-macroglobulin protein superfamily (A2M), the thiolester-containing protein family (TEP), and the complement factor proteins (C3, C4, C5) using the Neighbor-Joining method. The optimal un-rooted tree with the sum of branch length = 7.532 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site.

References:

Armstrong, P.B., and J.P. Quigley. 1999. α_2-macroglobulin: an evolutionarily conserved arm of the innate immune system. Developmental and Comparative Immunology 23:374-390.

Baldwin, I.T., R. Halitschke, A. Paschold, C.C. von Dahl, and C.A. Preston. 2010. Volatile signaling in plant-plant interactions: "talking trees" in the genomics era. Science 311: 812-815.

Berenbaum, M. R. 2002. Postgenomic chemical ecology: from genetic code to ecological interactions. Journal of Chemical Ecology 28: 873-896.

Blandin, S., and E.A. Levashina. 2004. Thioester-containing proteins and insect immunity. Molecular Immunology 40:903-908.

Burks, R.L., and D.M. Lodge. 2002. Cued in: advances and opportunities in freshwater chemical ecology. Journal of Chemical Ecology 28(10):1901-1917.

Connell, J. 1961. The influence of interspecific competition and other factors on the distribution of the barnacle Chthamalus stellatus. Ecology 42:710-723.

Dayton, P. 1971. Competition, disturbance, and community organization: the provision and subsequent utilization of space in a rocky intertidal community. Ecological Monographs 41:351-389.

De Boer, J.G., M.A. Postumus, and M. Dicke. 2004. Identification of volatiles that are used in discrimination between plants infested with prey or nonprey herbivores by a predatory mite. Journal of Chemical Ecology 30:2215-2230.

Dodds, A. 2002. Evolution of the Complement System. Immunobiology 205:340-354.

Dreanno, C., K. Matsumura, N. Dohmae, K. Takio, H. Hirota, R.R. Kirby, and A.S. Clare. 2006. An alpha2-macroglobulin-like protein is the cue to gregarious settlement of the barnacle Balanus amphitrite. Proceedings of the National Academy of Sciences of the United States of America 103:14396-401.

Kaddis, C.S., S.H. Lomeli, S. Yen, B. Berhane, M.I. Apostol, V.A. Kickhoefer, L.H. Rome, and J.A. Loo. 2007. Sizing large proteins and protein complexes by electrospray ionization mass spectrometry and ion mobility. Journal of the American Society for Mass Spectrometry 18:1206-1216.

Kaddis, C.S., and J.A Loo. 2007. Large flying proteins with ESI. Analytical Chemistry 3:1779-1784.

Kumar, S., M. Nei, J. Dudley, and K. Tamura. 2008. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Briefings in Bioinformatics 9:299-306.

Kvitek, R.G., and C. Bretz. 2004. Harmful algal bloom toxins protect bivalve populations from sea otter predation. Marine Ecological Progress Series 271:233-234.

Lambert, W.J., C.D. Todd, and J.D. Hardedge. 1997. Partial characterization and biological activity of a metamorphic inducer of the dorid nudibranch *Adalaria proxima* (Gastropoda: Nudibranchia). Invertebrate Biology 116:71–81.

Marin, F., and G. Luquet. 2004. Molluscan shell proteins. Comptes Rendus Palevol 3(7):469-492.

Matsumura, K., M. Nagano, and N. Fusetani. 1998. Purification of a Larval Settlement-Inducing Protein Complex (SIPC) of the Barnacle, *Balanus amphitrite*. Journal of Experimental Zoology 281:12-20.

Murdoch, W.W. 1969. Switching in general predators: experiments on predator specificity and the stability of prey populations. Ecological Monographs 39:335-354.

Paine, R. 1966. Food web complexity and species diversity. The American Naturalist 100:65-75.

Paul, V.J., K.E. Arthur, R. Ritson-Williams, C. Ross, and K. Sharp. 2007. Chemical Defenses: From Compounds to Communities. Biological Bulletin, 213:226 -251.

Raffa, K.F. 2001. Mixed messages across multiple trophic levels: the ecology of bark beetle chemical communication systems. Chemoecology 11:49-65.

Roberts, R. 2001. A review of settlement cues for larval abalone (*Haliotis spp.*). Journal of Shellfish Research 2:571–586.

Tillman, J.A., S.J. Seybold, R.A. Jurenka, and G.J. Blomquist. 1999. Insect pheromones—an overview of biosynthesis and endocrine regulation. Insect Biochemistry and Molecular Biology 29:481-514.

Swanson, R.L., J.E. Williamson, R. De Nys, N. Kumar, M.P. Bucknall, and P.D. Steinberg. 2004. Induction of settlement of larvae of the sea urchin *Holopneustes purpurascens* by histamine from a host alga. Biological Bulletin 206:161-172.

Teegarden, G.J., and A.D. Cembella. 1996. Grazing of toxic dinoflagellates, *Alexandrium* spp., by adult copepods of coastal Maine: Implications for the fate of paralytic shellfish toxins in marine food webs. Journal of Experimental Marine Biology and Ecology 196:145-176.

Vos, M., L.E. Vet, F. Wackers, J.J. Middelburg, W.H. van der Putten, W.M. Mooij, C.H.R. Heip, and E. von Donk. 2006. Infochemicals structure marine, terrestrial and freshwater food webs: Implications for ecological informatics. Ecological Informatics 1:23-32.

Wendal, T., and F. Jüttner. 1997. Excretion of heptadecene-1 into lake water by swarms of *Polyphemus pediculus* (Crustacea). Freshwater Biology 38:203–207.

Zimmer, R.K., and C.A. Butman. 2000. Chemical signaling processes in the marine environment. Biological Bulletin 199:168-187.

Zimmer, R.K., and R.P. Ferrer. 2007. Neuroecology, Chemical Defense, and the Keystone Species Concept. Biological Bulletin 213:208 -225.

Zimmer-Faust, R.K., and M.N. Tamburri. 1994. Chemical identity and ecological implications of a waterborne larval settlement cue. Limnology and Oceanography 39:1075-1087.